The BRAVE project (Bee Research And Virology in Europe) was selected from the call for proposals FP6-2003-SSP3[1] where one of the objectives was the «Assessment of the level of risk and the likely consequences for bees and other closely related pollinators of the introduction of bee viruses to Europe. BRAVE was aimed at knowledge transfer between experts with a broad base of skills in insect virology, diagnosis, immunology, epidemiology, international trade and risk management, along with scientists involved in fundamental and applied research on bees and related pollinator species. More than 60 world experts exchanged their knowledge during a preliminary meeting in Sophia-Antipolis (France) in April 2005. Following this first meeting, a smaller panel of experts gathered in Tourtour (Les Treilles Foundation – France) in September 2005 and produced this book which, in addition to being an overview of current virology status of the honey bee, also proposes a framework for future research programmes on virology and the honey bee.

[1] Call for proposals for indirect RTD actions under the specific programmefor research, technological development and demonstration: 'integratingand strengthening the European Research Area'.

Publications Office
Publications.europa.eu

ISBN 92-79-00586-3

KI-NA-21937-EN-C

Virology and the Honey Bee

EUROPEAN COMMISSION

European Research Area

Virology and the Honey Bee

PROJECT REPORT

EUR 21937

Interested in European research?

Research*eu is our monthly magazine keeping you in touch with main developments (results, programmes, events, etc.). It is available in English, French, German and Spanish. A free sample copy or free subscription can be obtained from:

European Commission
Directorate-General for Research
Communication Unit
B-1049 Brussels
Fax (32-2) 29-58220
E-mail: research-eu@ec.europa.eu
Internet: http://ec.europa.eu/research/research-eu

How to obtain EU publications

Our priced publications are available from EU Bookshop (http://bookshop.europa.eu), where you can place an order with the sales agent of your choice. The Publications Office has a worldwide network of sales agents. You can obtain their contact details by sending a fax to (352) 29 29-42758.

EUROPEAN COMMISSION

Directorate-General for Research
Directorate E — Biotechnologies, Agriculture, Food
Unit E4 — Agriculture, Forestry, Fisheries, Aquaculture

Contact: Isabel Minguez Tudela

European Commission
B-1049 Brussels

Tel. (32-2) 29-92109
Fax (32-2) 29-63029
E-mail: isabel.minguez-tudela@ec.europa.eu

EUROPEAN COMMISSION

Virology and the Honey Bee

edited by

Michel AUBERT,
Brenda BALL,
Ingemar FRIES,
Robin MORITZ,
Norberto MILANI and
Iris BERNARDINELLI

SIXTH FRAMEWORK
PROGRAMME

2008 Directorate-General for Research EUR 21937 EN

Europe Direct is a service to help you find answers to your questions about the European Union

Freephone number (*)**:**

00 800 6 7 8 9 10 11

(*) Certain mobile telephone operators do not allow access to 00 800 numbers or these calls may be billed.

More information on the European Union is available on the Internet (http://europa.eu).

Cataloguing data can be found at the end of this publication.

Luxembourg: Office for Official Publications of the European Communities, 2008

ISBN 92-79-00586-3

Printed in Belgium

Printed on white chlorine-free paper

Contents

INTRODUCTION

Why a book on virology in relation with the honey bee?

Because honey bees must be protected …

The main reason for protecting honey bees is their importance as pollinators of many wild and cultivated plant species. Pollination by bees and other insects is the first step in the flowering/fruiting process resulting in the growth of essential foods such as vegetables, fruits and seeds that make up approximately 35% of the human diet. Without pollinating insects – among them honey bees play the major role – the ecosystem would fall apart. The economic value of honey and other hive products, although great, is low when compared to the historical and social values of apiculture, and even lower when compared to the contribution of the honey bee to agriculture and biodiversity.

… against virus diseases

Honey bees may carry several virus species which generally provoke no visible symptoms. However, these commonly observed silent states are not incompatible with detrimental effects. Firstly, symptoms in individual bees may be impossible to observe without applying specific protocols, whereas the accumulation of individual disorders can eventually lead to the death of the whole colony. Secondly, after several months of asymptomatic infection, virus multiplication may be triggered for unknown reasons resulting in overt disease. Undeniably, one dramatic outcome of virus infection in bees (chronic paralysis) has been observed since antiquity. Moreover, several other bee viruses, which were considered harmless for many years, were seen to be pathogenic during the recent introduction of *Varroa destructor*. This exotic parasitic mite, now a global pest (with the exception of Australia), has decimated feral honey bee colonies, and causes millions of dollars of damage each year to apiculture. *Varroa destructor* plays an active role in facilitating virus transmission in bees at earlier stages of development. Clearly, the increasing prevalence of bee diseases – with a significant role of bee viruses – contributes to the current decline in honey bee populations.

The first step towards protecting bees is to know more about bee viruses and their harmful effects

The first chapter describes the most recent knowledge on the **natural history and geographical distribution of honey bee viruses**. As in the other chapters of this book, the authors identify the limit of present knowledge and the most common pitfalls that should be avoided. Additionally, they propose a list of questions to be solved as a priority and the strategy they consider the most appropriate to address them. The patchy documentation on the geographical and temporal distribution of the 18 viruses isolated from the genus *Apis*, on their means of persistence and transmission calls for surveys covering large areas over one seasonal cycle at least. The relationships within the complex system "bee – viruses – other bee pathogens" strongly suggest that sampling strategy should simultaneously include all bee stages and all pathogen populations and should, therefore, be adapted to the dynamics of these populations. Newly developed molecular techniques for virus identification need to be tested and shared by the scientific community. But considering the alternate state of infection (latent *vs* overt), these methods should always be completed by quantitative techniques and infectivity tests on bees, parasites, and other species or material that compose the environment of bees, and by the simultaneous evaluation of the impact of these infections on bee health in individuals and colonies.

Our understanding of insect viruses is progressing rapidly, including that of bee viruses. To throw a bridge between experts within the wide field of insect virology and bee science was precisely one of the driving ideas of this project. Two experts, one from each field, wrote the chapter on **molecular characterisation of honey bee viruses**. Exposing the latest knowledge on RNA viruses (all bee viruses except one are RNA viruses), the authors focus on the unique genetic variability of RNA viruses and the quasi-species concept. Moreover, whilst traditional "picornavirus-like" agents of insects are being separated into new groups, it is unlikely that we have yet seen all the types of virus that occur in the honey bee. These developments are not just inconsequential discussions between specialists since they alert field observers to the fact that the honey bee will have to bear the brunt of new forms of viruses already lying in ambush.

Another principal idea of the project was to provide field and laboratory workers with a unique compendium that they could use to switch from fundamental science to very practical principles and methods for their research. Thus follows a very documented chapter on **diagnostic techniques for virus detection in honey bees**. The reader is guided in

how to implement a diagnostic strategy, including sampling, a step too often neglected. The chapter details the various tools currently available to detect viruses, as well as those that are under development and describes their relative merits. Considering that many examples are drawn from areas outside honey bee research, their future application to this organism is promising.

The three following chapters deal with virus-bee relationships. **The impact of virus infections in honey bees** reviewed in the fourth chapter has already been mentioned at the beginning of this introduction. A new graphical presentation of the incomparable field observations performed by Bailey *et al*. in the 80's enhances the reality of this impact. These studies required several years of observation, hence the scarcity of large-scale epidemiological field studies on bee diseases since that time. Amongst the many difficulties met when conducting such studies, is the alternation between latent and overt infections. These **infection strategies of insect viruses** are reviewed in the fifth chapter. Several examples of insect viruses, bee viruses included, provide some simple rules for defining the classes of infection. The conclusions of both chapters are convergent: pathogens may well be present in host populations as persistent, endemic infections, and as such may be difficult to detect. Nevertheless, they may exert considerable regulatory influences on host dynamics.

Confronted with the complexity of the interactions between the honey bee and numerous environmental factors, disconcerted by the apparent unpredictability of the dynamics of bee colonies and deceived by asymptomic infections, the observer needs guidance in identifying organisational traits among this apparent chaos. This is a prerequisite for designing coherent and useful field protocols. Under the title, **evolutionary epidemiology of virus infections in honey bees**, the sixth chapter starts with a review of factors related to the biology of parasites and hosts in general, and their interaction and how they are considered to play a role in the evolution of parasite virulence. Optimal virulence is that which guarantees the maximum reproductive success of the parasite. (The traditional view, according to which the amount of harm parasites do to their hosts decreases over evolutionary time, has been proven incorrect!). The authors then focus on the biology of honey bees in order to highlight host-specific traits involved in parasite virulence. The last section of the chapter bring these two themes together and asks what levels of virulence we might expect to find in viral parasites/diseases of bees. Identifying where our knowledge is insufficient to explain the observed levels of virulence, they suggest specific research programmes that should significantly further our understanding of the epidemiology of bee viruses.

The second step is to develop counteracting strategies and tools

Currently, it is not possible to control viral diseases of honey bees by chemotherapy and only preventive or indirect measures can be used. These consist in a) maintaining or enhancing the innate immuno-competency of the host, and b) preventing virus invasion into non-infected areas or non-infected apiaries, or their spread between colonies within apiaries.

a) maintaining and enhancing the innate defences of the honey bee

Other pathogens, toxicants, hunger or other types of stress resulting from meteorological conditions may trigger the pathogenicity of viruses. As strong colonies can maintain better homoeostasis, common sense dictates that good husbandry produces colonies with high immuno-competency. Apicultural science must go further and the honey bee must profit from the remarkable progress made concerning immunity in the fruit fly *Drosophila melanogaster,* an easy to handle insect model. This is reviewed under the title **innate immunity of insects to infection**. This chapter describes how fruit flies mobilise different cellular and /or molecular (humoral) responses based on various genetic mechanisms to recognise and control invaders. Interestingly, these mechanisms are somewhat adapted to the nature of the pathogens: bacteria, fungi, large parasites or viruses. Another striking result is the conservation between *Drosophila* and mammals of many molecules and signalling modules operating in innate immunity. As this indicates the ancient origin of host-defence mechanisms in animal evolution, it suggests that challenging questions for new research on insect immunity in general should have strong relevance for the honey bee.

Another approach is the selection of honey bee stock that is more tolerant to viruses and viral vectors: this is exposed in the chapter entitled **honey bee genomics and breeding for resistance to virus infections**. Until now, due to the lack of tools, swift selection for disease resistance programmes had only mixed success. With the recent availability of the complete genome of the honey bee *Apis mellifera,* development of swift molecular tools for confirming mating control and selection of resistant colonies may be proposed. Genes that control specific disease resistance in honey bees are already known and the great wealth of indigenous subspecies of the honey bee in Europe, Africa, and Western Asia is a vast potential to reveal mechanisms for disease resistance. Therefore there is valid optimism that these proposed plans will fit well into standard breeding schemes as revolutionary ways to improve bee health.

b) preventing virus invasion and spreading.

In recent years, overall world bee health has declined as a consequence of the accelerated global spread of pathogens, while global trade rocketed and trade regulations relaxed. Nowadays, just to keep colonies alive, chemicals (acaricides) must be used regularly resulting in increased residue in hive products… while the consumer demand for higher levels of food surveillance is increasing. For the author of the last chapter (**overview of the regulatory framework for apiculture)**, the present risks of new exotic pathogens (*Varroa destructor*, *Aethina tumida*, *Tropilaelaps* spp. … and what next?) call for effective and sound international rules. His vivid style miraculously makes the reading of regulations fascinating. This is not tongue-tied or diplomatic language. The limits of regulations are illustrated by examples: some regulations are missing, others have proved useless and others have been infringed by citizens (beekeepers!) or are not implemented by the authorities. For *developing* countries, the complex and sometimes incoherent international rules add to the many difficulties already met when trying to export honey to markets such as the EU. The author suggests several ways for improvement. Among them, that better compliance be obtained from the beekeeping industry through education programmes in partnership with beekeeping organisations.

Last words.

The ambition of the editors was to produce an easy-to-consult manual containing all the information required for a pluri-disciplinary approach to viral diseases in bees. They hope that copies of this manual will be found on the benches of virologists, insect specialists and bee experts, as well as on the desks of policy makers who are all ultimately preoccupied with protection of the honey bee.

Michel Aubert

August 2007

The BRAVE project

The BRAVE project resulted from a request by the European Commission's 6th Framework Research and Development Programme in the field of policy-orientated research. The primary focus assigned to this Specific Support Action was "*to assess the level of risk and the likely consequences for bees and other closely related pollinators of the introduction of bee viruses into European bee colonies and ecosystems, and to provide advice to the EC on appropriate protective measures to prevent further incursions and spread.*"

The steering committee of BRAVE comprised Dr Michel Aubert, AFSSA, France (coordinator); Brenda Ball, Rothamsted Research, UK; Prof. Ingemar Fries, Swedish University of Agricultural Sciences, Upsala; Prof. Norberto Milani, University of Udine, Italy; and Prof. Robin Moritz, University of Halle, Germany.

The aims of BRAVE were: a) to facilitate knowledge and skill transfer between researchers and advisors within the European Research Area of bee virus diseases, and b) to identify significant gaps in the essential scientific knowledge required to support the formulation and integration of policy on the endemic and emergent diseases of bees. The project duration was one year (2005) and included two meetings.

The first scientific meeting held at Sophia-Antipolis, Provence, France, 24-26th April 2005, was attended by 55 scientists, advisors and policy makers. Their expertise ranged from insect virology, virus taxonomy, immunology, epidemiology, disease risk assessment and international trade, to fundamental and applied research on pollinators and their pathogens. Delegates represented 15 European countries as well as Australia, Canada, Lebanon, Mexico, and the USA, together with representatives of the European Commission and the OIE (Office International des Epizooties). Young scientists and students had the opportunity to apply for additional places, thus encouraging a better-balanced age and gender participation.

The first sessions of the meeting explored the complex and evolving taxonomy of bee viruses and covered the range of diagnostic techniques now available for virus detection. Further sessions covered aspects of genetics, physiology and behaviour of honey bees in relation to their resistance to viral infections. The genetic basis of disease resistance in bees was explored. Several papers considered the persistence of virus at sub-lethal levels in honey bees. The question of viruses being "triggered" by mite feeding and the association of virus infections with other parasites such as *Varroa destructor* and *Nosema apis* was investigated.

The evolutionary epidemiology of viral diseases and their virulence was considered in relation to the different routes of transmission.

Current information on the incidence, distribution and impact of honey bee viruses was reviewed, revealing large gaps in our knowledge. The management of bee diseases was also considered, mainly in relation to *V. destructor* including the non-intentional effects of acaricides including the behaviour and efficiency of bees and the development of tolerance in bees to mites and resistance of mites to acaricides.

The final session concerned the current regulatory mechanisms governing the movement of honey bees into the EU and the assessment of the risk of pathogen introduction related to trade issues.

Every session was followed by a working sessions which produced short and comprehensive recommendations for the focus of future research efforts. These recommendations are found in the annex at the end of this book.

The proceedings of this meeting have been published. They include the scientific contributions and recommendations drawn up by the working groups and approved in plenary sessions. All the printed copies of the proceedings have been distributed, however electronic copies can be obtained from the librarian of the Sophia-Antipolis laboratory (f.bautey@afssa.fr).

A smaller **workshop meeting** took place later (2-7 September 2005) uniting authors selected from the first meeting. Their task was to prepare a synthesis of current knowledge in the different subject areas covered during the first meeting and also to propose a framework for future research programmes for protecting the honey bee and related pollinator insects from viral diseases. This book is the result of this task.

The editorial lines

Coherence between chapters, their logical sequence and progression were a major editorial preoccupation and a source of long discussions and … reversing decisions. Each chapter can stand alone, separated from the rest of the book since it has its own reference list and for clarity, contains also summarised information that is extensively exposed elsewhere in the book.

Each chapter was reviewed by the authors of other chapters. The editorial work was greatly supported by Norberto Milani, then by Iris Bernardinelli when Norberto's health was temporally but severely impaired by a mountain accident.

A special thanks to "Fondation Les Treilles"

The second BRAVE meeting was supported by and held at the "Fondation Les Treilles" (http://www.les-treilles.com). All participants warmly thanked the foundation for providing wonderful accommodation in Provence and the opportunity to participate in fruitful scientific exchange.

The participants of the second BRAVE meeting at «Fondation Les Treilles». *From left to right: back row:* Magali Ribière, Norberto Milani, Elke Genersch, Michel Aubert, Brenda Ball and Joachim de Miranda. *Front rank:* Robin Moritz, Ingemar Fries, Jean-Luc Imler, Rosie Heils, Mike Carter and Mark Brown.

The authors and editors

Michel F. A. AUBERT
AFSSA (French food safety agency) – Laboratory on small ruminant and bee pathology
BP 111 - 06902 Sophia-Antipolis Cedex - France

Brenda V. BALL
Rothamsted Research -Harpenden - Hertfordshire
AL5 2JQ -United Kingdom

Iris BERNARDINELLI
Dipartimento di Biologia e Protezione delle Piante - Università degli Studi di Udine
Via delle Scienze, 208 - 33100 Udine - Italy

Mark J.F. BROWN
School of Natural Sciences - Department of Zoology - University of Dublin Trinity College
Dublin 2 - Ireland

Michael BROWN
National Bee Unit - Central Science Laboratory -
Sand Hutton - York YO41 1LZ – United Kingdom

Mike J. CARTER
Faculty of Health and Medical Sciences - University of Surrey
GU2 7XH - United Kingdom

Joachim R. DE MIRANDA
Institutionen för Ekologi - Sverige Lantbruksuniversitet
PO Box 7044 - Uppsala 750-07 - Sweden

Catherine DOSTERT
CNRS-UPR9022 - Institut de Biologie Moléculaire et Cellulaire - 15, rue René Descartes
67000 Strasbourg - France

Jay D. EVANS
USDA Bee Research Laboratory BARC-East Bldg. 476
Beltsville, MD USA

Ingemar FRIES
Department of Entomology - Swedish University of Agricultural Sciences
Box 7044, 750 07 Uppsala - Sweden

Delphine GALIANA-ARNOUX
CNRS-UPR9022 - Institut de Biologie Moléculaire et Cellulaire - 15, rue René Descartes
67000 Strasbourg - France

Elke GENERSCH
Institute for Bee Research Hohen Neuendorf - Friedrich-Engels-Str. 32
16540 Hohen Neuendorf - Germany

Rosemary S. HAILS
Centre for Ecology and Hydrology Mansfield Road, Oxford
OX1 3SR – United Kingdom

Jean-Luc IMLER
CNRS-UPR9022 - Institut de Biologie Moléculaire et Cellulaire - 15, rue René Descartes
67000 Strasbourg - France

Norberto MILANI
Dipartimento di Biologia e Protezione delle Piante - Università degli Studi di Udine
Via delle Scienze, 208 - 33100 Udine - Italy

Robin F.A. MORITZ
Institut für Zoologie, Martin Luther-Universität Halle-Wittenberg
Hoher Weg 4, 06099 Halle/Saale, Germany

Magali RIBIÈRE
AFSSA (French food safety agency) – Laboratory on small ruminant and bee pathology
BP 111 - 06902 Sophia-Antipolis Cedex - France

Chapter 1

NATURAL HISTORY AND GEOGRAPHICAL DISTRIBUTION OF HONEY BEE VIRUSES

Magali RIBIÈRE, Brenda BALL and Michel F. A. AUBERT

Table of Contents

1. Introduction

Since 1963, when chronic bee paralysis (CBPV) and acute bee paralysis virus (ABPV) were first isolated, a total of eighteen viruses have been identified and characterised from the genus *Apis*. Only those identified in *Apis mellifera* from Europe will be discussed in this chapter. The basic physico-chemical properties of these viruses are summarised in Table I. With the exception of the large filamentous virus (FV) which has a DNA genome, all are single-stranded RNA viruses mostly isometric in shape. However, the particles of chronic bee paralysis virus (CBPV) are very distinctive and readily identified by electron microscopy being anisometric, mostly ellipsoidal in outline and often with an irregular protuberance at one end. A wide range of shapes and sizes of CBPV particles occur, including rings, figure of eights, branching rods and lengths up to 640 nm (Fig. 1). Similarly, the distinctive size and shape of the filamentous virus allow it to be easily recognised morphologically (Fig. 2). The remaining RNA viruses are isometric particles that can be separated into three size classes: 17, 30 and 35 nm (Fig. 3; Tab. I). Most are 30 nm in diameter and morphologically indistinguishable from each other by electron microscopy (Fig. 3). However, they do differ in their buoyant density in CsCl which ranges from 1.34 to 1.37g/ml, their sedimentation coefficient which ranges from 151 to 176S and their capsid protein profiles (Tab. I).

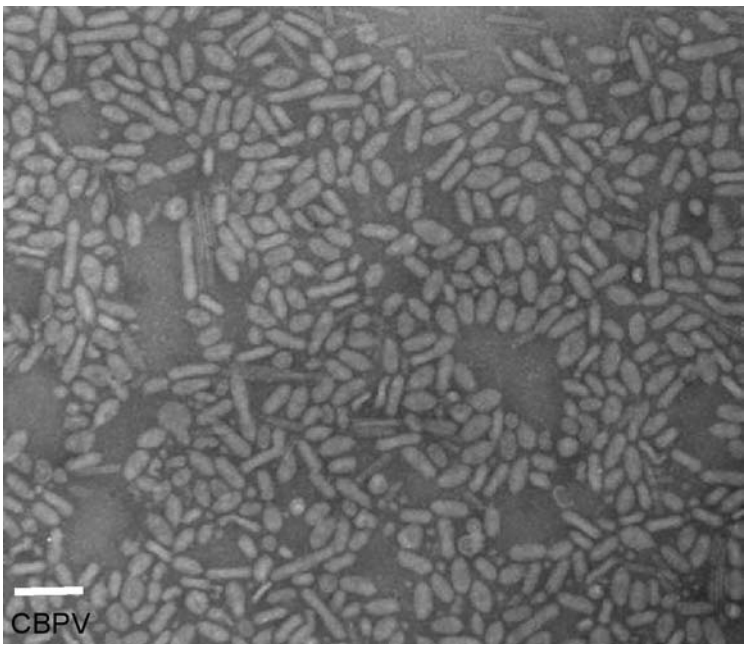


Figure 1. Electron micrograph of CBPV particles 20 x 30 to 60 nm. Scale bar = 100 nm. © B. Ball, Rothamsted Research.

Table I. Properties of honey bee viruses

Virus name	Capsid	Size (nm)	Coefficient S20W	Density in CsCl	Proteins (kDa)	Genome	Size (kb)
Filamentous virus (FV)	Ellipsoidal	450 x 150	ND	1.28	13-70*	ds DNA	20°
Chronic bee paralysis virus (CBPV)	Anisometric	20 x 30 to-60	80 to 130	1.33	23.5		4.2; 2.8; 3X(1.1)
Chronic bee paralysis virus associated particles (CBPVA)	Minute isometric	17	41	1.38	15		3X(1.1)
Cloudy wing virus (CWV)			49	1.38	19		1.4°
Acute bee paralysis virus (ABPV)		30	160	1.37	24, 33, 35		9.4
Black queen cell virus (BQCV)			151	1.34	6, 29, 32, 34		8.8
Deformed wing virus (DWV)			165	1.37	27, 45, 46**	ss RNA	10.1
Kashmir bee virus (KBV)	Isometric		172	1.37	25, 33, 36, 40, 44***		9.5
Sacbrood virus (SBV)			160	1.35	26, 28, 31		8.8
Slow bee paralysis virus (SBPV)			176	1.37	27, 29, 46		ND
Bee Virus X (BVX)		35	187	1.355	52		ND
Bee Virus Y (BVY)			187	1.347	50		ND

Coefficient S20W : sedimentation coefficient; **Density in CsCl** : buoyant density in Caesium chloride

* about 12 proteins

** three major (variable) and 2-3 minor

*** the molecular weight is variable

ds : double strain DNA size in kilobase pair (kbp)

ss : single strand RNA size in kilobase (kb)

° size deduced from the molecular weight (Bailey and Ball, 1991)

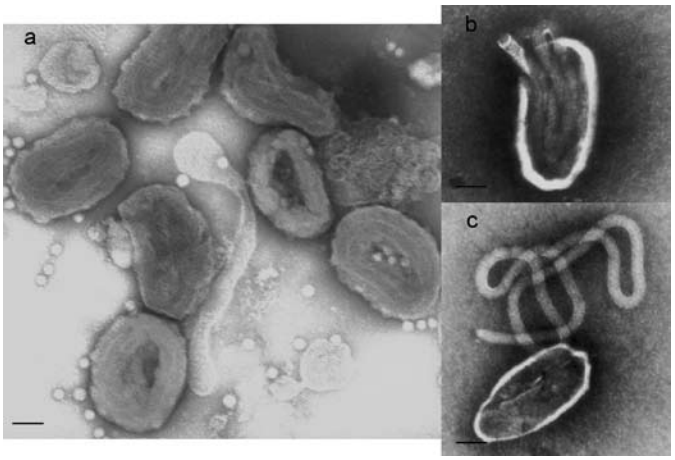


Figure 2. Electron micrographs of FV particles: (a) filamentous virus particles (b) with ruptured envelope releasing (c) the single flexuous rod, or nucleocapsid which contains DNA. Scale bars = 100 nm. © B. Ball, Rothamsted Research.

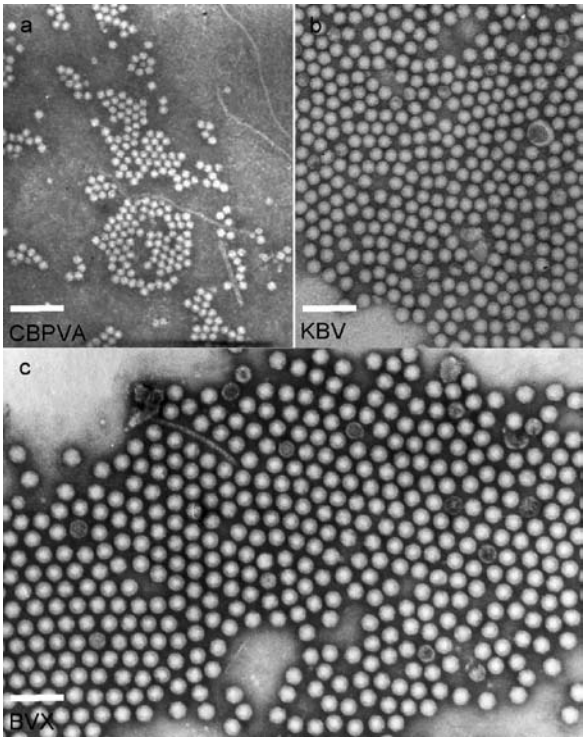


Figure 3. Electron micrographs of representative types of virus isometric non-occluded particles from bees: (a) particles 17 nm in diameter (CBPVA, CWV); (b) particles 30 nm in diameter (ABPV, BQCV, DWV, KBV, SBV, SBPV); (c) particles 35 nm in diameter (BVX, BVY). Scale bars = 100 nm. © B. Ball, Rothamsted Research.

Some bee viruses may depend on but do not invariably accompany the common microsporidian midgut parasite *Nosema apis*, and some are detected in and associated with *Varroa destructor* and may be transmitted by it. Table II summarises the possible associations and routes of transmission of these viruses.

In common with many mammalian viruses, most, if not all, of the honey bee viruses described here persist in the population as a low-level, occult or inapparent infection in live individuals (Bailey, 1965a; Dall, 1985; Anderson and Gibbs, 1988) (see Chapter 5). These infections have been called "inapparent" due to the lack of obvious symptoms or pathology in the individuals harbouring them and the fact that, in general, the amount of virus present cannot be readily detected by serological tests. However, the term inapparent is not precise; it can be applied to describe different types of viral persistence and our knowledge of these mechanisms in bees is rudimentary. The virus may be truly latent and confined within certain cells or tissues with no active replication and no disruption of cellular function, or it may be replicating at low levels in permissive cells but in non-vital sites or in honey bee life stages that do not exhibit any symptoms or obvious pathology. Many honey bee viruses commonly occur in bee populations from colonies that continue to appear healthy even when several different viruses are present (Anderson and Gibbs, 1988; Chen *et al.*, 2004c). Such low-level, chronic, persistent infections may be maintained in populations for many generations causing little or no harm, yet in certain circumstances they may be stimulated or activated to replicate rapidly or to infect sensitive stages or organs and initiate acute and often fatal infections.

Historically, inapparent infections have been revealed in so-called infectivity tests, by injecting extracts of live bees from apparently healthy colonies into other bees from the same colony (Bailey and Gibbs, 1964) or foreign proteins or large volumes of buffer solution into honey bee pupae (Dall, 1985; Anderson and Gibbs, 1988; Hung *et al.*, 1996). Whether these experimental inoculations induced or introduced the virus, the subsequent virus multiplication resulted in overt infections readily detectable in individuals by serological tests such as immunodiffusion (see Chapter 3). However, as a method to detect inapparent infections these techniques have some limitations. In the case of induction, if more than a single virus type is present the replication rate of one may result in competitive exclusion of the other (Anderson and Gibbs, 1988). Detection based on infectivity tests also excludes those viruses unable to multiply by injection into adult bees or pupae.

Until the past 15 years or so, studies on the natural incidence and

prevalence of honey bee viruses have been undertaken by the use of direct serological and indirect infectivity tests. The former are relatively insensitive and appropriate for the detection of overt, damaging infections and the latter are very sensitive but not appropriate to all viruses (Bailey, 1967; Allen and Ball, 1996) (see Chapter 3). The recent advances and more widespread use of molecular techniques and the availability of genome sequence data are now making the detection of several honey bee viruses possible at inapparent levels of infection (Ribière *et al.*, 2002; Tentcheva *et al.*, 2004b) (see Chapter 3). This will permit the detection of viruses not revealed by infectivity tests and provide information on the presence and relative amounts of several viruses simultaneously. At present, little is known of how viruses establish inapparent infections in their hosts, how they become activated, in what form they persist and where, or of interactions between viruses at inapparent levels. These different points are discussed in Chapter 5.

Many bee viruses are extremely common, and although able to cause severe and often fatal diseases they most frequently multiply, spread and persist within populations without causing obvious sickness or mortality. There are more different types of viruses than there are of the other known bee parasites and pathogens. For this reason, viruses have probably always been prime sources of confusion and error in the diagnosis and management of diseases and this could be compounded by the increased sensitivity of detection techniques (see Chapter 3). The mere presence of virus in an individual or population does not inevitably lead to an overt, damaging infection and thorough quantitative studies coupled with accurate evaluation of the pathological effects are now required to further our knowledge of the natural history, epidemiology and impact of these infections.

What follows is a review of the natural history of the twelve honey bee viruses so far identified in *Apis mellifera* in Europe. We shall present what is known or can be inferred on their mode and route of transmission, observations on their seasonality and current information on their geographical distribution. However, in most instances this highlights our lack of detailed knowledge in these fields. The effects of these viral infections on different life stages of honey bees will be described where known, but the question of their broader impact will be discussed in greater depth in Chapter 4.

Table II (part 1 of 2). The principal honey bee viruses in Europe: probable routes of transmission are indicated, susceptible life stages, consequence of infection (observed or inferred), seasonal incidence and field symptoms. Modes of transmission refer to within colony transmission only.

Virus	Modes of transmission- and association with other pathogen(s)	Susceptible life stage(s) and consequences	Seasonal incidence and field symptoms
Acute bee paralysis virus (ABPV)	Horizontal *per os.* Vectored by *V. destructor*[1]	Covert infection in adults and brood Death of brood and adults	Summer as covert infection Overt late summer and autumn with *V. destructor.* No reliable field symptoms
Black queen cell virus (BQCV)	Horizontal transmission *per os*. In workers depends on *N. apis*. In brood food to larvae.	Death of queen larvae and sometimes worker brood. Early death of adult workers	Spring and early summer with *N. apis.* No symptoms in adults, larvae fail to pupate.
Bee virus X (BVX)	Horizontal transmission *per os* often associated with *M. mellificae*	Earlier death of adults	Late winter. No symptoms
Bee virus Y (BVY)	Horizontal transmission *per os* often associated with *N. apis*	Earlier death of adults	Early summer. No symptoms
Chronic bee paralysis virus (CBPV)	Horizontal transmission by contact and *per os.* Potential vertical transmission?	Death of adults	Periodically occurring trembling symptoms and inability to fly. Black shiny bees and bees with distended abdomen.
Cloudy wing virus (CWV)	Not demonstrated but horizontal *per os* inferred. Potential vectored by *V. destructor*	Infected brood emerges normally. Earlier death of adults	No strong seasonality and no reliable symptoms

Table II (part 2 of 2).

Virus	Modes of transmission- and association with other pathogen(s)	Susceptible life stage(s) and consequences	Seasonal incidence and field symptoms
Deformed wing virus (DWV)	Not demonstrated but horizontal inferred, potential vertical transmission? Vectored by *V. destructor*	Deformation of emerging bees and earlier death of adults	Autumn and winter.Deformed newly emerging bees
Filamentous virus (FV)	Horizontal *per os*, associated with N. apis	Earlier death of adults	Spring and summer with *N. apis* and winter No symptoms
Kashmir bee virus (KBV)	Horizontal and potential vertical transmission? Vectored by *V. destructor*	Death of brood and adults	Association with *V. destructor*. No reliable field symptoms
Sacbrood virus (SBV)	Horizontal transmission *per os*. Potential vertical transmission?	Death of larvaeand earlier death of adults	Spring and early summer. Sac-like appearance of diseased larvae
Slow bee paralysis virus (SBPV)	Not demonstrated but horizontal inferred. Vectored by *V. destructor*	Death of brood and adults	No reliable field symptoms

2. Natural history and geographical distribution of honey bee viruses.

2.1. Acute bee paralysis virus.

2.1.1. Discovery

Acute bee paralysis virus (ABPV) was originally discovered during laboratory work on the identification of the causative agent of 'bee paralysis': chronic bee paralysis virus (CBPV) (Bailey *et al.*, 1963). Both viruses when fed to, sprayed on, or injected into healthy bees, caused them to become trembly and paralysed within a few days. However bees infected with ABPV die earlier (acute paralysis) than bees infected with CBPV (chronic paralysis). Bees injected with about 100 particles of ABPV usually first show symptoms of paralysis, abnormal trembling of wings and bodies within 2-4 days and then die within 1 or 2 days (Bailey *et al.*, 1963).

2.1.2. Acute bee paralysis virus before Varroa destructor

Until the relatively recent global spread of *V. destructor*, this virus had never been directly detected by serology in dead adult bees or brood, or associated with disease or mortality in nature. It was known to commonly persist in low concentrations as an inapparent infection in adult bees in Britain, especially during the summer (Bailey and Gibbs, 1964; Bailey *et al.*, 1981a; Allen and Ball, 1996). ABPV and CBPV both commonly occur at low levels in apparently healthy bees, but only CBPV particles are numerous in diseased bees from colonies naturally affected with 'paralysis' (Bailey *et al.*, 1963). In contrast to CBPV, ABPV exhibited a strong cycle of annual incidence (Bailey *et al.*, 1981a) and was not only readily detected by infectivity tests in honey bees but also in bumbles bees (Bailey and Gibbs, 1964). Bailey and colleagues (1963) observed that "*bees infected with ABPV are common*", he "*had not found a colony without some infected individuals*".

In the laboratory, about 10^{12} particles of ABPV can be extracted from a single acutely paralysed bee following injection with 10^{2} particles. The virus accumulates in the head, brain and hypopharyngeal glands of acutely paralysed bees, (Bailey and Milne, 1969) and their faeces also contain infectious ABPV particles (Bailey and Gibbs, 1964). Naïve bees fed by bees infected with pathogenic doses of ABPV did not become obviously diseased, but exhibited a temporary increase in the virus content of their tissues. About 10^{11} particles are needed to cause paralysis *per os*. However, extracts of the thoracic salivary glands of inapparently infected pollen-col-

lecting bees and of their pollen loads frequently contain sufficient virus to cause infection by infectivity tests (Bailey, 1976). In nature the virus may be spread as an inapparent infection via the salivary gland secretions of adult bees and the food to which these secretions are added (Bailey, 1976). Moreover, viral sequences have been recently detected in semen sampled from inapparently infected drones indicating that ABPV may be vertically transmitted (Yue *et al.*, 2006).

2.1.3. Acute bee paralysis virus and Varroa destructor

In contrast to the incidence of ABPV before the arrival of *V. destructor* in Europe, large amounts of virus were detected by serological tests in individual dead adult bees and diseased brood from collapsing colonies shortly after the establishment of *V. destructor* (Ball, 1985; Carpana *et al.*, 1990; Faucon *et al.*, 1992). Dead brood and adult bees from mite-infested colonies in Germany and Russia contained as much virus as bees killed by injection of ABPV in the laboratory (Ball, 1983). During late summer, frequent cases of brood disease were observed in severely infested colonies, the symptoms resembling those of American or European foulbrood depending on the stage at which the larvae or pupae died (Ball, 1983). Uncharacteristically, dead unsealed and sealed brood was often present at the same time, making diagnosis by symptoms difficult for the beekeepers, and the absence of bacteria difficult for the advisors to explain.

ABPV detection in samples of dead adult bees from *V. destructor*-infested colonies in Germany and the Netherlands was closely correlated with the degree of mite infestation. Moreover, the peak incidence of ABPV in dead bees in late summer coincided with a sharp decline in the adult bee population (Ball and Allen, 1988) and reflected the natural seasonal occurrence of inapparent infections (Bailey *et al.*, 1981a). At the same time a similar increase in overt ABPV infections was not detected in samples of dead bees from uninfested colonies in Britain. As a result, ABPV was implicated as the cause of mortality of severely infested colonies in many countries in Europe and, more widely, with the damaging effect of *V. destructor* (Ball, 1983; Allen and Ball, 1996).

Laboratory experiments demonstrated that adult female *V. destructor* could act as virus vectors transferring ABPV from infected individuals inoculated in the laboratory, to healthy white-eyed pupae (Ball, 1983; Wiegers, 1986). Some mites were able to transmit ABPV more than 36 hours after removal from an infected host and some were able to transmit to several subsequent hosts. It therefore seems unlikely that virus transmission is merely effected by contamination of the mouthparts of the parasite. However, the fact that transmission efficiency declines with

successive transfers and the lack of a noticeable latent period between acquisition and transmission, suggests that there is no virus replication in the mite. Despite the lack of evidence for viral multiplication in the mite, effective transmission of ABPV does occur. It may be that the virus is ingested with the haemolymph of systemically-infected bees and accumulates in the alimentary tract. Infection could take place by regurgitation of the gut contents during feeding, as occurs with some plant viruses transmitted by beetles (Wiegers, 1986). Only about 100 particles of ABPV are required to cause infection by injection into the haemolymph of bees but almost a million times this amount of virus was detected by indirect ELISA in individual female mites collected from naturally infested, infected honey bee colonies (Allen *et al.*, 1986). Most of this virus would be harmlessly travelling through the digestive system of the mite but a small proportion introduced into the haemolymph of other bees, or pupae, during feeding could infect new hosts.

A modelling approach to examine the role of *V. destructor* and viral pathogens in the collapse of honey bee colonies in greater detail, suggested that ABPV transmitted by mites might only be able to kill the colony if a large mite population was already present when an overt ABPV infection occurred (Martin, 2001). It was concluded that as the virus is rapidly fatal, its persistence implies that it is acquired and transmitted at a greater rate than the loss of infected hosts. However, this does not take into account the generally increased virus load in the bee population through contact and food exchange with infected individuals which could facilitate the induction of virus replication, or the great mobility of viruliferous mites between bees.

In Hungarian apiaries, ABPV was implicated in the collapse of several infested colonies at the end of summer (Bekesi *et al.*, 1999). Another 2 year survey conducted in Hungary detected only a low prevalence of ABPV in bees from seemingly healthy colonies: virus was detected in 14 out of 114 samples and only 8% of the colonies were positive (Bakonyi *et al.*, 2002). The detection of ABPV was inconsistent: some colonies and apiaries were found to be negative in one season and became positive in the next, but when few individuals in a population are virus carriers sampling errors may be considerable.

2.1.4. Geographical and temporal distribution, impact

The virus is widespread in most countries in Europe, as before *V. destructor* invasion, and is detected in live adult bees on every continent, without any associated disease or mortality (Allen and Ball, 1996). A recent study using molecular detection techniques investigated ABPV incidence

and seasonal occurrence in both honey bee and *V. destructor* populations in France. Tentcheva and colleagues (2004b) aimed to determine the prevalence of ABPV and 5 other honey bee viruses, in adult bees, pupae and mites, in 10 colonies from 36 apiaries located in different regions. In these 360 apparently healthy colonies, ABPV was found at least once in the adult bee population in 58% of the apiaries, and in pupae in 23%. However, ABPV infections were most often found in a limited number of colonies in individual apiaries: for example, ABPV was found in adult bees in approximately 15% of the colonies and only during the summer. The virus was detected in *V. destructor* in 10% of the colonies in 36% of apiaries. The virus was more prevalent in infested colonies in late summer and autumn, coinciding with the peak population development of *V. destructor,* and supporting the hypothesis that the mite has a role in transmitting this virus although the infection is not always apparent. These data indicate a wide distribution but a low prevalence of ABPV in French apiaries, probably correlated with effective virus transmission by *V. destructor*. However, ABPV was also present in bee samples from apiaries where none was detected in mites, supporting the idea that ABPV transmission also occurs between bees without the aid of the mite, as was probably always the case before the spread of *V. destructor* (Tentcheva *et al.*, 2004b).

2.1.5. Conclusion

Although ABPV seems to be widespread in honey bee colonies in Europe it is not very prevalent and has a strong seasonal incidence. Infection is generally inapparent and without any evident effect, and transmission of overt fatal infections is probably closely related to *V. destructor*. The early increase in the impact of ABPV with the establishment of the mite and the subsequent decline in virus prevalence are indicative of other changes occurring in the ecology of virus infections. These changes and some of the contributing factors are discussed at the end of this chapter.

2.2. Black queen cell virus.

2.2.1. Discovery

Black queen cell virus (BQCV) was first isolated from field collected dead queen larvae and prepupae found partially decomposed within the darkened cells in which they were being reared. Cell walls appeared almost black in patches, hence the name of the virus (Bailey and Woods, 1977). In the early stages of infection diseased larvae have a pale yellow appearance and tough saclike skin, resembling those killed by sacbrood virus (SBV, see section 2.11.) (Ball and Bailey, 1997).

2.2.2. *Pathogenicity and relationship with* Nosema apis

BQCV, like sacbrood virus, multiplies abundantly in the laboratory when injected into pupae which then fail to develop. However unlike sacbrood virus, infectivity tests on larval and adult bees usually failed (Bailey and Woods, 1977) and BQCV injected into adult worker bees or drones does not multiply to detectable levels. Again in contrast to SBV, BQCV does not readily multiply when ingested by young worker larvae or when fed alone to young adult bees. The virus was totally dependent on *N. apis* for infection of adult bees by ingestion and shortened their life more than that of adult bees infected only with the parasite (Bailey *et al.*, 1983a). Surprisingly, bees fed only washed spores also became infected with the virus. Either the spores had remained contaminated with virus particles or the young bees already contained low levels of BQCV which gained access to vulnerable tissues when *N. apis* initiated infection in the gut cells. Although the true nature of the intimate association between this virus and *N. apis* infection remains unknown, BQCV seems nevertheless more dependent on the Microsporidian for infection than the two other viruses also associated with the parasite, namely filamentous virus and bee virus Y (Bailey *et al.*, 1983a).

BQCV is intimately associated with the microsporidian gut parasite *N. apis* both in nature and in the laboratory. In Britain it follows the same regular cycle of annual incidence as the parasite, with peak infections in spring and early summer (Bailey *et al.*, 1983a). Adult bees in colonies that are used to rear queens are often older individuals and more likely to be severely infected with *N. apis*. (Allen and Ball, 1996) assume that queen larvae probably become overtly infected by ingesting food into which large amounts of the virus have been secreted by infected adult bees. In nature worker brood seems rarely to become similarly infected with BQCV, probably because such larvae receive less brood food for a shorter period of time than queen larvae and do not ingest sufficient virus to cause a fatal infection. However, the virus was detected as an inapparent infection in worker pupae from seemingly healthy Australian colonies (Anderson and Gibbs, 1988). The authors demonstrated that when honey bee pupae from seemingly healthy colonies were injected with various salt solutions or incubated at 35°C for 3 days, multiplication of BQCV could be activated to serologically detectable titres.

A very recent study using molecular detection techniques to investigate BQCV in 10 individual queens and their offspring has indicated that BQCV may be vertically transmitted. The virus sequence was detected in all queens and in all pools of 50 eggs of these queens (10/10), but only in 25% of the 100 individual larvae and in 4% of the 100 individual adults

workers analysed (Chen *et al.*, 2006b). BQCV was detected in the gut and ovaries of the queens but not in the haemolymph, spermatheca, head or eviscerated body.

2.2.3. Geographical and temporal distribution, impact

A survey of 25 colonies in Britain sampled monthly from 1977 to 1979 showed that up to 90% of spring samples of dead bees contained sufficiently large amounts of BQCV to be detected directly by serological tests, and that these samples were also infected with *N. apis*. Both virus and parasite followed the same annual cycle of incidence with an increase in infection in late winter reaching a peak in May or June and declining rapidly in August (Bailey *et al.*, 1981a). The prevalence of viruses and other pathogens in colonies found dead during late winter to early spring (about 200 examined each year from 1978 to 1982) indicated a significantly higher prevalence of BQCV in dead bees from dead colonies, than in dead bees from live colonies. The authors conclude that a combination of *N. apis* and the virus may be more harmful than *N. apis* alone (Bailey *et al.*, 1983a) (see Chapter 4).

During a one year survey conducted by Tentcheva and colleagues (2004b) using molecular techniques, BQCV was more frequently detected in adult bees (in 86% of the 36 apparently healthy apiaries) than in brood (only 23% of the apiaries). Furthermore, BQCV was detected in the majority of colonies in a given apiary, indicating the common occurrence of this virus, which was detected in up to 58% of the adult bee population during summer. This virus persisted in some of the apiaries throughout the year. However, the frequency of BQCV infection was lower in pupae, with a maximum of 2% in summer. As in previous studies, BQCV was not detected in *V. destructor*. However the sensitivity of real-time PCR has contradicted this (Chantawannakul *et al.*, 2006). Moreover, contrary to the believed absence of any association between BQCV and *V. destructor*, Davidson (2003) wrote: *"BQCV has been implicated in increased brood mortality in African honey bee colonies infested with* V. destructor. *Large numbers of uncapped cells with dead, pink-eyed pupae were seen in honey bee colonies with severe mite infestation. These pupae had amounts of virus much greater than that in healthy pupae removed from the same colonies, suggesting that BQCV replication was the cause of this mortality"* (Davison *et al.*, 2003). Unfortunately, we did not find the initial data that led Davidson to conclude to a relationship between *V. destructor* infestation and BQCV infection. BQCV does multiply after injection into pupae and it is possible that mites could acquire the virus from infected adult bees and transmit it to brood during feeding. However, the inability of BQCV to

replicate after injection into adult bees would be a limiting factor in virus spread in the bee population.

In Australia, this virus has been considered the most common cause of death of queen larvae as it was detected serologically in 19% of diseased samples and at low levels in all apparently healthy adult queens examined by infectivity tests (Anderson, 1993). BQCV was also commonly detected in honey bee colonies not showing obvious problems in Denmark (Nordström *et al.*, 1999).

In Germany in 2001, BQCV was detected by PCR in diseased drone larvae. The authors considered the damage symptomatic of BQCV infection of the drone brood (Siede and Buchler, 2003) though if this were the case, much less sensitive tests would have been adequate to detect the virus.

2.2.4. Conclusion

BQCV seems to be prevalent and widespread in honey bee colonies in Europe with a regular cycle of annual incidence in the adult population closely related to *N. apis* infection. Its transmission is largely independent of *V. destructor*. BQCV appeared to enhance the pathogenic effect of *N. apis* in laboratory experiments (Bailey *et al.*, 1983a) and both have been associated with the death of colonies. However, BQCV does not cause any visible symptoms of infection in adult bees and there is little information on which to estimate the current impact of BQCV in Europe. Reports by beekeepers of problems with queen rearing and of over-wintering losses in France (Faucon *et al.*, 2002) might be related to this virus and *N. apis* infection.

2.3. Bee virus X

2.3.1. Discovery

Bee virus X (BVX) was discovered during laboratory infection experiments with another virus, Arkansas bee virus (Bailey and Woods, 1974). Nothing is known of the natural history of this virus except that it had been isolated from adult bees from Arkansas. Large amounts of BVX were extracted from colony bees collected during the over-wintering period for laboratory use. The 35 nm size of the particles distinguished them from other bee viruses known at the time.

2.3.2. Pathogenicity and relationship with Malpighamoeba mellificae

BVX particles do not multiply when injected into adult bees or pupae, but do so readily when fed to newly emerged bees, and only when these are maintained at 30°C and not at 35°C. Experimentally infected

bees appear outwardly as healthy as uninfected control bees. In bees fed with BVX in the laboratory or from the field most particles were found in the abdomen and were concentrated in the gut (Bailey and Woods, 1974).

In common with many other honey bee viruses there are no characteristic symptoms associated with natural infections of BVX, but the life span of infected bees has been shown to be significantly reduced in the laboratory (Bailey *et al.*, 1983a). However in a 4 year study in Britain of about 200 colonies that were found dead annually in late winter to early spring, no statistically significant association of BVX with colony mortality was apparent, in contrast to other parasites and pathogens (Bailey *et al.*, 1983a) (see Chapter 4). Interestingly during this study, BVX was frequently found in association with the protozoan parasite *Malpighamoeba mellificae* in dead bees in late winter and, in laboratory infection experiments, the life spans of bees fed both parasite and virus were reduced in comparison to individuals fed one or other alone. Nevertheless, BVX is not dependent on the parasite in the same way that BQCV depends on *N. apis*: it can infect bees and occurs naturally in the absence of *M. mellificae* (Bailey and Woods, 1974). In the field the virus and parasite might become associated assuming they can be both transmitted by the same route. They could be ingested during cleaning activities subsequent to comb faecal contamination (Ball and Bailey, 1997).

2.3.3. Geographical distribution, impact and conclusion

BVX is not very pathogenic; it may multiply slowly, so that it needs the longer life of over-wintering bees to establish infection. Thus, it might be assumed that BVX is unlikely to be found in countries whose climate allows colonies to remain active and rear brood throughout the year. However, it has been detected in Australia and New Zealand where mild winters can occur and its apparent absence from Asia and most of Africa may more accurately reflect the lack of effort and ability in its recognition (Ellis and Munn, 2005).

BVX has been detected in most countries in Europe (Ellis and Munn, 2005) and, at least in Britain, it appears to be widely distributed. A study undertaken on colonies in Germany indicated that BVX incidence and prevalence was similar there to other temperate countries (Ball and Allen, 1988).

BVX is not rapidly fatal: bees experimentally infected on emergence can still live for many weeks, but their longevity is reduced, either only slightly, or more significantly if BVX is in association with other pathogens. In nature, this may become important when the longevity of adult bees is crucial for the survival of bee colonies, as in late winter (Bailey and

Woods, 1974). BVX probably accelerates the death of worker bees infected with it and with *M. mellificae* and because young bees are not reared in winter to replace these losses, infected colonies may dwindle and die in early spring (Allen and Ball, 1996).

In recent years, there have been no published reports of winter colony losses attributed to BVX infection, but few observations could have been made as the means of virus detection are not readily available. However, increasing over-wintering losses (Finley *et al.*, 1996; Faucon *et al.*, 2002) often associated with the spread of *V. destructor* infestation may have had a significant negative impact on the prevalence of this virus.

2.4. Bee virus Y

2.4.1. Discovery

Bee virus Y (BVY) was first isolated from dead adult honey bees collected in the field in Britain. The virus was extracted from adult bees found moribund or dead beneath live colonies in early summer, but the samples were part of a regular pathogen surveillance programme and no specific symptoms can be ascribed to this infection (Bailey *et al.*, 1980b). BVY is distantly related serologically to BVX, is similarly infective only by feeding and when fed bees are maintained at 30°C but not at 35°C.

2.4.2. Relationship with Nosema apis

Like BQCV this virus is intimately associated with the microsporidian parasite *N. apis* and follows the same regular cycle of annual incidence, with peak infections in spring and early summer (Bailey *et al.*, 1983a). However, in contrast to BQCV, BVY can infect young bees when fed alone to them, but more infections result when the virus is fed with *N. apis* spores. The nature of its association with *N. apis* is not known, but like BVX, the multiplication of BVY may be largely restricted to the gut of adult bees and the parasite may lower the resistance of gut cells to infection or aid entry of the virus. To a lesser degree than BQCV, BVY enhanced the pathogenic effect of *N. apis* in laboratory experiments and the presence or absence of one or both viruses may explain the large variation in virulence reported for the parasite (Bailey *et al.*, 1983a).

2.4.3. Geographical distribution and conclusion

BVY has been detected in several European countries and may be more common than documented (Ellis and Munn, 2005) since *N. apis* is prevalent throughout the continent. As with most bee viruses, data are lacking to evaluate its impact, particularly its possible contribution to the winter mortality of colonies (Bailey *et al.*, 1983a; Faucon *et al.*, 2002).

2.5. Chronic bee paralysis virus.

2.5.1. Discovery

Although the symptoms of "paralysis" were probably recognised more than two thousand years ago by Aristotle as he described hairless black bees that he called "thieves", the causative agent was not confirmed until 1963 when Bailey and colleagues isolated and characterised chronic bee paralysis virus (CBPV). Some twenty years earlier, Burnside (1945) in the USA, had succeeded in reproducing the disease in caged bees following spraying, feeding or injection with bacteria-free extracts of paralysed bees, and concluded that the responsible agent was a virus (Burnside, 1945).

2.5.2. Paralysis disease symptoms and aetiology

Paralysis symptoms of trembling, flightless clusters of bees crawling at the hive entrance have long been recognized by beekeepers (Ball and Bailey, 1997). One of these symptoms, crawling bees, was often attributed to infection with *N. apis*, *Malpighamoeba mellificae* or *Acarapis woodi* (Ball and Bailey, 1997). Bailey, (1967) demonstrated that 70% of crawling bees at the hive entrance that were not infected with these common macroscopic parasites had chronic paralysis. Moreover, some bees severely parasitized by *Acarapis woodi* also had CBPV (Bailey, 1967) and although independent, both mite and virus are contagiously transmitted and tend to increase in prevalence under similar circumstances (Bailey *et al.*, 1983b) (see section 2.5.5).

Paralysis is the only common disease of adult bees that has striking, well defined signs with two distinct sets of symptoms (syndromes) (Fig. 4) (Bailey and Ball, 1991; Ball and Bailey, 1997). Type 1 syndrome, seemingly the most common in Britain, includes an abnormal trembling motion of the wings and bodies of affected bees (paralysis). These bees fail to fly and often crawl on the ground and up the stems of grass, sometimes in masses of thousands of individuals. Frequently they huddle together on top of the cluster in the hive. They often have bloated abdomens and partially spread, dislocated wings. The bloated abdomen is caused by distension of the honey sac with fluid which accelerates the onset of so-called "dysentery". Sick individuals die within a few days following the onset of the symptoms. Severely affected colonies suddenly collapse, particularly at the height of the summer, typically leaving the queen with a few workers on neglected combs. All these symptoms are identical to those attributed to the "Isle of Wight disease" in Britain at the beginning of the century.

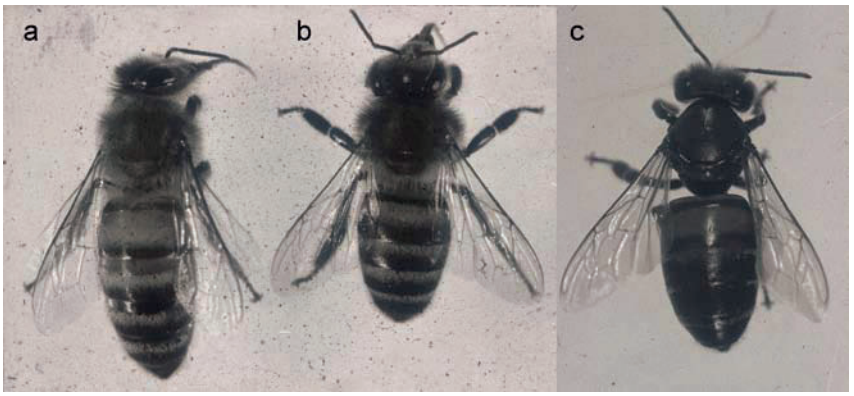


Figure 4. Bees with chronic paralysis: (a) Type 1 syndrome; (b) healthy individual; (c) Type 2 syndrome. © B. Ball, Rothamsted Research.

The other syndrome (Type 2) has been given a variety of names: "black robbers" and "little blacks" in Britain; *Schwarzsucht*, *mal noir*, *maladie noire* or *mal nero* in continental Europe and "hairless black syndrome" in the United States (Rinderer and Rothenbuhler, 1976). At first the affected bees can fly, but they become almost hairless, appearing dark or almost black, which makes them seem smaller than healthy bees, with a relatively broader abdomen. They are shiny, appearing greasy in bright light, and they suffer nibbling attacks by healthy bees of their colony, which makes them seem like robber bees. In a few days they become flightless, trembly, and soon die. Both syndromes can occur in the same colony (Ball and Bailey, 1997). On the basis of these different symptoms a visual clinical diagnosis can be made (Ball, 1999a).

During a study on the occurrence of chronic bee paralysis virus in bees outside Britain, it was clearly demonstrated that all samples of bees suffering from Type 2 syndrome contained as much CBPV as bees suffering from paralysis (Type 1 syndrome) diagnosed in Britain. In addition, CBPV and the virus causing hairless black syndrome have been demonstrated to be serologically indistinguishable (Rinderer and Green, 1976). This supports the assertion that CBPV is the essential cause of this disease and that the different signs of infection may be caused by secondary factors (Bailey, 1965b).

2.5.3. Virus transmission and paralysis

In laboratory experiments, adult worker bees are susceptible to CBPV infection by injection, topical application and feeding. Very few particles are necessary to induce paralysis by inoculation, more particles are required to infect bees by topical application and yet more particles

are necessary to infect bees *per os* (Bailey, 1965a; Ball, 1999a). The estimated infective doses corresponded to approximately 100 particles by inoculation (Bailey *et al.*, 1963), to10^7 CBPV genome copies (estimated by real-time PCR) by topical application, (Ribière *et al.*, 2004) and to more than 10^{10} particles by ingestion (Bailey, 1976).

Workers injected with CBPV usually show symptoms of paralysis about 5 or 6 days later: they seem feeble and have the same trembly movements as those occurring in naturally infected bees and they die a few days later (Bailey *et al.*, 1963; Ribière *et al.*, 2000).

Although the infective dose of CBPV by injection into adult bees is similar to that of ABPV, no similar association has been reported between outbreaks of chronic paralysis and *V. destructor* infestation (Ball and Allen, 1988) and unlike ABPV the virus has not been detected in this parasite (Ball, 1999a; Tentcheva *et al.*, 2004b; Chantawannakul *et al.*, 2006). This suggests that the contribution of *V. destructor* to the dissemination of this virus, if any, is small.

CBPV is readily transmitted to adult bees experimentally by application to the surface of cuticle freshly denuded of its hairs or when healthy bees are crowded in cages with overtly infected individuals (which induces the bees to frequently brush against each other). The virus probably leaves and invades the bodies of adult bees via the epidermal cytoplasm which is exposed when the hairs are broken (Bailey *et al.*, 1983b). Moreover, paralysed bees excrete CBPV particles in their faeces (Bailey, 1965a) and these contaminated faeces have been recently proven to provoke CBPV infection and overt disease in naive bees placed in cages previously occupied by contaminated individuals (Ribière *et al.*, 2006a; Ribière *et al.*, 2006b). The risk of virus transmission is increased by further contacts provoked by confinement during the usually active season (sudden failures of nectar flows, periods of inclement weather) or "*when too many colonies are kept for the available nectar*" (Ball and Bailey, 1997). The effects of these non-seasonal factors may explain the irregular occurrence of the disease observed in Britain (Bailey, 1967).

Infection *per os,* even less effective, may also contribute to the dissemination of this virus via food sharing. Bees infected with CBPV by injection transmitted the virus in the food that they passed to normal bees (Bailey, 1965a), but the number of particles transferred in this way was insufficient to cause disease (Bailey *et al.*, 1983b). Bailey reports that the distended honey sacs of paralysed bees can contain up to about 10^{11} particles of CBPV. Moreover, the virus is probably added to stored pollen in the same way as sacbrood virus (Bailey, 1976) (see section 2.11.2). In addition,

the sensitivity of bees to ingested virus is increased by the admixture of broken hairs to the food (Rinderer and Rothenbuhler, 1975b). This observation correlates with the fact that honey bees attacking paralysed bees were found to have ingested hairs: this behaviour may enhance transmission of the virus within the colony (Rinderer and Rothenbuhler, 1975b).

Another way that CBPV might be transmitted is vertically via the queen. Although chronic paralysis is a disease of adult bees the virus can be propagated by injecting the queen, drone pupae or adults (Ball *et al.*, 1985) or by feeding young queens (Kulincevic and Rothenbuhler, 1989). Moreover, the susceptibility of bees to paralysis seems to be related to inherited factors associated with the queen (see section 2.5.4.). Observations by Kulincevic and colleagues (1973) revealed that sealed brood removed from colonies and allowed to emerge in an incubator sometimes developed paralysis symptoms as adults irrespective of whether the original colonies showed signs of disease. These results indicate that bees may become infected at an immature stage even in apparently healthy colonies (Kulincevic *et al.*, 1973). Direct detection of CBPV using sensitive molecular techniques in queens (Chen *et al.*, 2005a; Chen *et al.*, 2006b) and in all development stages of their offspring including eggs, has recently been reported (Chen *et al.*, 2006b) which raises the possibility of vertical transmission. CBPV was detected in 4 queens out of 10, in 3 pools of 50 eggs from these queens and in 10% of individual larvae (10/100). The virus was detected in the eviscerated body of the queens and in the haemolymph, but not in the other samples (head, gut, ovaries, spermatheca and feces) (Chen *et al.*, 2006b). However, more information is required on sample origin and status (apparently healthy or symptomatic colonies) to interpret and compare the epidemiological significance of vertical transmission and horizontal transmission by contact.

Paralysis, in nature, is known to be a disease of adult workers (Ball, 1999a). Nevertheless, Bailey and Ball (1991) note that *"occasionally pupae are killed by the virus at a late stage in their development in colonies suffering severely from paralysis"*. Large amounts of virus are required to cause infection by feeding in adults, so the overt infection of brood would only occur when many individuals in the population were paralysed. A recent study using real-time PCR detection to quantify CBPV genomic loads in different categories of bees from a symptomatic hive confirmed the detection of CBPV loads in all adult bee and brood samples (Blanchard *et al.*, 2007). However the CBPV genomic load in pupae, larvae and eggs did not exceed 2×10^3 CBPV copies per individual and remained significantly lower than in adult bees: up to 1.9×10^{13} CBPV copies in guard, symptomatic and dead bees and up to 3.4×10^6 CBPV copies in forager,

drones and workers sampled inside the hive. In addition, the measured genomic loads in haemolymph samples collected from experimentally- and naturally-paralysed bees were 2×10^{10} and 4.1×10^{9} copies of CBPV per µl respectively. These results indicate that the virus can infect immature stages in symptomatic colonies, but losses of brood directly due to chronic paralysis, if any, are probably limited. Tentcheva and colleagues did not detect CBPV in samples of pupae collected in apparently healthy colonies from 360 hives in France, during spring, summer and autumn of 2002. However the virus was detected in 4% of adult bees during the summer (Tentcheva *et al.*, 2004b). The sporadic detection of this virus during the year in adults but not in brood suggests a predominant horizontal transmission, and mirrors the sporadic nature of the infection.

In conclusion, although the persistence of CBPV in colonies remains poorly understood, several results suggest that a) it might result from the transmission of a few particles between bees by food exchange, contact and even transovarial transmission, and that b) transmission by contact may be predominant during disease outbreaks.

2.5.4. Susceptibility of bees and the "queen effect"

It was suggested that susceptibility of bees to paralysis was linked with genetic differences between bees. Rinderer and Rothenbuhler, (1975a) and Kulincevic and Rothenbuhler, (1975) were able to select strains of bees seemingly more susceptible to Type 2 syndrome than usual. Moreover, research in the 1960's indicated that susceptibility to paralysis in Britain might be influenced by hereditary factors, seemingly associated with queens (Bailey, 1965a; Bailey, 1967). Indeed, Bailey (1965a) reported that overt disease rapidly disappeared when queens of naturally diseased colonies were replaced with others from apparently healthy colonies. This suggests that queens heading colonies with paralysis either transmit the virus itself (see section 2.5.3) or pass on susceptibility to chronic paralysis, or both, to their offspring.

Kulincevic and colleagues (1973) observed that paralysis symptoms occurred sooner when bees were deprived of their queen. Such bees reduce their foraging activities and also become agitated, so perhaps are suffering more physical damage than usual within the colony. However, it is difficult to distinguish between this effect and the known reduction of life span experienced by adult bees when deprived of their queen. More developments of these early results are still needed to conclude on the genetic contribution of bee susceptibility to CBPV infection.

2.5.5. Inapparent infection and factors favouring overt disease

Infectivity tests and laboratory experiments to investigate the incidence and prevalence of CBPV in Britain indicated that the virus was endemic in many apparently healthy colonies with no regular seasonal cycle of occurrence (Bailey, 1967).

At present it is possible with molecular diagnosis to detect inapparent CBPV infections in individuals without the need for complex and time consuming infectivity tests (Ribière *et al.*, 2002; Tentcheva *et al.*, 2004b). By combining serological and molecular detection it was possible to more accurately characterise the CBPV infection status of French colonies demonstrating the presence of CBPV in apparently healthy colonies and allowing to differentiate them from non infected and from overtly diseased colonies in the same apiaries (Ribière *et al.*, 2002).

Studies undertaken between 1977 and 1979 showed that the mean percentage of 25 colonies overtly infected with CBPV varied from over 50% to less than 5% during the period of investigation (Bailey *et al.*, 1981a). These infections were apparently localised in two groups of colonies suggesting that unknown environmental factors might influence disease outbreaks in apparently healthy colonies where CBPV persists. In some regions of mainland Europe outbreaks of paralysis occur when colonies are taken to forested areas to forage on honey dew (Giauffret and Lambert, 1972). The Black Forest region of Germany, for example, has been historically associated with symptoms corresponding to those caused by CBPV (Ball and Allen, 1988).

The number of colonies showing signs of paralysis declined in Britain from about 8% of samples submitted by beekeepers when records began in 1947, to less than 2% by 1966. The rate of decrease is very closely and significantly associated with the decline in number of bee colonies during the same period (Bailey *et al.*, 1983b). This also corresponds exactly with the decline of *Acarapis woodi* (Bailey *et al.*, 1982), which is similarly transmitted by close bodily contact between live bees. It contrasts with the unchanging or somewhat increasing incidences during the same period of common pathogens that do not require such contact because they have resistant spore stages (Bailey, 1985). This significant positive regression of paralysis outbreaks on diminishing numbers of colonies in England and Wales is interpreted as a result of relatively increased foraging activity by the remaining colonies which led to fewer contacts between healthy and infected individuals within enzootically infected colonies (Bailey *et al.*, 1983b). This may also help to explain the higher prevalence of the disease in areas such as the Black Forest, where colony density is very high com-

pared to other areas of West Germany or Britain (Ball and Allen, 1988).

The clustering of individuals during winter has been considered less significant for the transmission of paralysis because bees are then least active within the colony and less likely to damage each other (Ball, 1999a). This assumption does not necessarily imply that overt disease will not entail honey bee mortality during winter: as illustrated by serological detection of the virus in 19% of apiaries during honey bee mortality in the winters of 1999 and 2000 in France (Faucon *et al.*, 2002).

CBPV, like many other bee viruses, persists at a low level in live individuals without any apparent effect and disease outbreaks are sporadic. Either bees possess a natural degree of resistance to spread and multiplication of the virus under ordinary circumstances, or the means of viral dissemination may not be very efficient.

2.5.6. Geographical and temporal distribution

CBPV has been directly detected by serology in extracts of dead adult *Apis mellifera*, confirming the presence of chronic paralysis on every continent (Bailey, 1965b; Bailey, 1967) except South America, but has not been confirmed in the Caribbean region (Allen and Ball, 1996). The virus has recently been detected by molecular techniques in honey bee samples collected during December in Uruguay. The presence of CBPV was apparently associated with episodes of bee mortality but without the trembling and crawling symptoms typical of paralysis outbreaks (Antúnez *et al.*, 2005). In the absence of information on the amount of virus detected in individuals it is difficult to be certain that CBPV was responsible for the observed mortality; the virus may have been present at inapparent levels of infection and bee mortality may have had other causes.

In the mid 1990's, CBPV was detected as a cause of unusual and severe adult bee mortality in the Canary Islands. More than 75% of samples of dead adult bees collected in spring and early summer contained large amounts of virus, sufficient to be detected serologically in immunodiffusion tests. The increased prevalence of CBPV in these islands may have been associated with high colony densities or possibly with increased susceptibility to infection due to inbreeding (Ball, 1999a).

In France, CBPV has been detected by serology in extracts of dead adult *Apis mellifera,* thus confirming that overt disease can be present at any time of the year (Faucon *et al.*, 2002; Faucon *et al.*, unpublished data). However, the highest rates of bee mortality occurred in spring and summer (Giauffret *et al.*, 1967; Ribière *et al.*, 2002; Ribière, unpublished data).

Sensitive molecular detection of CBPV performed on the same colo-

nies at various periods of the year produced successive positive or negative results: apparently healthy live individuals were found to harbour CBPV in July but not in October (Ribière *et al.*, 2002). Tentcheva *et al.*, (2004b) also reported sporadic detection of CBPV over the year in adult bees sampled from 360 apparently healthy colonies in different regions of France. The virus was always detected at low frequencies (in at most 4% of colonies during summer). Despite these low frequencies, CBPV was found in adult bees in 28% of the 36 surveyed apiaries (Tentcheva *et al.*, 2004b). Thus, these molecular studies lead us to ask how CBPV persists and spreads in bee populations at such low frequencies and whether the detection of inapparent endemic infections can help to predict the probability of overt infections. Moreover, these studies based on the analysis of bees sampled inside the hive could have underestimated virus occurrence as CBPV infection seems more prevalent in the oldest bees (sampled at the hive entrance). Indeed, the CBPV genomic loads from a symptomatic colony can attain 1.9×10^{13} CBPV copies in guard and symptomatic bees sampled at the hive entrance and only 3.4×10^{6} CBPV copies in workers sampled inside the hive (Blanchard *et al.*, 2007). Thus to be representative of the whole colony, the appropriate material for studying this infection should include at least 2 samples: one of adult bees sampled inside the hive and one of adult bees sampled at the hive entrance (Ball, 1999a; Blanchard *et al.*, 2007).

2.5.7. Conclusion

CBPV is a widespread though uncommon virus infection which is probably present at low levels in a few individuals in most colonies at some time of the year. This means that it is crucial to obtain appropriate samples when studying the infection. Infected colonies are usually considered healthy by beekeepers, although they may occasionally experience the loss of many individuals from paralysis (Ball, 1999a). The virus has been detected at any season and sporadic occurrence of paralysis disease has been reported in many countries. However, the Type 1 syndrome from Britain characterised by *"severely affected colonies that suddenly collapse, particularly at the height of the summer season"*, and observations from France report that paralysis outbreaks are more prevalent in spring and summer. This may be linked with environmental factors such as unseasonal bad weather, which may favour disease development by increasing the frequency of contacts between infected and non-infected individuals, food exchange and exposure to contaminated faeces. The same situation may occur when too many colonies are kept for the available local forage as is sometimes the case for commercial honey production in intensive

agricultural areas. Other environmental factors may be involved in the development of disease outbreaks such as foraging in forests for honey dew. There is also some indication that over-wintering losses may have been associated with CBPV infection (Faucon *et al.*, 2002.; Antúnez *et al.*, 2005). Demonstrating the presence of CBPV as either an inapparent or an overt infection in honey bee colonies in Europe (Ball, 1999a; Ribière *et al.*, 2000; Ribière *et al.*, 2002) illustrate the need for further information on the real impact of the disease or a better understanding of its epidemiology.

2.6. Chronic bee paralysis virus associate.

During investigations of CBPV, which has irregular anisometric particles (Tab. I; Fig. 1), Bailey (1976) occasionally observed large amounts of a minute isometric particle about 17 nm in diameter. Most of these associated particles seemed to be localised in the abdomen of infected adults. When isolated and purified, these particles were found to be serologically unrelated to CBPV (Tab. I; Fig. 1), but contained three single stranded RNA components with similar oligonucleotide fingerprints, after digestion with T1 ribonuclease, to the three smallest RNA's of CBPV (Overton *et al.*, 1982).

When highly purified preparations of CBPV are injected into queen bees (adults or pupae) both types of particle frequently multiply in large amounts, whereas they have only occasionally been extracted in similar amounts from worker bees and only CBPV usually multiplies in pupae. CBPVA will not multiply in any type of bee when injected alone, but when added to CBPV inocula CBPVA interferes with replication of the virus decreasing the amount, particle lengths and maximum sedimentation coefficients of CBPV (Ball *et al.*, 1985). As the longest particles of CBPV appeared the most infective, CBPVA may have a role in defence mechanisms, especially of queens.

Due to its frequent association with CBPV in naturally infected bees, it was called chronic bee paralysis virus associate (CBPVA) (Bailey *et al.*, 1980a). This is the first record of a satellite virus in insects and it is classified as the only member of the subgroup *Chronic bee-paralysis virus associated satellite virus* distinct from the other subgroup of satellite viruses *Tobacco necrosis satellite virus-like* (Fauquet *et al.*, 2005).

2.7. Cloudy wing virus.

2.7.1. Discovery

Cloudy wing virus (CWV) particles were discovered in extracts of

adult bees that were being maintained in the laboratory for work on other viruses. The bees showed a marked loss of transparency of their wings and most, including the controls, died within a few days and much earlier than expected from previous experiments (Bailey *et al.*, 1980a).

2.7.2. Pathogenicity

The association of CWV infection with the loss of wing transparency and early death is inconsistent. The authors state that *"cloudy wing symptoms do not seem to be invariably or specially associated with severe infection by CWV. We have failed to detect the particles in some bees that were dead and had cloudy wings when collected in field and, in contrast, have detected many of the particles in others not showing any external signs of disease"* (Bailey *et al.*, 1980a).

There were no obvious histopathological differences between the thoracic muscle of bees infected with CWV and uninfected muscle, but particles in crystalline array were observed between sarcolemmae of fibres of infected individuals, especially in the region of the tracheoles (Bailey *et al.*, 1980a). CWV, like chronic bee paralysis virus associate (CBPVA), is the smallest viral particle isolated from honey bees being about 17 nm in diameter (Tab. I). Most CWV appears to be in the head and thorax of infected individuals as extracts of these tissues gave strong reactions to CWV antiserum in immunodiffusion tests whereas extracts of the abdomens gave no or feeble reactions. However, little more is known of the multiplication regions of CWV as the small size of these particles makes their identification uncertain in ultrathin tissue sections except when they form crystalline arrays (Bailey *et al.*, 1980a).

The simultaneous occurrence of cloudy wings and early deaths in all of many groups of caged bees in one incubator but not in parallel groups in another and the accumulation of crystalline arrays of virus in the thorax where the main inhalatory spiracles occur, suggested that infection might be airborne over a short distance (Bailey *et al.*, 1980a). However, numerous laboratory infection tests have given equivocal results. In attempts to infect caged bees by spraying them or rubbing their thoraces with semi-purified preparations of CWV all the inoculated individuals died after incubation periods of 10 to 14 days and contained much virus (Bailey *et al.*, 1980a). Other seemingly identical attempts failed and CWV did not multiply when fed to caged bees nor when injected into adult bees or pupae. Possibly, unknown factors that are essential for infection or pathogenesis may have been eliminated from some preparations in these experiments (Bailey *et al.*, 1980a).

An alternative explanation for these earlier results is that CWV persists as an inapparent infection in some colonies. Indeed, the testing by ELISA of several hundred individual larvae, prepupae and pupae from a number of colonies throughout the year showed that CWV was a very common infection in honey bee brood. Although the tested brood came from colonies infested with *V. destructor* CWV infection was as prevalent in non-infested brood as in brood from cells in which mites were present (Ball, unpublished data). This suggests that larvae acquire the virus from the food secreted by infected adult bees or that the virus may be vertically transmitted. Moreover, the brood continues to develop and emerge, though the life-span of individuals may be reduced. In some colonies CWV was detected in large amounts in 30% to 80% of the brood and adult bees throughout the year, which implies that the virus is not highly pathogenic.

2.7.3. Geographical distribution and impact

CWV seems to be widely distributed throughout Europe and it has been detected on every other continent (Allen and Ball, 1996). In Britain, CWV was detected serologically in the extracts of dead bees collected from about 15% of colonies, but this fluctuated widely during the year, although there was no apparent seasonal cycle of incidence (Bailey *et al.*, 1981a).

Studies on pathogen incidence in colonies infested with *V. destructor* soon after the mite was first detected in England, showed that CWV was more prevalent in infested colonies than in mite-free colonies elsewhere in Britain (Ball, 1997). The virus was detected by serology in a large percentage of dead adult bee samples from all study colonies throughout the season and in pupae during the summer. It seemed that CWV might be transmitted by the feeding activities of *V. destructor* as it was detected for the first time in infested pupae and in individual mites. Later observations proved this assumption to be false (see previous section). The occurrence of CWV seemed to be independent of mite infestation levels, although it was associated with the death of some of the study colonies in Britain (Ball, 1997) and in the former Yugoslavia (Martin *et al.*, 1998). Similarly, during a study of virus infections in colonies in Nordic countries (Denmark, Finland, Norway, Sweden), CWV was the most prevalent virus in all apiaries irrespective of the mite infestation level. Despite its high prevalence, it was not associated with colony collapse (Nordström *et al.*, 1999).

2.7.4. Conclusion

Little is known of the means of persistence and spread of CWV infection in nature and field data on the real impact of the virus are lacking.

However, the common occurrence and prevalence of this infection in both brood and adult bees suggests that it is not highly pathogenic, although colonies in which most individuals are infected may become inactive and die (Ball and Bailey, 1997). The symptom of cloudy wings is unreliable for diagnosis and the association with *V. destructor* has not been substantiated.

2.8. Deformed wing virus.

2.8.1. Discovery and aetiology of deformed wings

Deformed wing virus (DWV) was initially isolated from adult honey bees from Japan, from colonies infested with *V. destructor* (Ball, 1983). It was subsequently identified as a cause of brood and adult bee mortality in *V. destructor* infested colonies in many countries (Ball, 1983; Ball, 1989b; Bailey and Ball, 1991). It was first called Japanese strain of Egypt bee virus (EBV) because of its distant serological relationship with this virus (Ball, 1989b), but after the production of an antiserum it is now considered sufficiently different to be a distinct virus type (Ball, unpublished data). EBV has been detected only in adult bees from Egypt (Bailey *et al.*, 1979), and nothing is known of its natural history (Ball and Bailey, 1997).

The name given to the virus originates from the characteristic symptom of deformed or poorly developed wings in the newly emerged bees from affected colonies (Fig. 5) (Ball, 1993). Wing deformity and reduced size of emerging adult bees were previously attributed solely to the feeding activities of *V. destructor* (Akratanakul and Burgett, 1975; Koch and Ritter, 1991). Indeed, these symptoms only became evident after the mite became established indicating the strong association between the mite and the deformities. However, bees emerging from cells with very few or occasionally no mites, can exhibit the same deformity as highly parasitized bees, while highly parasitized bees without any deformity have also been

Figure 5. Characteristic symptom of deformed or poorly developed wings in a newly emerged bees with the *Varroa destructor* from the same cell.
© J.P. Faucon, Afssa Sophia-Antipolis.

recorded (Marcangeli *et al.*, 1992; Ball, 1993). This strongly suggested that mite parasitization was not the primary cause of the wing malformation. In fact, there is a systematic association between deformed honey bees and DWV infection (Bowen-Walker *et al.*, 1999; Chen *et al.*, 2005b; Tentcheva *et al.*, 2006) and Allen and Ball, 1996 indicate (without further details) that they have experimentally confirmed the aetiology of this symptom: "*injection of DWV into pupae in the laboratory can cause the wing malformations that have been attributed to parasitization*" (Allen and Ball, 1996). Moreover, the virus has also been detected in non infested bumble bees (*Bombus terrestris, Bombus pascuorum*) exhibiting wing deformities (Genersch *et al.*, 2006). Nevertheless, bees with wing deformity do not systematically contain the largest amounts of virus and highly infected bees do not always have deformed wings (Chen *et al.*, 2005b; Tentcheva *et al.*, 2006). Indeed, no physical signs of infection are apparent when bees are infected post-emergence.

2.8.2. DWV infection, pathogenicity and Varroa destructor *infestation*

Since the discovery of DWV in bees with deformed wings, evidence has been accumulated that the virus is closely associated with *V. destructor* (Ball, 1989b; Bowen-Walker *et al.*, 1999; Nordström *et al.*, 1999).

The virus is widely distributed and prevalent in colonies infested by the mite. In samples collected from two severely infested colonies in the USA, DWV was detected in 92% of the mites (22/24), in all of the 24 adult worker bees with deformed wings, 92% of the pupae (22/24), 80% of the larvae (24/30), 75% of the apparently normal worker bees (18/24) and 47% of the adult drones (14/30) (Chen *et al.*, 2004b; Chen *et al.*, 2005b). The close association between *V. destructor* infestation and DWV infection was confirmed in pupae collected from the sealed cells of the same infested colony. Pupae from cells with mites had significantly larger amounts of DWV than pupae from non-infested cells, and the number of mites within the cell was positively correlated with the incidence of morphological deformity and death (Bowen-Walker *et al.*, 1999), and with the amount of DWV detected in individual pupae (Shen *et al.*, 2005b). Interestingly, Shen *et al.*, (2005b) using dot blot analysis to quantify DWV in a 2-year-old colony highly infested with *V. destructor,* detected DWV in 78% (36/46) of mite infested pupae and never in mite-free pupae (0/36). The complete absence of DWV detection in mite-free pupae from a highly infested colony is contradicted by studies using real-time PCR (Tentcheva *et al.*, 2006) (Chen *et al.*, 2005b). However, this could reflect the different virus yields in the colonies: the one studied by Shen and colleagues *"exhibited little or no symptoms of viral infections"* (with no details given on these symptoms)

in contrast to the above mentioned studies where symptomatic deformed bees were analysed. In any case, a comparison of such studies will require further information on the origin and status of the colonies, the detection threshold of the technique and the protocol used.

DWV, like ABPV, has also been detected in individual *V. destructor* females and the ability of the mite to transmit the virus from severely infected to healthy brood during feeding activities has been demonstrated experimentally (Ball, 1989b; Bowen-Walker *et al.*, 1999; Nordström, 2003; Shen *et al.*, 2005b).

The results correlating the detection of DWV within *V. destructor* to the virus content of host bees are further supported by the work of (Bowen-Walker *et al.*, 1999). These authors confirmed a highly significant association between bee deformity or bee DWV infection and the presence of DWV in the parasitising mites. Moreover, Tentcheva *et al.*, (2006) demonstrated that the amounts of DWV recorded in drone prepupae and mite samples from the same cell were positively correlated to the number of mites in the cell. Other work by (Nordström *et al.*, 1999) reported that the majority of mites feeding on pupae that would later emerge with deformed wings, contained DWV. The authors concluded that "*both adult and immature mite progeny most likely acquire DWV from DWV-infected host bees and not from their mother mite*" (Nordström *et al.*, 1999).

There is some circumstantial evidence that *V. destructor* feeding may induce or activate viral replication (Ball, 1983; Bailey and Ball, 1991) (see Chapter 5). Although the biochemical components of mite saliva have not yet been characterized, the introduction of foreign proteins during mite feeding may suppress both humoral and cellular immune responses of honey bees. Parasitization by *V. destructor* may have an immunosuppressant effect on honey bees by depressing the expression of immunity-related genes (Gregory *et al.*, 2005). DWV viral RNA titres have been shown to be negatively correlated with the expression of immunity-related enzymes (Yang and Cox-Foster, 2005). Likewise, the immunosuppressive effect of mite saliva may contribute to the activation of latent persistent viral infections in honey bees.

The acquisition and transmission of viral particles, after activation of viral replication and the establishment of systemic virus infection in bees, is facilitated by the ease of movement of mites between different life stages and different individuals within the bee population.

It seems unlikely that DWV transmission is effected merely by contamination of the mouthparts of the mite because individual *V. destructor* serially transferred on to healthy white eyed pupae up to four days

after removal from an infected host were able to transmit infection (Ball, 1989b). It has been suggested that the virus ingested with the haemolymph of the bee accumulates within the gastric caeca of the mite and that transmission of infection could take place by regurgitation of the gut contents just before feeding (Wiegers, 1986). In this instance DWV, or indeed any other honey bee virus detected in *V. destructor*, merely "reflects" the established viral infections in the bee population. The prevalence of DWV in individual *V. destructor* in autumn (98%), which is greater than the prevalence of the virus in pupae (54%) or in adult bees (85%) sampled during the same period (Tentcheva *et al.*, 2004b), might result from the accumulation and concentration of haemolymph meals taken from several infected hosts throughout its life.

Alternatively, others have suggested that this indicates that DWV may be replicating in *V. destructor*. Apparently the amounts of virus contained in adult female mites obtained from honey bee pupae naturally infected with DWV were many times in excess of those found in their host and beyond that which might be expected from a concentration effect (Bowen-Walker *et al.*, 1999)However, there are limitations in quantifying an unstable particle such as DWV by ELISA which makes the estimation of virus yields more than usually unreliable (Ball, unpublished data). Nevertheless, further support of DWV replication in *V. destructor* has been provided by the detection of negative sense DWV RNA in the parasite using a selective RT-PCR (Ongus *et al.*, 2004; Yue and Genersch, 2005). Work by Ongus and colleagues (2004) implies that both DWV and the very similar *V. destructor* virus can replicate in mites but the evidence for two different viruses seems slight and intra cellular replication of either virus in mites has yet to be unequivocally proven. Yue and Genersch (2005) also demonstrated the multiplication of DWV in mites and this detection was shown to be correlated with the occurrence of wing deformity in the parasitized host. Moreover, electron microscopy studies of *V. destructor* tissue (in histological sections) showed "*accumulations of para-crystalline structures of 27 nm picorna-like virus particles scattered in the cytoplasm giving the apparent indication of a virus replicating in the mite*" (Ongus, 2006). However, using immunohistochemmistry DWV antigen accumulation was observed only in the midgut lumen of *V. destructor* (Santillan-Galicia, 2006). Thus localisation of DWV replication site in histological sections needs further investigations.

Nevertheless, *V. destructor* is predominantly implicated in DWV transmission. The close relationship between *V. destructor* infestation and DWV prevalence in honey bee colonies has also been observed in France. In their survey of 360 colonies, Tentcheva and colleagues (2004 a and b)

established that DWV and *V. destructor* were present everywhere except on Ouessant Island. This island has remained mite-free because no new bee colonies have been introduced since the early eighties. Young larvae were introduced for queen production but only after careful checking that no mites were present with the brood. Considering the prevalence and widespread distribution of DWV in French apiaries, these larvae probably originated from DWV infected colonies. It is likely therefore that they were inapparently infected with the virus, but in the absence of *V. destructor* or other triggering factors, this apparently did not result in the establishment of the virus on the island. This suggests that DWV might not be vertically transmitted (Tentcheva *et al.*, 2004a).

It has been stated that until induced to multiply and vectored by the mite, DWV may not cause noticeable pathology and virus titres may remain low or undetectable (Bowen-Walker et al., 1999) However, overt infections of DWV occasionally leading to queen and colony mortality in Britain and South Africa were (serologically) detected before the mite became established, so transmission routes other than by mites must exist (Ball, 1989a; Ball, 1989b). Interestingly, in 1963, before the aetiology of deformed wings had been recognised and before the arrival of V. destructor in Europe, Fyg published photographs of queens with such deformities in a review on abnormality and queens' diseases, and explained that similar malformations could be observed on workers in spring (Fyg, 1963).

In addition, vertical transmission through the queen is implied by the molecular detection of virus in all developmental stages including eggs (Chen *et al.*, 2004b; Chen *et al.*, 2005b; Chen *et al.*, 2006b). DWV was detected in the whole body and organs of queens (gut, spermatheca and ovaries) (Chen *et al.*, 2005a; Chen *et al.*, 2006b) and recently the detection of DWV was reported in sperm sampled from drones (Yue *et al.*, 2006). Moreover, DWV was also detected in larval food (Yue and Genersch, 2005) indicating a possible horizontal oral route of virus transmission.

These observations indicate that although DWV transmission and wing deformities are strongly associated with parasitization, this association is not an absolute rule. Indeed, wing deformity is probably dependent on infection at a specific life stage and/or of particular tissues as post-emergence morphological changes are unlikely (Bailey and Ball, 1991). Interestingly, a recent study reports the molecular detection of DWV in 100% of both deformed and asymptomatic bees from mite infested colonies but a significant difference in virus localisation, DWV being detected in all body parts of deformed bees, but never in the heads of symptomless bees (Yue and Genersch, 2005).

Moreover, DWV contamination at the right stage, even in association with *V. destructor,* may be insufficient to systematically induce deformities as not all bees from a colony with highly DWV infected pupae (Chen *et al.*, 2006a) and from infested and infected colonies (Tentcheva *et al.*, 2004a; Yue and Genersch, 2005) systematically developed abnormalities. Virus titre might also be important. Indeed, mite infested deformed bees invariably had DWV ELISA optical densities considerably in excess of those of mite infested apparently healthy bees from the same colony (Bowen-Walker *et al.*, 1999). In other infested colonies, deformed bees contained at least 10 to 100 times more DWV particles than symptomless ones (Tentcheva *et al.*, 2004a). However, Chen and colleagues (2005b), using real-time RT-PCR found a maximum difference of only 1 logarithm in the amount of DWV between deformed bees and the less infected apparently healthy individuals (adult drones) and similar virus amounts in deformed bees and in apparently healthy pupae collected from two colonies heavily parasitized with *V. destructor* in the USA (Chen *et al.*, 2005b). Surprisingly the authors considered these close amounts of virus as significantly different.

In conclusion, other routes of transmission and complementary factors beneficial to *V. destructor* must exist which contribute to DWV infection dynamics that may or may not induce deformities. Observations and experiments have demonstrated that, in a mite infested colony, a small proportion of pupae infected with DWV at a specific stage of development (perhaps the white-eyed stage) may die, some will develop into short lived adult bees showing a reduction in emergence size, shortened abdomens and characteristic morphological deformities (DeJong *et al.*, 1982; Bailey and Ball, 1991; Bowen-Walker *et al.*, 1999), but most will emerge as apparently normal, but overtly infected individuals. Because most infected pupae continue their development and emerge, it was assumed that the virus multiplied slowly (Ball, 1993), but this is not true as pupae (some at white-eyed stage) were found to contain as much virus as deformed bees (Chen *et al.*, 2005b; Tentcheva *et al.*, 2006). The large amounts of virus in newly emerged individuals, the large proportion of severely infected individuals in the bee population resulting in a small number of deformed bees in infested colonies, and the almost ubiquitous occurrence of DWV, all indicate that the pathogenicity of this virus is either low or insidious.

2.8.3. Geographical and temporal distribution, impact

DWV has been detected in honey bees from colonies in Europe, Africa, Asia (Allen and Ball, 1996) and North and Central America (Ellis and Munn, 2005). It is the most prevalent virus infection in colonies in the UK, being detected in 94% of mite samples in a national survey (Ball, 2001)

and where it has also been linked to colony collapse (Martin *et al.*, 1998).

During a study of virus infections in colonies in the Nordic countries, the serological detection of DWV was associated with colony collapse in one apiary. Severe infestation by *V. destructor* was indicated by an average daily mite mortality of more than 60 in that apiary. Both live and dead bee samples always gave positive reactions to DWV antiserum (in immunodiffusion tests) indicating large amounts of virus (see Chapter 3). However, DWV was similarly detected serologically in bees from colonies with lower mite infestation rates (Nordström *et al.*, 1999).

In the 360 colonies analysed for viruses by molecular techniques in France, DWV was by far the most frequently detected virus, both in adult bee samples (97% of apiaries) and in pupae (94% of apiaries). The virus was also detected in 98% of *V. destructor* samples. A progressive increase was observed throughout the year both in the numbers of colonies and the percentage of bees within them, in which DWV was detected. The seasonal variations in DWV incidence ranged from 16% to 54% in pupae and from 56% to 85% in adult bees from spring to autumn (Tentcheva *et al.*, 2004b). The greatly increased prevalence of DWV described in the UK and France is probably reflected all over Europe. Certainly in Poland, DWV was detected serologically in 69 % of samples of dead but non deformed bees collected from mite-infested colonies in mid-summer (Topolska *et al.*, 1995).

In the theoretical modelling approach of host-parasite-pathogen interactions described by Martin (2001), simultaneous infestation by *V. destructor* and infection by DWV has little or no impact on the colony in the early stages of infection. However, during late summer, as both the mite population and the number of mites that are vectors of DWV increase, the proportion of infested and infected brood also increases. Most pupae infected with DWV during their development emerge as apparently normal adult bees but their longevity is reduced. This creates an imbalance in the age structure of the over-wintering bee population which could result in eventual death of the colony during winter or spring (Martin, 2001; Ball, 2001). However, some authors stating that usually only a few bees in colonies severely infested by *V. destructor* display deformed wings, consider that DWV is poorly pathogenic (Tentcheva *et al.*, 2004a; Tentcheva *et al.*, 2004b). This view seems to be at odds with the increasing evidence that DWV replication not only causes losses of deformed emerging bees but also reduces the life span of young bees and may be implicated in losses of brood and adult bees which are important parameters under critical conditions (Allen and Ball, 1996; Genersch, 2005).

2.8.5. Conclusion

Despite the almost ubiquitous occurrence of DWV in colonies infested with *V. destructor* throughout Europe, the virus has not yet been detected in some areas of the world. Studies in New Zealand in 2001 and 2002 revealed that DWV was neither detected in living bees sampled in colonies before demise nor in dead bees (Ball *et al.*, 2005). Thus, colonies may collapse when they are infested by *V. destructor*, even in the absence of DWV. Conversely, in the absence of the parasite, DWV has been implicated in losses of queens, brood, adult workers and even colonies in Britain and South Africa (Allen and Ball, 1996). The spread of *V. destructor* has been implicated in a dramatic increase in the prevalence of DWV (Allen and Ball, 1996) and it is likely that this virus will become predominant in infested areas. Although there is increased interest and capability in the detection of DWV, more information is required to fully understand the contribution of DWV infection in association with *V. destructor* to colony decline. The role of this parasite in DWV transmission and the appearance of wing deformities in newly emerged bees also deserve more intensive investigation.

2.9. Filamentous virus.

2.9.1. Discovery

Filamentous virus (FV) is the only DNA virus known to naturally infect *Apis mellifera* (Bailey *et al.*, 1981b). It was first identified in the milky white haemolymph of adult bees in the USA (Clark, 1977; Clark, 1978). Some of these bees exhibited the signs and symptoms of the bee rickettsiosis described by Wille (Wille and Pinter, 1961; Wille, 1967). The crawling bees on the ground beneath the entrance to the hives had milky white haemolymph which Wille described as densely filled with small bodies that could only be seen with phase contrast microscopy at a magnification of at least 1000x. Wille referred to this blood disease as "Anomaly-R" and reported having found it often in crawling bees and in bees infected with other disease agents. However, later observations by Clark (1977) confirmed that the etiological agent was not a *Rickettsia* but a filamentous virus.

2.9.2. The virus and its properties

Clark (1978) described milky haemolymph that contained many particles whose size was near the limit of resolution for light microscopy. These particles ranged from almost spherical to rod shaped and contained filaments considered to be nucleocapsids of virions (Fig. 2). Although viri-

ons with folded nucleocapsids were present in all the examined tissues, virogenesis was seen only in fat body and ovarian tissues (Clark, 1978). Newly emerged workers injected or fed with the viral suspension began to die 5 days after treatment, after their haemolymph became opalescent (Clark, 1978). The physicochemical properties of the filamentous virus were later described by (Bailey *et al.*, 1981b) (Table I).

2.9.3. Pathogenicity and relationship with Nosema apis

Particles could multiply when concentrated preparations were injected into adult bees but infected bees appeared outwardly as healthy as uninfected control bees (Bailey *et al.*, 1983a). Attempts to infect newly-emerged adult bees by feeding with FV alone failed and Bailey et al (1983a) demonstrated that infectivity of FV *per os* was associated with *Nosema apis*, but that there was no difference between the life spans of groups infected with both FV and *N. apis* or with *N. apis* alone. Infection is symptomless in adult honey bees and the virus appears less harmful than either black queen cell virus or bee virus Y, both of which are also associated with *N. apis*.

2.9.4. Geographical and temporal distribution, impact

Particles similar to the ones described by Clark (1978) were seen in ultrathin sections of tissues of bees in Britain (Bailey and Milne, 1978) and Italy (Vidano and Arzone, 1978). The virus has been detected by electron microscopy in extracts of bees from most continents (Allen and Ball, 1996; Ellis and Munn, 2005), although its apparent absence from South America and Canada should be viewed with caution, given the distribution of *N. apis* and the close association of the virus with this gut parasite (Bailey *et al.*, 1983a).

Surveys in Britain have shown that FV is probably the most common but the least pathogenic of all the honey bee viruses isolated (Bailey *et al.*, 1983a). FV particles were abundant only in individuals infected with *N. apis*. The peak of FV infection, in colonies sampled on a regular basis over three years, was 80% during spring and early summer, falling to 10% or less during winter, which coincides with the annual incidence of *N. apis* (Bailey *et al.*, 1983a). A one year study in the Netherlands and Germany gave considerably lower mean percentages of infection: approximately 15% and 20% of colonies respectively compared to the mean percentage of 50% of colonies in Britain (Ball and Allen, 1988). However, the virus was more common in dead adult bee samples collected during September throughout Southern Finland than in samples from colonies in Britain collected at the same time of year (Varis *et al.*, 1992). During the winter of 1985-86

the loss of large numbers of hives exhibiting crawling bees was reported in the northern part of Greece (Sitaropoulou *et al.*, 1989). Although colony mortality was attributed to FV infection, the wide distribution and common occurrence of this virus suggest that the infection simply coincided with the primary cause of the losses, which remained unidentified.

2.9.5. Conclusion

Like other bee viruses, the pathogenicity of the filamentous virus in nature has been viewed very differently. In Britain it is considered the least harmful of all the honey bee viruses, but in Greece it was said to be responsible for episodes of severe mortality. This underlines the need for more detailed field studies of this and other viruses, including tests for all other known parasites and pathogens. Such investigations should also be supported by laboratory infection experiments.

2.10. Kashmir bee virus.

2.10.1.Discovery

Kashmir bee virus (KBV) was discovered in 1974 as a contaminant in preparations of *Apis* iridescent virus extracted from the Asian hive bee *Apis cerana* sent from northern India (Bailey and Woods, 1977). Adult *Apis mellifera* bees died when injected with these particles, which the authors called Kashmir bee virus after the area from which the bees originated (Bailey and Woods, 1977). However, serologically related strains of KBV were later detected in *A. mellifera* from Australia (Bailey *et al.*, 1979).

2.10.2. KBV a related but distinct viral species to ABPV

KBV is serologically, biologically and genetically related to ABPV (Anderson, 1991; Allen and Ball, 1995; Evans, 2001; de Miranda *et al.*, 2004). However, the VP4 proteins of ABPV and KBV particles are serologically distinct (Stoltz *et al.*, 1995), the capsid protein profiles differ (Allen and Ball, 1995), and the viruses can be readily distinguished by RT-PCR (Evans, 2001). Finally, phylogenetic analysis confirms that KBV and ABPV are close but distinct viral species (de Miranda *et al.*, 2004) (see Chapter 2). Serological and molecular characterisations indicate that KBV strains are a heterogeneous group (Allen and Ball, 1995; Hung *et al.*, 2000; de Miranda *et al.*, 2004) (see Chapter 2).

2.10.3. Pathogenicity

In the laboratory, KBV like ABPV, is rapidly lethal to adults and pupae by injection and in larger doses by feeding to newly emerged bees

and larvae. However in contrast to ABPV, KBV does not provoke typical symptoms of paralysis when experimentally injected in adult bees. KBV seems the most virulent of all known honey bee viruses as few particles are required to infect both adult bees and pupae by injection and the virus multiplies rapidly causing death within three days (Bailey *et al.*, 1979). The LD_{50} of KBV by injection into pupae has been calculated to be 35 particles, with virus yields reaching their highest level within 24 h of inoculation and host eye pigmentation ceasing development 48 to 60 h post-inoculation (Dall, 1985; Dall, 1987).

Anderson and Gibbs (1989) reported that when diluted preparations of KBV particles were fed to larvae the resulting pupae continued their development and emerged as inapparently infected adult bees. Moreover, in the laboratory natural inapparent infections can be activated to multiply to lethal levels by injecting the larvae with various salt solutions or foreign proteins, or by incubation at 35°C for 3 days (Bailey and Gibbs, 1964; Dall, 1985; Anderson and Gibbs, 1988; Hung *et al.*, 1996).

In nature, KBV has been detected in dead adult bees and brood collected from different areas of Australia, in amounts sufficient to have caused mortality (Bailey *et al.*, 1979) but overt KBV infection has never been associated with any specific clinical symptoms (Hornitzky, 1987).

2.10.4. KBV transmission with and without Varroa destructor

The activation of KBV replication by experimental injection of various solutions or proteins (see previous paragraph) suggests a possible role of *V. destructor* in the initiation of KBV replication by the introduction of foreign proteins during feeding or by disrupting the normal immunosuppressive mechanisms as described above for DWV (Yang and Cox-Foster, 2005). Furthermore, *V. destructor* may transmit KBV between adult bees and to pupae as the virus has been detected in the parasite (Hung and Shimanuki, 1999; Hung *et al.*, 2000; Chen *et al.*, 2004a; Shen *et al.*, 2005b) and in its saliva (Shen *et al.*, 2005a). When mites carrying KBV were transferred to healthy pupae from non infected colonies, 70% of the pupae became infected with the virus, indicating its successful transmission by mites (Chen *et al.*, 2004a).

The strong association between *V. destructor* and KBV infection has been shown not only under experimental conditions, but also in nature. KBV RNA (evaluated by dot blot and analysis of hybridisation signals) and structural proteins (evaluated by ELISA) were found in significantly greater amounts in pupae from mite infested cells than in pupae from non infested cells. Moreover, the number of mites present in individual brood cells and the amount of KBV RNA in the corresponding pupa were highly

correlated (Shen *et al.*, 2005b). However, the detection of KBV by RT-PCR, in adult female mites and in bee pupae from the same sealed cell did not always correspond: KBV was detected in 3 adult mites from 7 non-infected bee pupae and in only 3 adult mites from 12 parasitized pupae infected with KBV. Residual KBV in the mite gut from a previous meal on an infected host could explain the former. However, KBV has not been successfully detected in mite-free pupae which could have explained the latter. Moreover, the reported detection of KBV in *V. destructor* nymph and its absence in adult mites and in the bee nymph (all taken from the same cell) question the origin of the virus and require further investigation.

Other possible routes of KBV transmission do not involve *V. destructor*. Horizontal transmission is very likely between adult bees and from adult workers to larvae, either through contaminated faeces when bees clean the hive (Hung, 2000) or through contaminated food: the virus has been detected in all food sources (brood food, honey, pollen and royal jelly) and in all larval stages (Shen *et al.*, 2005a). A transovarial route has also been suggested as KBV RNA was detected by RT-PCR in queens (Chen *et al.*, 2005a) and their eggs (Shen *et al.*, 2005a) but not in their offspring larvae and adults (Chen *et al.*, 2006a).

2.10.5. Geographical distribution and host origins of KBV

The geographical distribution and host origins of KBV are still a matter of discussion. Initially, direct serological detection of KBV in three samples of diseased adult *Apis cerana* from India in amounts sufficient to have been responsible for their mortality, indicated that the virus was an established infection in this bee species (Bailey *et al.*, 1979). Later, other observations revealed that KBV was not only a natural infection in *Apis cerana* but also in *Apis mellifera*. Serologically closely related strains of KBV were subsequently detected in dead adults, larvae and prepupae of *Apis mellifera* from South Australia, New South Wales and Queensland, and other KBV strains were later isolated from dead adult bees in New Zealand, Canada, Fiji (Anderson, 1985; Anderson, 1990).

Although Australian honey bees are descendants of bees imported from Europe and North America, there was no evidence at this time that KBV occurred naturally in *Apis mellifera* in Europe (Bailey *et al.*, 1979). Moreover, in contrast to many of the bee viruses that exhibit little variation in protein profiles between isolates from different countries, KBV isolates from the same region were highly variable (Allen and Ball, 1995). This could be indicative of the adaptation of KBV to a new host. The absence of *Apis cerana* from Australia could indicate either that KBV had been derived from other insect species native to both Australia and south-

east Asia, or that strains of KBV occur naturally in *A. mellifera* but might multiply more in these regions than in others due to environmental factors (Bailey *et al.*, 1979). Bees of the genus *Trigona*, common to Australia and south-east Asia, were examined for KBV but the virus was not found in such colonies (Anderson, 1991) and indeed KBV can persist within *A. mellifera* colonies without depending on re-infection from other insect species (Dall, 1985). Interestingly, Anderson (1991) indicated that KBV has been found in *Vespula germanica* colonies. However, as no information was given on the geographic origin of the samples this observation provides no clue as to whether the origin of KBV would be European or Australasian. As a matter of fact, the German wasp is native to Europe, North Africa and Asia, but reached Tasmania in 1959 (Crosland, 1991), and soon became well established in New Zealand and Australia (Clapperton *et al.*, 1989).

Subsequently, KBV has been detected in field collected adult *Apis mellifera* from Canada, the USA and Spain (Stoltz *et al.*, 1995; Bruce *et al.*, 1995; Allen and Ball, 1995), and from Africanised honey bee colonies infested with *V. destructor* in Costa Rica (Calderon *et al.*, 2003). Infectivity tests (Bruce *et al.*, 1995; Hung *et al.*, 1996) and molecular detection in apparently healthy adult bees have shown that KBV is a widespread and common inapparent infection in the USA (Hung *et al.*, 1996; Hung and Shimanuki, 1999).

Molecular detection was recently used to determine the prevalence and distribution of KBV in mainland Europe. KBV was first detected in France (Tentcheva *et al.*, 2004b) and in Germany in colonies from Hesse (Siede and Büchler, 2004). A more extensive survey of 281 apiaries in six different countries in central Europe detected KBV in Germany and Luxembourg but failed to detect it in Austria, Hungary, Poland and Slovenia (Siede *et al.*, 2005).

2.10.6. Incidence and impact

In Eastern Australia, between 1980 and 1983, KBV was detected in just 21 (5%) of 418 samples of bees and brood considered abnormal and sent for diagnosis by apiary inspectors and beekeepers (Hornitzky, 1987). A peak of KBV infection was detected serologically in September 1983 in adult bees from 11 of 20 colonies from an apiary in New South Wales monitored from August 1982 to October 1983. However, KBV was detected in brood samples taken from the same colonies at most times of the year but never in more than 3 hives at any one time and this detection of KBV in brood was never associated with any clinical signs of disease (Hornitzky, 1987).

Surveys of virus presence by infectivity tests confirmed that KBV was common as an inapparent infection in pupae in Australia (Dall, 1985; Anderson and Gibbs, 1988; Bruce *et al.*, 1995; Hung *et al.*, 1996), but the prevalence of these inapparent KBV infections, investigated during three consecutive summers in two widely separated locations, varied considerably from year to year (Dall, 1985).

During a recent survey in mainland Europe, KBV was detected by molecular techniques in 23 of 127 apiaries sampled in Germany and in two of the three apiaries sampled in Luxembourg (Siede *et al.*, 2005). Beekeepers reported anomalies in 20 of these 25 apiaries detected positive: 15 suffered severe winter mortality and five showed other disorders such as spring dwindling, crawlers and poor populations. However, in the absence of information on the amount of virus detected, it is difficult to be certain that KBV was responsible for the observed anomalies. The virus may have been present at inapparent levels of infection, as in the remaining five KBV infected apiaries free of anomalies, and the bee mortality and disorders may have had other causes.

Tentcheva and colleagues (2004b) investigated the prevalence and seasonal variations of six honey bee viruses in apparently healthy colonies in 36 apiaries throughout France. Overall, KBV was the least frequently detected virus being found in adult bees and pupae in only 17% and 6% of the apiaries respectively. The virus was detected in *V. destructor* in only 1 out of 21 samples of mites collected. This initial detection of KBV in France shows similarities with ABPV, but the seasonal incidence seems slightly different. Although the prevalence of both viruses is low, ABPV occurs more commonly in summer in association with *V. destructor*, whereas KBV is more prevalent in spring and declines in autumn (Tentcheva *et al.*, 2004b).

2.10.7. Conclusion.

KBV appears to be widely distributed throughout the world but, with the probable exception of Australasia and North America, its prevalence is generally low. The impact of KBV infection on honey bee colonies and its role in the mortality of colonies infested with *V. destructor* are still poorly understood (Anderson, 1991; Hung *et al.*, 1996). Some authors consider that its detection in dead adult bees and brood in the absence of obvious or specific symptoms, deserves more attention, while with no more convincing arguments, other authors consider that KBV is a *"relatively harmless virus"* (Anderson, 1991). Perhaps the recent detection of *V. destructor* in New Zealand, where KBV is endemic, will shed further light on the nature of this relationship between the virus, the honey bee and the mite.

2.11. Sacbrood virus

2.11.1. Discovery

Sacbrood is an infectious disease of the honey bee causing losses and characteristic symptoms in developing brood: a sac-like appearance of diseased larvae (Fig. 6) (Ball, 1999b).

Sacbrood was the first honey bee disease attributed to a virus. In the United States in 1917, G. F. White succeeded in causing sacbrood by inoculating larvae with heat-sensitive filtrates of watery extracts of diseased larvae and was the first to suggest the viral nature of this disease (White, 1917). Sacbrood virus (SBV) was also one of the earliest non-occluded insect viruses to be detected, but this was long before these types of virus particles could be seen by electron microscopy. Bailey and colleagues (1964), showed that isometric particles measuring 28 nm in diameter (Fig. 3) were associated with the infectivity of preparations from sacbrood larvae, thus presenting unequivocal evidence for the establishment of sacbrood as a virus disease (Bailey *et al.*, 1964).

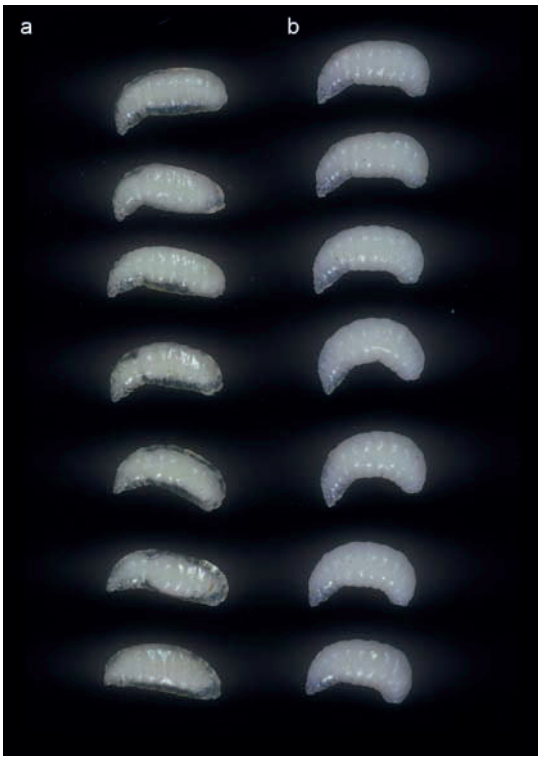


Figure 6. (a) Characteristic symptom of sacbrood disease on larvae compare to (b) normal ones: fluid accumulates between the body of the larva and its unshed skin, forming the sac after which the condition is named.

2.11.2. Disease, virus transmission and persistence

Normal honey bee larvae pupate 4 days after they have been sealed in their brood cells by the workers. Diseased larvae fail to pupate: fluid then accumulates between the body of the larva and its unshed skin, forming the sac after which the condition is named (Fig. 6). The larva changes from its usual pearly white colour to pale yellow, finally becoming dark brown from the anterior end, when it dies a few days later, and eventually drying down to a flattened scale (Bailey *et al.*, 1964). Sacbrood disease was transmitted experimentally to honey bee larvae of all ages by contaminating their food with the virus, but 2-day old larvae were the most susceptible (Hitchcock, 1966).

Although primarily a disease of larvae, SBV multiplies in adult bees without inducing obvious symptoms. Young adult bees were readily infected when fed with concentrated preparations of SBV, and they seem a likely means of perpetuating the infection in nature (Bailey, 1968). By contrast, serologically detectable amounts of SBV were not found in similarly fed bees more than 4-8 days old, suggesting that susceptibility to infection is age related (Bailey, 1969). SBV multiplied much more in the heads of infected adult bees than in other parts of the body and large amounts of virus occurred in the hypopharyngeal glands (Bailey, 1968; Bailey, 1969; Mussen and Furgala, 1977). Experimentally infected adult bees, in which the virus is actively multiplying, do not exhibit obvious signs of disease but may have a decreased life span (Bailey, 1969; Bailey and Fernando, 1972).

Drones may also be experimentally infected by injection (Bailey and Fernando, 1972). Their susceptibility to infection by feeding decreased with age at about the same rate as it did for worker bees (Bailey, 1969) and infected drones, like worker bees, remained apparently healthy though much virus accumulated in their brains (Bailey and Fernando, 1972).

In Australia, SBV was reported to persist as a serologically undetectable inapparent infection in 43 % of seemingly healthy worker pupae, and to replicate to serologically detectable concentrations, when such pupae were injected with various buffer solutions (Dall, 1985; Anderson and Gibbs, 1988). These inapparent infections of SBV in pupae were also seemingly activated merely by incubating pupae at 35°C (Anderson and Gibbs, 1988). When diluted preparations of SBV particles were fed to larvae, the resulting pupae became inapparently infected and these later emerged as inapparently infected worker bees (Anderson and Gibbs, 1989). Sacbrood virus may persist in larvae at very low levels or particles may be restricted to non permissive tissues. Similarly the virus may persist at very low levels

in adult bees, but in contrast to larvae, when the virus does actively replicate, though it may cause behavioural changes there is no obvious pathology (Bailey, 1981; Dall, 1985). This seems to be confirmed by the fact that SBV has been detected serologically only rarely in extracts of dead adult bees (Bailey *et al.*, 1981a).

Infection with SBV can persist in colonies from year to year. Inapparent infections were detected in pupae by infectivity tests at a fairly constant frequency during three consecutive summers in Australia (Dall, 1985). The rapid loss of infectivity of the virus outside the living host (White, 1917; Hitchcock, 1966; Bailey, 1968), and the absence of larvae in many bee colonies in winter indicate that the continuity of infection from one year to the next, is probably provided by the maintenance of a low level inapparent infection in the adult bee population. The youngest worker bees are the most susceptible to infection and are most likely to acquire the virus when they are engaged in their earliest duties of cell cleaning. When young bees remove larvae killed by sacbrood, they ingest a little of the virus rich ecdysial fluid and readily become infected, as is easily demonstrated experimentally. Within one day, much SBV begins to accumulate in the hypopharyngeal glands of these individuals (Bailey, 1969), and they probably transmit the virus when they become nurse bees and feed larvae with their glandular secretions. Larvae and newly emerged bees may also be infected by pollen loads freshly collected by infected foragers whose glandular secretions used for forming the pollen loads, contain much virus. The horizontal virus transmission between adult bees and from adults to larvae through contaminated food is substantiated by the molecular detection of SBV in all food resources; brood food, honey, pollen and royal jelly (Shen *et al.*, 2005a).

Sacbrood is common in colonies (Bailey, 1967), but large numbers of diseased larvae are rarely seen. This is because adult bees are very efficient at detecting and removing most larvae in the early stages of infection and there are a number of other factors that limit virus spread within the colony. Infection of young bees with the virus accelerates the normal sequence of hive duties: they stop eating pollen, cease feeding and tending larvae and start foraging much earlier than usual (Bailey and Fernando, 1972). Moreover, these foragers almost all fail to collect pollen, which would be a prime source of infection for other individuals. Disease outbreaks most commonly occur in spring and early summer when there are fewer young bees, forage is limited and the usual division of labour according to age is least well developed. Transmission of SBV from infected adults to larvae decreases when foraging increases later in the year during the major nectar flows, at which time most colonies recover spontaneously

(Ball and Bailey, 1997).

Another possible route of transmission of SBV could involve *V. destructor*. Indeed the most convenient means of propagating sacbrood virus in the laboratory seems to be the injection of very few particles into white eyed pupae (Bailey, 1968; Bailey and Fernando, 1972) and SBV has been detected in mites and their saliva (Shen *et al.*, 2005a). Furthermore, the ability of the mite to transmit SBV from severely infected to healthy pupae has been demonstrated experimentally (Ball, 1989b). The mortality of pupae due to the multiplication of SBV occasionally occurs in colonies infested with *V. destructor* (Ball, 1999b), but whether the mite plays a significant role in the transmission of SBV to adult bees in nature has not yet been demonstrated. Most records of SBV are of overt disease in brood, but the virus was detected serologically in dead bees from two out of 18 colonies in Germany (Ball and Allen, 1988) and in one out of 29 colonies in Poland (Topolska *et al.*, 1995). Although the colonies were infested with *V. destructor*, SBV was the least common of all the bee viruses detected and occurred only during May and June which reflects the natural prevalence of infection in larvae. Using molecular techniques to detect bee viruses in French apiaries, Tentcheva and colleagues (2004b) found SBV in *V. destructor* from only 14% of colonies, whereas it was very common in summer in adult bees (64% of samples) and less common in pupae (18% of samples). The greater frequency of virus detection in adults than in pupae or mites is consistent with the conception that the virus persists in colonies through the symptomless infection of adult bees. Although the authors also demonstrated a positive correlation between SBV in mites and in adult bees and pupae collected during summer (Tentcheva *et al.*, 2004b) this again may only reflect the natural occurrence of overt SBV infection. In addition, Shen and colleagues (2005b) found no significant difference between the amount of SBV detected in mite-free pupae and naturally infested ones from the same colony (Shen *et al.*, 2005b). There is no proof of the replication of SBV in *V. destructor*, thus the detection of the virus in mites may be merely a consequence of the acquisition of virus from infected bees.

SBV might also be transmitted through the queen, but direct attempts to show this have failed (Ball, 1999b). The virus was injected into laying queens or fed to young individuals which successfully mated and produced larvae. None of the queens transmitted serologically detectable SBV, although infectivity and serological tests with extracts of their heads showed that the virus had multiplied within them (Ball, 1999b). However, the direct detection of SBV in naturally infected queens and in all developmental stages, even eggs, has been reported (Chen *et al.*, 2005a; Shen *et*

al., 2005a; Chen *et al.*, 2006b). SBV was detected in 6 queens out of 10 and in 100% of the pooled eggs from these queens. However only 25% of the larvae and 10% of the adult worker offspring of the infected queens were SBV positive. In this study SBV was detected in the ovaries of the queens but not in their spermatheca (Chen *et al.*, 2006b).

These results might suggest a transovarial transmission of the virus although the probable primary route of infection still seems to be by ingestion.

2.11.3. Geographical, temporal distribution and impact

In Britain, over 80% of diseased larvae that were free of bacterial pathogens were infected with SBV and up to 30% of apparently healthy colonies contained a few larvae killed by the virus (Bailey, 1967). In Australia, SBV was reported to be the most common honey bee virus, occasionally causing serious losses of brood (Dall, 1985). It was detected as an inapparent infection in more than 40% of healthy worker pupae (Anderson and Gibbs, 1988), and as an overt infection in more than 90% of colonies (Anderson, 1983). In Fiji, SBV caused some brood mortality in 30% of colonies in August (Simpson, 1983) and in 70 % during October and November (Anderson, 1990), which suggests that in Oceania and elsewhere the seasonal incidence of sacbrood disease may follow a pattern similar to that seen in temperate regions, with outbreaks occurring most commonly in spring and early summer when brood rearing increases (Ball, 1999b).

In Britain, the prevalence of inapparent infections of SBV in adult honey bees was greatest in summer, when it was detected in most colonies. Sacbrood virus was detected by infectivity tests in 11 out of 12 symptom-free bee colonies by injecting healthy test pupae with extracts of live adult bees (Bailey *et al.*, 1981a). In France, analysis of samples from 360 apparently healthy colonies confirmed that the frequency of SBV-infected colonies increased from spring to summer and declined in autumn in both adults and pupae (Tentcheva *et al.*, 2004b).

2.11.4.Conclusion

Sacbrood virus is widely distributed, occurring in colonies of *Apis mellifera* on every continent (Allen and Ball, 1996). In several areas, it is considered to be the commonest bee virus infection (Hornitzky, 1987). However, this may be due to the fact that sacbrood, unlike most other bee viruses, may be reliably diagnosed by the distinctive and specific symptoms of infection in larvae (Ball, 1996) (see Chapter 3). In any case, the almost ubiquitous occurrence of SBV and its persistence in bee populations

as a symptomless infection of adults calls for more study of its impact on colonies.

2.12. Slow bee paralysis virus

2.12.1. Discovery

Slow bee paralysis virus (SBPV) was incidentally isolated during laboratory experiments performed in the context of a field survey of bee virus X (Bailey and Woods, 1974). When injected with preparations of SBPV, adult bees died after about 12 days typically suffering paralysis of the two anterior pairs of legs for a day or two before death. The virus was called slow bee paralysis virus to differentiate it from the comparatively quick-acting acute and chronic bee paralysis viruses.

2.12.2. Prevalence before and after Varroa destructor

Before the detection of *V. destructor* in Britain slow bee paralysis virus was rare, being detected indirectly (by infectivity tests) in the extracts of only four out of 96 samples of live bees from symptom-free colonies in 1976-1977 (Bailey *et al.*, 1981a). This fact and its complete absence by serological detection in extracts of dead bees over a number of years suggests it is either a very uncommon infection of bees, or one that is permanently inapparent, and similar to ABPV (Bailey *et al.*, 1981a). During a field study, when *V. destructor* first became established in the south-west of England, SBPV was detected directly by serology in dead adult bees and brood from six out of 21 infested colonies but was still not detected in colonies that had never been infested. The detection of SBPV in dead adult bees and brood coincided with the peak of mite population development (Ball, 1997). Following this study, Ball (1997) confirmed that SBPV infection became established initially in colonies with the largest mite populations and that the virus could be detected in mites two months before its detection in live or dead adult bee and brood samples, implicating *V. destructor* in virus spread. Detection of SBPV as a primary cause of brood mortality in October also suggested that virus transmission increased when brood rearing decreased as there were then relatively more parasites available to invade the fewer brood cells (Ball, 1997). SBPV has also been associated with colony collapse in the United Kingdom (Martin *et al.*, 1998).

More recently, detailed observations were made on a small number of *V. destructor* infested colonies throughout a whole year in the UK. Adult bees, brood and mites were sampled every 21 days, and individually assayed for SBPV by ELISA. The results showed that SBPV first appeared in those colonies with the largest mite populations, and virus infection was

most prevalent late in the season when multiple infestation of brood cells frequently occurred. Colony death often followed, although where virus infection declined, colonies could recover and over-winter successfully (Carreck *et al.*, 2005).

2.12.3. Conclusion

SBPV seems to primarily persist as an inapparent infection in the same way as ABPV and may be activated to multiply by the feeding activities of *V. destructor*. Only limited data, regarding its distribution and impact outside the United Kingdom, are available, presumably because its detection is problematical and overt infections are rare.

3. Multiple viral infections.

Multiple viral infections have been reported in honey bee colonies or in individuals by a number of authors (Bailey *et al.*, 1981a; Ball and Allen, 1988; Anderson and Gibbs, 1988; Evans, 2001; Tentcheva *et al.*, 2004b; Chen *et al.*, 2004c; Shen *et al.*, 2005b; Chen *et al.*, 2006b). However, due to considerable differences between methodologies in the thresholds of detection (see Chapter 3), the reported occurrence of coincident infections at the apiary, colony and individual level must be considered with great care.

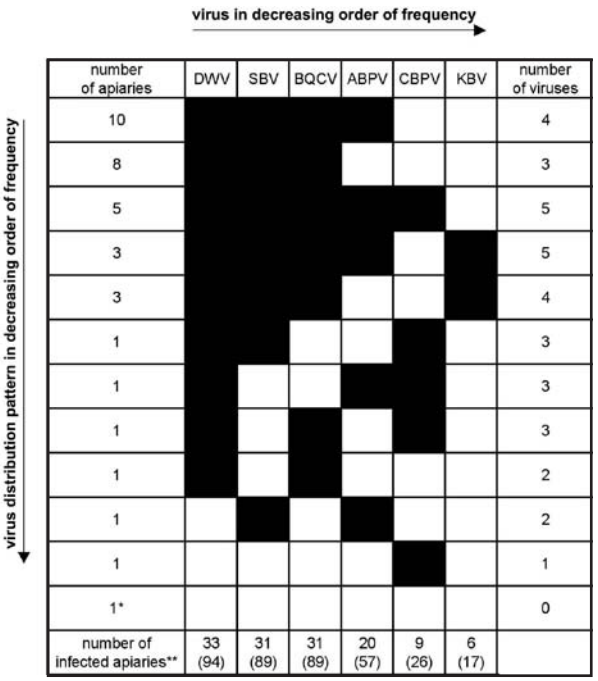
3.1. Multiple viral infections in colonies

Studies on approximately 25 colonies over several years in Britain reported coincident infections with BQCV + SBV, BQCV + BVY + CWV or BVX, and BVY + CWV in samples of adult bees (Bailey *et al.*, 1981a). Both indirect (infectivity) and direct (serology) methods were used for virus detection, the former being many thousand times more sensitive. However, both techniques have some limitations (see Chapter 3).

Tentcheva *et al.*, (2004b) used PCR detection to study the prevalence and geographic distribution of ABPV, BQCV, CBPV, DWV, KBV and SBV in 36 apparently healthy apiaries throughout France. At least one virus, and frequently several, were detected in all but one apiary. The only apparently virus-free apiary was located on the Isle of Ouessant which until now has not been invaded by *V. destructor*. Most of the other apiaries (all were mite infested) were found to be harbouring several viruses: 34/35 had at least two viruses and 32/35 had at least 3 viruses (Tentcheva *et al.*, 2004b). We elaborated Figure 7 from these original data for describing the details of the patterns of virus distribution in apiaries (how many

and which viruses) and the number of apiaries with the same patterns of virus incidence. The most frequently detected virus was DWV (33/35) and the least common was KBV (6/35). However, although this provides useful information on virus prevalence and distribution, the detection technique, although very sensitive, was not quantitative so inapparent or latent infections could not be distinguished from overt infections. We used the detailed results of these authors to compare the frequency of the simultaneous presence (or absence) of viruses in the same apiaries with a totally random theoretical distribution of these viruses based on the probability multiplication theorem: there is no statistically significant aggregation of viruses – nor any exclusion between viruses in apiaries (data not shown).

Chen and colleagues (2004c) examined 56 colonies for BQCV, DWV,



number of apiaries	DWV	SBV	BQCV	ABPV	CBPV	KBV	number of viruses
10	■	■	■	■			4
8	■	■	■				3
5	■	■	■	■	■		5
3	■	■	■	■		■	5
3	■	■	■			■	4
1	■	■			■		3
1	■			■	■		3
1	■		■		■		3
1	■		■				2
1		■		■			2
1					■		1
1*							0
number of infected apiaries**	33 (94)	31 (89)	31 (89)	20 (57)	9 (26)	6 (17)	

Figure 7. Prevalence pattern of 6 bee viruses in 36 apiaries in France revealed by RT-PCR (interpreted from Tentcheva et al., 2004b). Data given by the authors have been gathered for showing the infection of adults or pupae sampled during spring, summer or autumn 2002: a black box indicates that the corresponding virus has been diagnosed in one sample at least of adults and/or pupae. Virus (columns) and patterns of virus infection (rows) are ordered in decreasing order of frequency.

* This apiary was located in the Island of Ouessant which is Varroa free.

** Between parenthesis : infected among continental apiaries per cent.

KBV and SBV by RT-PCR, and detected one, or more than one virus, in 42 (75 %) of the colonies. The percentage of colonies in which none up to four viruses were detected was 25%, 28.5%, 37.5%, 5.4% and 3.6% respectively.

Both of these recent studies serve to demonstrate that honey bee colonies may harbour several different viruses simultaneously, probably as the simple result of the broad geographic distribution of bee viruses and their ability to persist in populations without exhibiting obvious pathological symptoms (Chen *et al.*, 2004c).

3.2. Multiple viral infections in individuals

Multiple viral infections demonstrated at the colony level is also true for individual bees (brood, adult workers and queens). The experimental inoculation of pupae with extracts of live bees showed that BQCV + SBV or CBPV + BQCV, frequently multiplied in the same individuals (Bailey *et al.*, 1981a). These studies also revealed that ABPV did not multiply in test pupae in association with BQCV or SBV.

Anderson and Gibbs (1988) demonstrated that when honey bee pupae from seemingly healthy colonies were injected with various salt solutions, BQCV, KBV and SBV could be simultaneously activated to serologically detectable titres: these three viruses were detected in 2.6% of the pupae. Double infections with KBV + SBV were detected in 14.6% of pupae, SBV + BQCV in 3.9% and BQCV+ KBV in 3.4%. The authors noticed that there was no evidence of interference between the viruses in the establishment of inapparent infections in the pupae, but clear evidence of competitive exclusion during or after activation. When pupae with combinations of inapparent BQCV, KBV and SBV infections were injected with buffer and held at 35°C, only KBV replicated to detectable concentrations, presumably because of its significantly more rapid replication rate. Only suppression of KBV replication by the injection of specific neutralising antisera permitted the multiplication and detection of the other viruses (Anderson and Gibbs, 1988).

Evans (2001) demonstrated the co-incident occurrence of ABPV and KBV in healthy individual worker bees sampled from a colony known to harbour both viruses. KBV was detected in 19 of the 23 adult bees tested and 5 of them were also carrying ABPV (Evans, 2001). The authors noted that the bees testing positive for one or both viruses did not look any different from those in which no virus was detected.

In detailed field studies in colonies infested with *V. destructor*, Shen and colleagues (2005b) used dot blot analysis to quantify KBV and DWV in individual pupae from cells with and without mites. KBV was detected

in 80% (37/46) and DWV was detected in 78% (36/46) of mite infested pupae. It was also found that 70% (32/46) of individual pupae were infected by both KBV and DWV which was in sharp contrast with mite-free pupae in which neither virus was detected (Shen *et al.*, 2005b). Interestingly, the quantitative detection method used also indicated that the amount of DWV present in individuals was far greater than that of KBV. Another study by Chen and colleagues (2004c) using RT-PCR to detect BQCV, DWV, KBV and SBV showed that individual bees could harbour all four of these viruses simultaneously.

Multiple viral infections revealed by molecular techniques, have also been recently reported in queens. Chen and colleagues (2006b) simultaneously investigated ABPV, BQCV, CBPV, DWV, KBV and SBV in 10 individual queens and their offspring. The 10 queens were infected by 2 to 5 viruses: all were positive for BQCV and DWV, 6 were also positive for SBV, 4 for CBPV and 2 for KBV whereas ABPV was never detected. However there may be gaps in this transmission to the offspring as KBV, for example, was detected in 50 egg polls but not in larvae and adult workers (Chen *et al.*, 2006b).

Moreover, ABPV and DWV sequences have recently been detected in semen polls sampled from 4 to 5 inapparently infected drones, indicating that drones may be infected by several viruses (Yue *et al.*, 2006).

3.3. Conclusion

These results indicate that any individuals of a colony, even the queen, can be infected by several viruses. However, in the absence of information as to what constitutes a covert (inapparent or latent) or overt (actively replicating or systemic) infection it is difficult to determine the significance of some of the observations of multiple viral infections. This is even more difficult when quantitative data are missing.

This serves to highlight the considerable gaps that still exist in our knowledge of the nature of possible virus interactions and the effects of mixed virus infections on pathogenic processes in honey bees.

Competition between viruses present in the same colony or in the same individual may be one reason for the variation in virus prevalence. As previously stated, ABPV does not multiply in association with BQCV or SBV when experimentally injected into pupae (Bailey *et al.*, 1981a). Another example is that of DWV which multiplies to very high titres even in symptomless bees (Chen *et al.*, 2005b). This systemic but not rapidly fatal overt infection may nevertheless inhibit the replication of other viruses, as may be the case during co-infection with KBV in pupae parasitized

by *V. destructor* (Shen *et al.*, 2005b). It remains to be determined whether different viruses, when present in the same host, occupy separate niches (perhaps due to their mode of transmission) or share the same microenvironment and compete for the host resources (Chen *et al.*, 2004c).

Thus, the diversity of bee viruses, their ability to persist seemingly indefinitely in populations and their almost ubiquitous distribution strongly suggest that individual characteristics such as mode of transmission, seasonality, life stage infected, site of replication and other factors separate them sufficiently in time and space to ensure their survival.

Currently, it is not know whether mixed virus infections could lead to genetic recombination between coexisting viruses and whether such recombination could result in the emergence of new viruses (Chen *et al.*, 2004c). Although there is no indication at present that novel recombinant viruses have suddenly emerged in bees, the lability of ssRNA genomes and the establishment of new routes of virus transmission should encourage us to remain vigilant.

4. *Varroa destructor* and honey bee viruses

4.1. *Varroa destructor* as a vector of viruses

As reviewed in the previous sections, *V. destructor* can act as a vector of several different honey bee viruses, although it should not be assumed that the detection of viruses within mites necessarily indicates effective transmission. However, the mechanism of transfer of these viruses between individuals and to different life stages of bees, whether it is active or passive, and whether any multiplication of these viruses takes place within the mite, deserves further investigation. To summarise, each virus probably represents a different situation because they each have unique characteristics and ecology or it may depend on the length of the parasite pathogen association. In agreement with the fact that some mites have been shown to transmit ABPV and DWV several hours after removal from an infected host or to several successive new host bees, it seems unlikely that the transmission of viruses is merely effected by contamination of the mouthparts of the parasite (Ball, 1983; Wiegers, 1986; Ball, 1989b). Laboratory studies with ABPV indicated that transmission efficiency by mites decreased with successive transfers and the lack of a noticeable latent period between acquisition and transmission, suggests that there is no need for virus replication in *V. destructor* (Ball, 1983; Wiegers, 1986).

Several authors have hypothesised that the feeding activities of

V. destructor could induce or activate the replication of inapparent and normally non pathological infections (Ball, 1983; Bailey and Ball, 1991; Bowen-Walker *et al.*, 1999) Parasitism by *V. destructor* was shown to depress the expression of genes that govern both humoral and cellular immune responses in honey bees. In addition, the negative correlation between DWV titres and the expression of immunity-related enzymes indicated that the immunosuppressive effect of mite saliva could be responsible for the activation of latent viral infections in honey bees (Yang and Cox-Foster, 2005).

After the activation of viral replication in bees, *V. destructor* can ingest large amounts of virus with the haemolymph during feeding which are retained in the alimentary tract. Infection of new individuals could occur by regurgitation of the gut contents just before feeding, as described in beetles (Wiegers, 1986). In such a process, the detection of viruses in *V. destructor* would merely reflect the type and prevalence of virus infections already established in the bee population. With no evidence of viral multiplication in the mite, this is potentially the means of SBV dissemination which has been detected in mites and their saliva (Shen *et al.*, 2005a). However SBV was only detected in 14% of mites compared to 86% of adult bees and 80% of pupae from infested apiaries (Tentcheva *et al.*, 2004b) which suggests that for this virus at least, other means of transmission are more important. Nevertheless, the ease of movement of mites between the different life stages of the honey bee, the potential for progeny mites to acquire virus by feeding on pupae infected by their mother and the numerous individuals in the mite population may favour the acquisition and transmission of numerous virus particles.

The process may be quite different for any honey bee virus that can replicate within *V. destructor* as has been reported for DWV (Ongus *et al.*, 2004; Yue and Genersch, 2005) (see section 2.8 DWV).

4.2. Impact of *Varroa destructor* on the prevalence of honey bee viruses

There is increasing evidence that the global spread of *V. destructor* has resulted in a significant change in the type and prevalence of viruses causing mortality in honey bee colonies. A good example is ABPV, which until the spread of *V. destructor,* had never been detected in association with disease or mortality in nature but was considered an important cause of adult bee and brood mortality in mite infested colonies after the mite spread across mainland Europe. However, after more than a decade of mite infestation during which the impact of ABPV was enhanced, there seem to be no further reports of ABPV in association with honey bee

colony losses in Europe. Are we experiencing a co-adaptation of the virus and its host, or is this the result of selective pressure exerted by the mite in facilitating new routes for virus transmission which overcome the normal constraints on virus spread?

Several studies, in different countries suggest that similar patterns of virus succession in infested colonies have occurred. When mites first enter an area, the viruses that initially show a marked increase in prevalence are those that are infective by injection into both adults and pupae (eg: ABPV, KBV, SBPV) and that have a short incubation period. They are readily transmitted by mites to all life stages, replicate rapidly and are fatal. This tends to exclude the development of the other virus infections that are not so easily transmitted by injection into the haemolymph of either adult bees or pupae (eg. BQCV, CWV) or multiply more slowly (BVX, BVY). However, there are disadvantages to killing the host quickly: to maintain the infection, the rate of virus acquisition and transmission by mites has to be greater than the loss of infected individuals from the population (Martin, 2001). Ultimately, either the entire population of bees is killed or virus transmission decreases to such an extent that it can no longer be sustained. In both cases the virus may decline. Firstly, killing all of the hosts can also lead to the extinction of the virus. Secondly, any surviving colonies are less likely to contain significant numbers of individuals carrying these viruses. It might be the scenario that prevailed with ABPV. A much more successful strategy for the survival and persistence of honey bee viruses in mite infested colonies would be to multiply within the host without causing a rapidly fatal infection. The longer a severely infected individual survives in the population, the more opportunities the mite has to feed on that individual and acquire and transfer the virus to a new host. Such a scenario may explain the now almost ubiquitous occurrence of DWV worldwide (Ball, 2004) (see Chapter 6).

5. Summary

The existence, occurrence and distribution of honey bee viruses have been revealed and documented mainly by the examination of diseased or dead individuals of all development stages and sometimes following comprehensive surveys.

Although we may consider that most of the viruses that currently infect the European honey bee have been discovered, much information is still lacking on their geographical and temporal distribution. Perhaps more importantly, their natural history, means of persistence and trans-

mission are still poorly documented. The records collected here, as for other reviews of honey bee diseases, often reflect the location of individual research workers or localized outbreaks of disease when and where bee specialists and appropriate detection techniques were available. It should also be remembered that apparent differences in the distribution of certain infections may more accurately reflect differences between the sensitivity of the detection techniques used, the time of observation and sampling strategy or the duration of the studies.

Surveys that cover at least one twelve-month period provide the most detailed information on virus incidence over a seasonal cycle. However, for such surveys that analysed samples of apparently healthy bees, virus detection was often inconsistent even when they used relatively sensitive techniques. This may have been due to inappropriate sampling strategies. Sampling procedures should take into account the natural prevalence of the virus and the life stage most likely to be infected. In addition, the analysis of a very small proportion of the live population is unlikely to identify every infection. Using these strategies, viruses could frequently only be detected once a year in two or three samples from colonies (Ribière *et al.*, 2002; Bakonyi *et al.*, 2002; Tentcheva *et al.*, 2004a; Tentcheva *et al.*, 2004b). Considering the current lack of knowledge of the prevalence and distribution of viral infections in individual colonies, it would be risky to consider these results as unbiased reflections of real temporal variations (see Chapter 3). Specific studies of virus distribution and yields in bee and mite populations are prerequisites for defining an appropriate sampling strategy.

Nevertheless, the data presented here already indicate that several viruses are widely distributed in honey bee colonies, often in association with other parasites and pathogens and this warrants more intensive investigation to clarify their relationship with bee populations and to determine the consequences of these relationships in terms of bee health and apicultural production (see Chapters 4 and 6).

The increasing sensitivity of detection techniques means that the presence of relatively few particles will be revealed but the mere detection of a virus does not necessarily mean that a debilitating or fatal infection is, or will become established. Determination of the amount of virus per individual or the number of individuals harbouring the infection together with studies of their longevity or productivity, will provide a better understanding of the epidemiology and impact of these infections. Moreover, molecular methods should be complemented by laboratory tests of infectivity in comprehensive studies of viral transmission between hosts, parasites, their excreta and food.

Obviously, to progress on the knowledge of the biology of bee viruses, the following questions must be addressed:

- a better understanding of virus distribution and incidence within colonies (where and when ?) is a prerequisite for developing appropriate sampling strategies (number of individuals for each development stage and sex, quality, frequency),
- the detailed observation of colonies (method of observation of individuals – longevity behaviour, description of symptoms, health criteria) and virus detection techniques should be improved and standardized – the limits of detection for different levels of infection and their interpretation should be well established and shared by the scientific community.
- virus detection in colonies should ideally be undertaken simultaneously for all bee castes and developmental stages with

  a) quantification of the infection level and

  b) evaluation of the health status of individuals and colonies, to better evaluate the impact of virus multiplication and infection within populations.
- the demonstrated pathogenic synergy between parasites, fungi, bacteria and viruses strongly suggests that it is essential to simultaneously detect most (if not all) of the known pathogens of bees in experiments or surveys aimed to evaluate the impact of specific infections. Ideally, as many factors as possible that may depress natural bee immunity (environmental toxicants included) should also be assessed in such studies.
- virus detections in samples such as food, bee faeces, parasites and sperm … should be completed by infectivity tests to assess their epidemiological significance.
- finally, any hypotheses developed during observational field studies should be confirmed by experiments in the laboratory or in small colony units under controlled conditions.

References

Akratanakul P., Burgett M. (1975) *Varroa jacobsoni:* A prospective pest of honeybees in many parts of the world, Bee World 56, 119-121.

Allen M., Ball B.V. (1995) Characterisation and serological relationships of strains of Kashmir bee virus, Ann. Appl. Biol. 126, 471-484.

Allen M., Ball B.V. (1996) The incidence and world distribution of honey bee viruses, Bee World 77, 141-162.

Allen M.F., Ball B.V., White R.F., Antoniw J.F. (1986) The detection of acute paralysis virus in *Varroa jacobsoni* by the use of a simple indirect ELISA, J. Apic. Res. 25, 100-105.

Anderson D.L. (1983) Viruses of honeybees in north eastern Australia, The Australa. Beekeep. 84, 219-223.

Anderson D.L. (1985) Virus of New Zealand honey bees, New Zealand Beekeeper 188, 8-10.

Anderson D.L. (1990) Pests and pathogens of the honeybee (*Apis mellifera* L.) in Fiji, J. Apic. Res. 29, 53-59.

Anderson D.L. (1991) Kashmir bee virus - A relatively harmless virus of honey bee colonies, Am. Bee J. 131, 767-770.

Anderson D.L. (1993) Pathogens and queen bees, Australian Beekeeper 94, 292-296.

Anderson D.L., Gibbs A.J. (1988) Inapparent virus infections and their interactions in pupae of honey bee (*Apis mellifera* Linnaeus) in Australia, J. Gen. Virol. 69, 1617-1625.

Anderson D.L., Gibbs A.J. (1989) Transpuparial transmission of Kashmir bee virus and sacbrood virus in the honey bee (*Apis mellifera*), Ann. Appl. Biol. 114, 1-7.

Antúnez K., D'Alessandro B., Corbella E., Zunino P. (2005) Detection of chronic bee paralysis virus and acute bee paralysis virus in Uruguayan honeybees, J. Invertebr. Pathol. 90, 69-72.

Bailey L. (1965a) Paralysis of the honey bee, *Apis mellifera* Linnaeus, J. Invertebr. Pathol. 7, 132-140.

Bailey L. (1965b) The occurrence of chronic and acute bee paralysis viruses in bees outside Britain, J. Invertebr. Pathol. 7, 167-169.

Bailey L. (1967) The incidence of virus diseases in the honey bee, Ann. Appl. Biol. 60, 43-48.

Bailey L. (1968) The multiplication of sacbrood virus in the adult honeybee, Virol. 36, 312-313.

Bailey L. (1969) The multiplication and spread of sacbrood virus of bees, Ann. Appl. Biol. 63, 483-491.

Bailey L. (1976) Viruses attacking the honey bee, in: Lauffer, M. A., Bang, F. B., Maramorosch, K., and Smith, K. M. (Ed.), Adv. Virus Res. volume 20, Academic Press, New York, pp. 271-304.

Bailey L. (1981) Honey bee pathology, Academic Press, London and New York.

Bailey L. (1985) *Acarapis woodi*: a modern appraisal, Bee World 66, 99-104.

Bailey L., Gibbs A.J. (1964) Acute infection of bees with paralysis virus, J. Insect Pathol. 6, 395-407.

Bailey L., Milne R.G. (1969) The multiplication regions and interaction of acute and chronic bee paralysis viruses in adult honeybees, J. Gen. Virol. 4, 9-14.

Bailey L., Fernando E.F.W. (1972) Effects of sacbrood virus on adult honeybees, Ann. Appl. Biol. 72, 27-35.

Bailey L., Woods R.D. (1974) Three previously undescribed viruses from the honey bee, J. Gen. Virol. 25, 175-186.

Bailey L., Woods R.D. (1977) Two more small RNA viruses from honey bees and further observations on sacbrood and acute bee-paralysis viruses, J. Gen. Virol. 37, 175-182.

Bailey L., Milne R.G. (1978) Filamentous viruslike particles in honey bees in Britain, J. Invertebr. Pathol. 32, 390-391

Bailey L., Ball B.V. (1991) Honey bee pathology, Harcourt Brace Jovanovich, Sidcup, UK.

Bailey L., Gibbs A.J., Woods R.D. (1963) Two viruses from adult honey bees (*Apis mellifera* Linnaeus), Virol. 21, 390-395.

Bailey L., Gibbs A.J., Woods R.D. (1964) Sacbrood virus of the larval honey bee (*Apis mellifera* Linneaus), Virol. 23, 425-429.

Bailey L., Carpenter J.M., Woods R.D. (1979) Egypt bee virus and australian isolates of Kashmir bee virus, J. Gen. Virol. 43, 641-647.

Bailey L., Ball B.V., Carpenter J.M., Woods R.D. (1980a) Small virus-like particles in honey bees associated with chronic paralysis virus and with a previously undescribed disease, J. Gen. Virol. 46, 149-155.

Bailey L., Carpenter J.M., Govier D.A., Woods R.D. (1980b) Bee virus Y, J. Gen. Virol. 51, 405-407.

Bailey L., Ball B.V., Perry J.N. (1981a) The prevalence of viruses of honey bees in Britain, Ann. Appl. Biol. 97, 109-118.

Bailey L., Carpenter J.M., Woods R.D. (1981b) Properties of a filamentous virus of the honey bee (*Apis mellifera*), Virol. 114, 1-7.

Bailey L., Carpenter J.M., Woods R.D. (1982) The diminished incidence of *Acarapis woodi* (Rennie) (Acari: Tarsonelidae) in honey bee, *Apis mellifera* L. (Hymenoptera: Apidae), in Britain, Bull. Entomol Res. 72, 655-662.

Bailey L., Ball B.V., Perry J.N. (1983a) Association of viruses with two protozoal pathogens of the honey bee, Ann. Appl. Biol. 103, 13-20.

Bailey L., Ball B.V., Perry J.N. (1983b) Honeybee paralysis: its natural spread and its diminished incidence in England and Wales, J. Apic. Res. 22, 191-195.

Bakonyi T., Frakas R., Szendroi A., Dobos-Kovacs M., Rusvai M. (2002) Detection of acute bee paralysis virus by RT-PCR in honey bee and *Varroa destructor* field samples: rapid screening of representative Hungarian apiaries, Apidologie 33, 63-74.

Ball B.V. (1983) The association of *Varroa jacobsoni* with virus diseases of honey bees, Meeting of the EC Experts' Group, Wageningen, in: Wageningen, pp.21-23.

Ball B.V. (1985) Acute paralysis virus isolates from honeybee, *Apis mellifera*, colonies infested with *Varroa jacobsoni*, J. Apic. Res. 24, 115-119.

Ball B.V. (1989a) Evaluation of the risk of importing Kashmir bee virus into Britain in queen honey bees imported from New Zealand, MAFF report.

Ball B.V. (1989b) *Varroa jacobsoni* as a virus vector, in: Cavalloro, R. (Ed), Present status of varroatosis in Europe and progress in the varroa mite control, Udine, Italy, pp.241-244.

Ball B.V. (1993) The damaging effects of *Varroa jacobsoni*, in: Matheson, A. (Ed.), Living with Varroa, International Bee Research Association, Cardiff, pp. 9-16.

Ball B.V. (1996) Honey bee viruses: a cause for concern?, Bee World 77, 117-119.

Ball B.V. (1997) Varroa and viruses, in: Munn, P. and Jones, R. (Ed.), Varroa! Fight the mite, International Bee Research Association, Cardiff, pp. 11-15.

Ball B.V. (1999a) Paralysis, in: Colin, M. E., Ball, B. V., and Kilani, M. (Ed.), Bee disease diagnosis, Options Mediterannéennes, pp. 81-89.

Ball B.V. (1999b) Sacbrood, in: Colin, M. E., Ball, B. V., and Kilani, M. (Ed.), Bee disease diagnosis, Options Mediterannéennes, pp. 91-96.

Ball B.V. (2001) Viruses and Varroa, Bee Craft Nov, 20-22.

Ball B.V. (2004) The trouble with viruses, Bee World 85, 25.

Ball B.V., Allen M.F. (1988) The prevalence of pathogens in honey bee (*Apis mellifera*) colonies infested with the parasitic mite *Varroa jacobsoni*, Ann. Appl. Biol. 113, 237-244.

Ball B.V., Bailey L. (1997) Viruses, in: Morse, R. A. and Flottum, K. (Ed.), Honey bee pests, predators, & diseases, A.I. Root Company, Medina, pp. 11-32.

Ball B.V., Overton H.A., Buck K.W., Bailey L., Perry J.N. (1985) Relationships between the multiplication of chronic bee-paralysis virus and its associate particle, J. Gen. Virol. 66, 1423-1429.

Ball B.V., Todd J.H., de Miranda J.R. (2005) Searching for deformed wing virus in New Zealand honeybees, in: Scientific Programme Apimondia Ireland 2005, pp.48.

Bekesi L., Ball B.V., DobosKovacs M., Bakonyi T., Rusvai M. (1999) Occurrence of acute paralysis virus of the honey bee (*Apis mellifera*) in a Hungarian apiary infested with the parasitic mite *Varroa jacobsoni*, Acta Vet. Hung. 47, 319-324.

Blanchard P., Ribière M., Celle O., Lallemand P., Schurr F., Olivier V., Iscache A.L., Faucon J.P. (2007) Evaluation of a real-time two step RT-PCR assay for quantitation of Chronic bee paralysis virus (CBPV) genome in experimentally-infected bee tissues and in life stages of a symptomatic colony, J. Virol. Methods 141, 7-13.

Bowen-Walker P.L., Martin S.J., Gunn A. (1999) The transmission of deformed wing virus between honeybees (*Apis mellifera* L.) by the ectoparasitic mite *Varroa jacobsoni* Oud, J. Invertebr. Pathol. 73, 101-106.

Bruce W.A., Anderson D.L., Calderone N.W., Shimanuki H. (1995) A survey for Kashmir bee virus in honey bee colonies in the United States, Am. Bee J. 135, 352-355.

Burnside C.E. (1945) The cause of paralysis of honeybees, Am. Bee J. 85, 354-363.

Calderon R.A., Van Veen J., Arce H.G., Esquivel M.E. (2003) Presence of deformed wing virus and Kashmir bee virus in Africanized honey bee colonies in Costa Rica infested with *Varroa destructor*, Bee World 84, 112-116.

Carpana E., Vecchi M.A., Lavazza A., Bassi S., Dottori M. (1990) Prevalence of acute paralysis virus (APV) and other viral infections in honeybees in Italy, in: Ritter, W. (Ed), Proceedings of the international symposium on recent research on bee pathology, Ghent (Belgique), 155-165.

Carreck N.L., Ball B.V., Wilson J.K., Allen M.F. (2005) The epidemiology of slow paralysis virus in honeybee colonies infested by *Varroa destructor* in the UK. Scientific Programme 39th Apimondia International Apicultural Congress, Dublin, Ireland, 2005.

Chantawannakul P., Ward L., Boonham N., Brown M. (2006) A scientific note on the detection of honeybee viruses using real-time PCR (TaqMan) in Varroa mites collected from a Thai honeybee (*Apis mellifera*) apiary, J. Invertebr. Pathol. 91, 69-73.

Chen Y., Pettis J.S., Evans J.D., Kramer M., Feldlaufer M.F. (2004a) Transmission of Kashmir bee virus by the ectoparasitic mite *Varroa destructor*, Apidologie 35, 441-448.

Chen Y., Smith I.B., Collins A.M., Pettis J.S., Feldlaufer M.F. (2004b) Detection of deformed wing virus infection in honey bees, *Apis mellifera* L., in the United States, Am. Bee J. 557-559.

Chen Y., Zhao Y., Hammond J., Hsu H.T., Evans J., Feldlaufer M. (2004c) Multiple virus infections in the honey bee and genome divergence of honey bee viruses, J. Invertebr. Pathol. 87, 84-93.

Chen Y., Pettis J.S., Feldlaufer M.F. (2005a) Detection of multiple viruses in queens of the honey bee *Apis mellifera* L., J. Invertebr. Pathol. 90, 118-121.

Chen Y.P., Higgins J.A., Feldlaufer M.F. (2005b) Quantitative real-time reverse transcription-PCR analysis of deformed wing virus infection in the honeybee (*Apis mellifera* L.), Appl. Environ. Microbiol. 71, 436-441.

Chen Y., Evans J., Feldlaufer M. (2006a) Horizontal and vertical transmission of viruses in the honey bee, *Apis mellifera*, J. Invertebr. Pathol. 92, 152-159.

Chen Y.P., Pettis J.S., Collins A., Feldlaufer M.F. (2006b) Prevalence and transmission of honeybee viruses, Appl. Environ. Microbiol. 72, 606-611.

Clapperton B.K., Moller H., Sandlant G.R. (1989) Distribution of social wasps (Hymenoptera: Vespidae) in New Zealand in 1987, N. Z. J. Zool. 16, 315-323.

Clark T.B. (1977) Another virus in honey bees, Am. Bee J. 117, 340-341.

Clark T.B. (1978) A filamentous virus of the honey bee, J. Invertebr. Pathol. 32, 332-340.

Crosland M.W. (1991) The spread of the social wasp, *Vespula germanica*, in Australia, New Zealand Journal of Zoology 18, 375-388.

Dall D.J. (1985) Inapparent infection of honey bee pupae by Kashmir and sacbrood bee viruses in Australia, Ann. Appl. Biol. 106, 461-468.

Dall D.J. (1987) Multiplication of Kashmir bee virus in pupae of the honey bee, *Apis mellifera.*, J. Invertebr. Pathol. 49, 279-290.

Davison S., Leat N., Benjeddou M. (2003) Development of molecular tools for honeybee virus research: the South African contribution, Afr. J. Biotechnol. 2, 867-878.

De Miranda J.R., Drebot M., Tyler S., Shen M., Cameron C.E., Stoltz D.B., Camazine S.M. (2004) Complete nucleotide sequence of Kashmir bee virus and comparison with acute bee paralysis virus, J. Gen. Virol. 85, 2263-2270.

DeJong D., Morse R.A., Eickwort G.C. (1982) Mite pests of honey bees, Ann. Rev. Entomol. 27, 229-252.

Ellis J.D., Munn P.A. (2005) The worldwide health status of honey bees, Bee World 86, 88-101.

Evans J.D. (2001) Genetic evidence for coinfection of honey bees by acute bee paralysis and Kashmir bee viruses, J. Invertebr. Pathol. 78, 189-193.

Faucon J.P., Vitu C., Russo P., Vignoni M. (1992) Diagnostic de la paralysie aiguë : application à l'épidémiologie des maladies virales en France en 1990, Apidologie 23, 139-146.

Faucon J.P., Mathieu L., Ribière M., Martel A.C.M., Drajnudel P., Zeggane S., Aurières C., Aubert M. (2002) Honey bee winter mortality in France in 1999 and 2000, Bee World 83, 14-23.

Fauquet C.M., Mayo M.A., Maniloff J., Desselberger U., Ball L.A. (2005) Virus Taxonomy, eighth report of the international committee on taxonomy of viruses, Elsevier Academic Press, London.

Finley J., Camazine S., Frazier M. (1996) The epidemic of honey bee colony losses during the 1995-1996 season, Am. Bee J. 136, 805-808.

Fyg W. (1963) Anomalies et maladies des reines d'abeilles, Bull. Apic. VI, 37-151.

Genersch E. (2005) Development of rapid and sensitive RT-PCR method for the detecteion of deformed wing virus, a pathogen of the honeybee (*Apis mellifera*), The Veterinary Journal 169, 121-123.

Genersch E., Yue C., Fries I., de Miranda J.R. (2006) Detection of deformed wing virus, a honey bee viral pathogen, in bumble bees (*Bombus terristris* and *Bombus pascuorum*) with wing deformities, J. Invertebr. Pathol. 91, 61-63.

Giauffret A., Caucat M.J., Amargier A., Bres N. (1967) Etude histopathologique de la maladie noire de l'abeille, Bull. Apic. X, 133-144.

Giauffret A., Lambert M. (1972) Le diagnostic de la maladie noire de l'abeille, Recl. Méd. Vét. CXLVIII, 869-877.

Gregory P.G., Evans J.D., Rinderer T., de Guzman L. (2005) Conditional immune-gene suppression of honeybees parasitized by Varroa mites, Journal of Insect Science 5, 1-5.

Hitchcock J.D. (1966) Transmission of sacbrood disease to individual honey bee larvae, J. Econ. Entomol. 59, 1154-1156.

Hornitzky M.A.Z. (1987) Prevalence of virus infections of honeybees in Eastern Australia, J. Apic. Res. 26, 181-185.

Hung A.C.F. (2000) PCR detection of Kashmir bee virus in honey bee excreta, J. Apic. Res. 39, 103-106.

Hung A.C.F., Shimanuki H. (1999) A scientific note on the detection of Kashmir bee virus in individual honeybees and *Varroa jacobsoni* mites, Apidologie 30, 353-354.

Hung A.C.F., Shimanuki H., Knox D.A. (1996) Inapparent infection of acute virus and Kashmir bee virus in the U.S. honey bees, Am. Bee J. 136, 874-876.

Hung A.C.F., Peng C.Y.S., Shimanuki H. (2000) Nucleotide sequence variations in Kasmir bee virus isolated from *Apis mellifera* L. and *Varroa jacobsoni* Oud, Apidologie 31, 17-23.

Koch W., Ritter W. (1991) Experimental examinations concerning the problem of deformed emerging bees after infestation with *Varroa jacobsoni*, Zentralbl. Veterinärmed. 38, 337-344.

Kulincevic J.M., Rothenbuhler W.C. (1975) Selection for resistance and susceptibility to hairless-black syndrome in the honeybee, J. Invertebr. Pathol. 25, 289-295.

Kulincevic J.M., Rothenbuhler W.C. (1989) The effects of artificial infection with chronic bee paralysis virus on queens from strains of honeybees resistant or susceptible to hairless-black syndrome, J. Apic. Res. 28, 79-80.

Kulincevic J.M., Rothenbuhler W.C., Stairs G.D. (1973) The effect of presence of a queen upon outbreak of a hairless-black syndrome in the honey bee, J. Invertebr. Pathol. 21, 241-247.

Marcangeli L., Monetti L., Fernandez N. (1992) Malformations produced by *Varroa jacobsoni* on *Apis mellifera* in the province of Buenos Aires, Argentina, Apidologie 23, 399-402.

Martin S.J. (2001) The role of *Varroa* and viral pathogens in the collapse of honeybee colonies: a modelling approach, J. Appl. Ecol. 38, 1082-1093.

Martin S.J., Hogarth A., van Breda J., Perrett J. (1998) A scientific note on *Varroa jacobsoni* Oudemans and the collapse of *Apis mellifera* L. colonies in the United Kingdom, Apidologie 29, 369-370.

Mussen E.C., Furgala B. (1977) Replication of sacbrood virus in larval and adult honeybees, *Apis mellifera*, J. Invertebr. Pathol. 30, 20-34.

Nordström S. (2003) Distribution of deformed wing virus within honey bee (*Apis mellifera*) brood cells infested with the ectoparasitic mite *Varroa destructor*, Exp. Appl. Acarol. 29, 293-302.

Nordström S., Fries I., Aarshus A., Hansen H., Korpela S. (1999) Virus infections in Nordic honey bee colonies with no, low or severe *Varroa jacobsoni* infestations, Apidologie 30, 475-484.

Ongus J.R. (2006) *Varroa destructor virus* 1: a new picorna-like virus in *Varroa* mites as well as honey bees, Wageningen University, Thesis.

Ongus J.R., Peters D., Bonmatin J.M., Bengsch E., Vlak J.M., van Oers M.M. (2004) Complete sequence of a picorna-like virus of the genus *Iflavirus* replicating in the mite *Varroa destructor*, J. Gen. Virol. 85, 3747-3755.

Overton H.A., Buck K.W., Bailey L., Ball B.V. (1982) Relationships between the RNA components of chronic bee-paralysis virus and those of chronic bee-paralysis virus associate, J. Gen. Virol. 63, 171-179.

Ribière M., Faucon J.P., Pépin M. (2000) Detection of chronic bee paralysis virus infection: application to a field survey, Apidologie 31, 567-577.

Ribière M., Triboulot C., Mathieu L., Aurières C., Faucon J.P., Pépin M. (2002) Molecular diagnosis of chronic bee paralysis virus infection, Apidologie 33, 339-351.

Ribière M., Chaouch S., Lallemand P., Schurr F., Faucon J.P., Aubert M. (2004) Asymptomatic contamination of adult honey bee by the chronic bee paralysis virus (CBPV). Viral load relative quantification assay by real time-PCR., in: Bernardinelli, I. and Milani, N. (Ed), Proceeding of the first european conference of apidology, EurBee, Udine, Italy, pp.94-95.

Ribière M., Lallemand P., Iscache A.L., Schurr F., Celle O., Blanchard P., Olivier V., Faucon J.P. (2006a) Infectious Chronic bee paralysis virus (CBPV) excretion in honey bee (*Apis mellifera L.*) faeces: a way of spread, in: Veselý, V. and Titera, D. (Ed), Proceeding of the second european conference of apidology, EurBee, Prague, Czech Republic, pp.21-22.

Rinderer T.E., Rothenbuhler W.C. (1975a) Responses of three genetically different stocks of the honeybee to a virus from bees with hairless-black syndrome, J. Invertebr. Pathol. 25, 297-300.

Rinderer T.E., Rothenbuhler W.C. (1975b) The fate and effect of hairs removed from honeybees with hairless-black syndrome, J. Invertebr. Pathol. 26, 305-308.

Rinderer T.E., Green T.J. (1976) Serological relationship between chronic bee paralysis virus and the causing hairless-black syndrome in the honeybee, J. Invertebr. Pathol. 27, 403-405.

Rinderer T.E., Rothenbuhler W.C. (1976) Characteristic field symptoms comprising honeybee hairless-black syndrome induced in the laboratory by a virus, J. Invertebr. Pathol. 27, 215-219.

Santillan-Galicia M.T. (2006) Characterisation of deformed wing and slow paralysis virus transmission by *Varroa destructor*, University of Nottingham, Thesis.

Shen M., Cui L., Ostiguy N., Cox-Foster D. (2005a) Intricate transmission routes and intercations between picorna-like viruses (Kashmir bee virus and sacbrood virus) with the honeybee host and the parasitic varroa mite, J. Gen. Virol. 86, 2281-2289.

Shen M., Yang X., Cox-Foster D., Cui L. (2005b) The role of varroa mites in infections of Kashmir bee virus (KBV) and deformed wing virus (DWV) in honey bees, Virol. 342, 141-149.

Siede R., Büchler R. (2003) Symptomatic black queen cell virus infection of drone brood in Hessian apiaries, Berl. Munch. Tierarztl. Wochenschr. 116, 130-133.

Siede R., Büchler R. (2004) First detection of Kashmir bee virus in Hesse, Berl Munch. Tierarztl. Wochenschr. 117, 12-15.

Siede R., Derakhshifar I., Otten C., Berenyi O., Bakonyi T., Koglberger H., Büchler R. (2005) Prevalence of Kashmir bee virus in Central Europe, J. Apic. Res. 44, 129.

Simpson K. (1983) Situation report on honey bee disease and apiculture in Fiji survey 8-18 August 1983. Ministry of Agriculture and Fisheries Report; Oamaru,New Zealand.

Sitaropoulou N., Neophytou E.P., Thomopoulos G.N. (1989) Structure of the nucleocapsid of a filamentous virus of the honey bee (*Apis mellifera*), J. Invertebr. Pathol. 53, 354-357.

Stoltz D., Xue-Ren S., Boggis C., Sisson G. (1995) Molecular diagnosis of Kashmir bee virus infection, J. Apic. Res. 34, 153-160.

Tentcheva D., Gauthier L., Jouve S., Canabady-Rochelle L., Dainat B., Cousserans F., Colin M.E., Ball B.V., Bergoin M. (2004a) Polymerase chain reaction detection of deformed wing virus (DWV) in *Apis mellifera* and *Varroa destructor*, Apidologie 35, 431-439.

Tentcheva D., Gauthier L., Zappulla N., Dainat B., Cousserans F., Colin M.E., Bergoin M. (2004b) Prevalence and seasonal variations of six bee viruses in *Apis mellifera* L. and *Varroa destructor* mite populations in France, Appl. Environ. Microbiol. 70, 7185-7191.

Tentcheva D., Gauthier L., Bagny L., Fievet J., Dainat B., Cousserans F., Colin M.E., Bergoin M. (2006) Comparative analysis of deformed wing virus (DWV) RNA in *Apis mellifera* and *Varroa destructor*, Apidologie 37, 41-50.

Topolska G., Ball B., Allen M. (1995) [Identification of viruses in bees from two Warsaw apiaries], Medycyna Weterynaryjna 51, 145-147.

Varis A.L., Ball B.V., Allen M. (1992) The incidence of pathogens in honey bee (*Apis mellifera* L) colonies in Finland and Great Britain, Apidologie 23, 133-137.

Vidano C., Arzone A. (1978) Osservazioni al microscopio elettronicco di strane particelle presenti in api operaie malate, Apicoltore M. 69, 1-4.

White G.F. (1917) Sacbrood., US Department of Agriculture Bulletin N°431.

Wiegers F.P. (1986) Transmission of honeybee viruses by *Varroa jacobsoni* Oud. European research on varroatosis control, Udine, Italy, in: Cavalloro, R. (Ed), Commission of the European Communities, Joint Research Centre, Ispra, Luxembourg.

Wille H. (1967) Mischinfektionen in der Honigbiene (*Apis mellifica* L.) nach Ermittlungen in Schweizerischem Material der Jahre 1965/66, Z. Bienenforsch. 9, 150-171.

Wille H., Pinter L. (1961) Recherches sur les septicemies bactériennes de l'abeille adulte en Suisse, Bull. Apic. VI, 162-178.

Yang X., Cox-Foster D. (2005) Impact of an ectoparasite on the immunity and pathology of an invertebrate: evidence for host immunosuppression and viral amplification, Proc. Natl. Acad. Sci. U. S. A 102, 7470-7475.

Yue C., Genersch E. (2005) RT-PCR analysis of deformed wing virus in honeybees (*Apis mellifera*) and mites (*Varroa destructor*), J. Gen. Virol. 86, 3419-3424.

Yue C., Schröder M., Bienefeld K., Genersch E. (2006) Detection of viral sequences in semen of honeybees (*Apis mellifera*): evidence for vertical transmission of viruses through drones, J. Invertebr. Pathol. 92, 105-108.

Chapter 2

MOLECULAR CHARACTERISATION OF HONEY BEE VIRUSES

Mike CARTER and Elke GENERSCH

Table of contents

1. Introduction

Thanks to the development of molecular biology extraordinary progress has been made over the last fifty years in all fields of biomedical science. Although human, plant and animal virology also profited from this progress, bee virology is still in its infancy. Currently, we know the nucleotide sequences of some but not all bee viruses, but the identity of target cells or organs, pathogenic mechanisms, and virulence determinants remain elusive. Research might have been hampered by (i) the complex biology of bees as holometabolic insects, (ii) the lack of clear clinical symptoms in many infections, (iii) the lack of a cell culture system to easily propagate the virus, and (iv) the fact that most of the bee viruses are RNA-viruses. This review will focus on the unique genetic plasticity of RNA-based viruses, which led to adoption of the quasispecies concept by virologists. The concept of the quasispecies nature of RNA-based viruses will be introduced, the mechanisms generating genetic variability will be explained, and the issue of PCR-introduced errors in the evaluation of genetic divergence will be discussed. Against this background, the two taxonomic entities among honey bee viruses, Iflaviruses and Dicistroviruses, will be covered in detail and finally the applicability and validity of the quasispecies concept will be discussed for selected honey bee viruses.

2. Genetic variability and the quasispecies nature of RNA-based viruses

Genetic information is encoded by DNA and RNA. Most species use the chemically stable DNA to store their genetic information and the less stable RNA for its expression. The only known organisms using RNA as genetic material are RNA-based viruses, namely RNA viruses and retroviruses.

Viruses are obligate cellular parasites which normally use the host machinery for transcription, translation and replication. In the case of RNA-based viruses the very efficient and accurate host replication machinery cannot be used for replication. Instead they have to use their own RNA-dependent RNA polymerase (RNA viruses) or RNA-dependent DNA polymerase (retroviruses) to replicate their genomes. Since in contrast to DNA-dependent DNA polymerases these RNA-dependent viral replicases lack proofreading activity (3'-5' exonuclease activity), the mutation rate of the RNA virus genome is several orders of magnitude greater than that of DNA genomes. In fact, RNA-based viruses show the highest mutation rates among living beings (Drake *et al.*, 1998). Thanks to their error prone

replication RNA-based viruses reveal an enormous genetic plasticity and, therefore, have a unique ability to adapt to new situations (Fig. 1). In addition, large population sizes, short generation times and high replication rates also play a role in the fast evolutionary rates of RNA-based viruses.

The unusual heterogeneity of RNA viruses was first observed by Weissmann and co-workers in the late seventies, whose study object was phage Qβ, an RNA-based bacterial virus infecting *Escherichia coli* (Batschelet *et al.*, 1976; Domingo *et al.*, 1978). After repeatedly passaging a phage population they found that each viable phage genome differed in one or two positions from the average sequence (master sequence) of the parental population. Hence, this phage population was not at all homogenous but rather a pool of genetically distinct although related variants. Later on it became clear that this view holds true for all RNA-based viruses. To describe such a complex viral population structure the concept of viral quasispecies was introduced. The term quasispecies is used by virologists to describe the whole population of phylogenetically related variants which are present in a single infected organism. In this concept, the wild type is no longer considered an individual genome with a defined nucleotide sequence, but an ensemble of closely related genomes on which

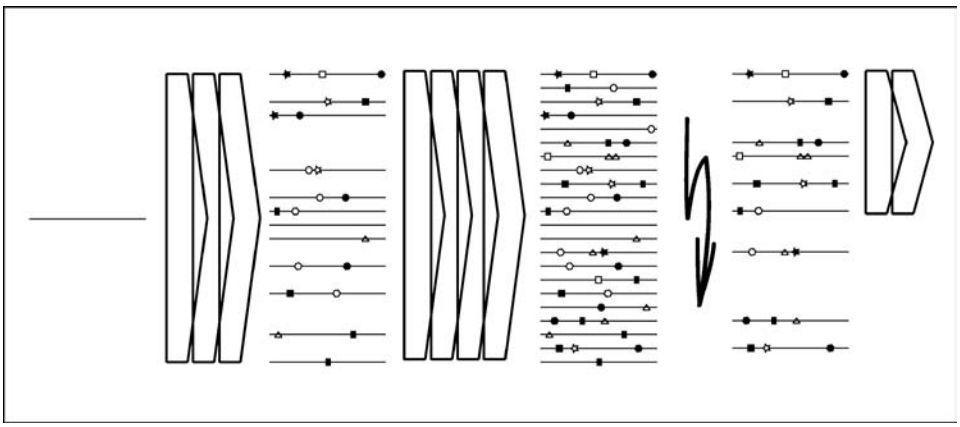


Figure 1. RNA-based viruses consist of complex distributions of mutant genomes termed viral quasispecies. Lines represent individual RNA genomes and symbols on the lines mutations. With each replication cycle new mutants arise until the population forms a mutant swarm having a huge adaptive potential. Because of their complexity at the population level, quasispecies are capable of responding rapidly and effectively to perturbations of the environment (indicated by lightning arrow) in which they replicate. There will always be mutants surviving the perturbation and even some being able to replicate under the new circumstances.

selective forces act. The target of selection, therefore, is not a single fittest genotype but rather a cloud of mutants (mutant spectrum or mutant swarm) distributed around a most frequent one (Eigen, 1996).

There is an ongoing debate about whether the term quasispecies is correctly used by virologists or is simply and inappropriately used as a surrogate for intrapopulation genetic variation (Eigen, 1996; Holmes and Moya, 2002; Domingo, 2002). Indeed, by applying this term to the mutant spectra observed for RNA-based viruses, the original concept (Eigen, 1971) is not rigorously used. Yet, from the current knowledge of the composition of mutant spectra, polymorphism does not seem an accurate term either (Domingo, 2002).

Most likely, the concept of quasispecies will also apply for at least some honey bee RNA-viruses. Therefore, for a certain viral infection we have to expect a population of viruses differing in their genomic sequence even within a single infected bee or larva. But as with all other RNA-based viruses, there will be a dynamic equilibrium in the individual bee/larva as well as in the colony, with viable mutants arising at high rates and then being selected by the host for infectious, well-replicating, well-adapted genotypes.

3. Mechanisms generating genetic variability

A systematic analysis of virus populations from infected organisms revealed a large number of modifications present in the RNA genomes of one population. In the most general terms, these modifications fall into two classes: point mutations and more complex rearrangements of genomic molecules. While point mutations result from error prone replication or cellular RNA-editing processes, rearrangement of viral RNA genomes are the product of RNA recombination or genetic reassortment.

RNA-based viruses fall into two main classes: retroviruses and RNA-viruses. For replication, retroviruses are reverse transcribed into dsDNA, integrated into the host genome and replicated by the host's replication system.

The genome of RNA-viruses can be composed of dsRNA or ssRNA. ssRNA genomes can be either of positive sense ((+)RNA) or negative sense ((-)RNA). The entire RNA-replication process is catalysed by virus encoded RNA-dependent RNA polymerases lacking proofreading activity. During the first step of replication the plus strand is used as template to produce the minus strand in plus-stranded RNA-viruses and vice versa in minus-stranded RNA-viruses. Then the newly obtained RNA strand of opposite sense is used to generate new RNA molecules which now have again the

correct orientation to be packed into virions. A number of distinct biochemical mechanisms may generate diversity. These are considered briefly below and comprise polymerase error, RNA editing and RNA recombination.

Genetic and biochemical investigations have indicated that the mutation rate μ_g (expressed per genome per replication) for lytic RNA viruses ranges between 0.8 (Poliovirus) and 6.5 (bacteriophage Qβ) (Drake *et al.*, 1998) resulting in 10^{-3} to 10^{-5} misincorporations per nucleotide copying event (Holland *et al.*, 1982; Drake, 1993). In contrast, spontaneous mutation rates μ_g average roughly 0.2 for retroelements, close to 0.0034 for DNA-based microbes (including both viral and cellular organisms), and around 0.01 for higher eukaryotes (Drake and Holland, 1999). When the frequency of viral genome mutation due to error prone replication was determined *in vivo* for influenza A virus and poliovirus it turned out to be 10^{-4} to 10^{-5} mutations per incorporated nucleotide (Parvin *et al.*, 1986; Smith and Inglis, 1987). Theoretically this means that around one nucleotide can be incorporated incorrectly during one to ten replication cycles of a genome approximately 10^4 nucleotides in length. Taking into account the massive number of viral particles produced during infection these numbers illustrate what an enormous evolutionary potential the error-prone replication of RNA-viruses gives to these viruses.

The most frequently observed mutations are nucleotide substitutions, mostly transitions (U to C and G to A changes). They result from the RNA-polymerase introducing U instead of C or G instead of A into the nascent strand. Since U can form a weak although stable base pair with G, the viral replication complex is not disturbed by this pairing and continues its task.

Before being packed into virions, viral genomic molecules can also serve as substrates for enzymes involved in cellular RNA editing. Some viral RNAs obviously are suitable substrates for adenosine deaminase (ADAR) which catalyses the deamination of adenosine to inosine (Maas *et al.*, 1997). Since inosine preferentially pairs with cytidine, this editing event results in the substitution of G for A.

Although, RNA recombination was described for picornaviruses as early as 1963 (Ledinko, 1963) it took more than forty years to establish that RNA recombination occurs in all RNA-based viruses (Lai, 1992; Plyusnin *et al.*, 2002). Viral RNA recombination takes place during viral replication via a so-called copy-choice mechanism (Fig.2). The underlying principle is that the polymerase engaged in replication switches from one RNA template to another. The proposed model of genetic recombination in picornaviruses assumes that template switching events occur preferentially during minus-strand synthesis. Exchange of genetic material can occur within a viral population, between different viral strains (Keck *et*

al., 1988) or different species (Smith *et al.*, 2000), and even with host RNA (Meyers *et al.*, 1991). Based on these data it is conceivable that RNA recombination is one of the major factors responsible for the generation of new, often dangerous viral strains or species.

Which role the outlined mechanisms play in the generation of genetic variability in honey bee RNA viruses is still an open question although infections of bees with multiple viruses (Chen *et al.*, 2004) makes it likely that even recombination between different viruses might have occurred at some time during the co-evolution of bees and viruses or still does occur.

4. Repeatability and reliability in the evaluation of genetic variability

To analyse the genetic variability of RNA viruses and the properties of such variable populations it is necessary to know the nucleotide sequence of the constituting genomes. There are two main methodologies for this. Both methods proceed through reverse transcription and PCR amplification first. Then the PCR amplified fragments are either subjected to direct sequencing or are cloned into appropriate vectors and transformed, before clones derived from a single DNA molecule are sequenced.

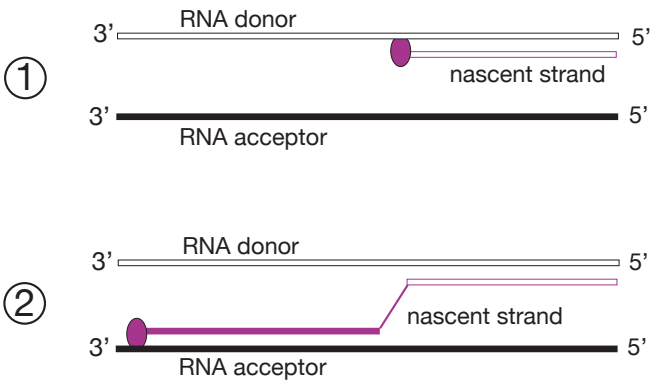


Figure 2. Copy-choice mechanism. Viral recombinants are generated during the replication process. Viral polymerase starts nascent strand synthesis on one template (RNA donor, step 1) and then it switches to another template (RNA acceptor). The resultant recombinant sequence derives from two different RNA templates. For homologous recombination, these templates are identical or very similar and belong to representatives of the same population, for non-homologous recombination RNA donor and acceptor are different, even from different species.

Sequencing reactions themselves are nowadays usually performed using PCR protocols. It is thus reasonable to ask whether data obtained in this way is repeatable when it is the product of one RT reaction and several independent PCR amplifications. Error rates of amplification polymerases vary (Tab. I). Those for *Taq* DNA polymerase which lacks 3'-5' exonuclease proofreading activity have been calculated to range from 0.2 to 2 x 10^{-4} errors per bp per cycle, whilst error rates for the *Pfu* DNA polymerase (which does possess 3'-5' exonuclease proofreading activity) have been reported to be three to ten times lower (Cline *et al.*, 1996; Lundberg *et al.*, 1991; Bracho *et al.*, 1998). In addition, reaction conditions can also influence the error rate of the thermostable polymerases used (Vartanian *et al.*, 1996). Consequently product fidelity depends on the polymerase, several reaction parameters (e.g. ionic components of the buffer, the relative concentration of nucleoside triphosphate substrates), and on the sequence context at the template site being copied. However, it is not just PCR amplification errors that have a direct impact on the estimation of genetic variability of RNA-based viruses. In addition, the retrovirus reverse transcriptases used to polymerize cDNA *in vitro* from viral RNA prior to PCR amplification do not work 100% accurately (Williams and Loeb, 1992). Hence, it is not possible to distinguish between misincorporations that occur during *in vitro* reverse transcription and heterogeneities already present in the virus population. In fact the error rate of Moloney murine leukemia virus reverse transcriptase, widely used in RT reaction protocols, on RNA templates *in vitro* is less than one error per 28 000 nucleotides. This is more or less the same error rate estimated for viral RNA polymerases *in vivo* (Ji and Loeb, 1992). Therefore, the *in vitro* RT reaction might introduce errors at the same rate as the viral replication *in vivo*.

Based on these considerations a few authors attributed the reported complexity of populations of RNA-based viruses to artifactual mutations

Table I. Substitution error frequencies for Taq- and Pfu-DNA polymerases expressed as errors per bp per cycle.

Polymerase error rate (x10^{-4})*			Reference
Taq	Pfu		
0.27	< 0.03	(a)	Bracho et al., 1998
0.08	0.013	(b)	Cline et al., 1996
0.2	0.01	(b)	Lundberg et al., 1991

* Error rates were obtained by: (a): sequencing of recombinant plasmids from a PCR product; (b): by reversion assay from a PCR product

introduced during the processes of reverse transcription and PCR amplification (Smith *et al.*, 1997). Studies have shown that *Taq*-polymerase-induced errors may indeed exaggerate the extent of RNA virus diversity, especially when used under non-optimized reaction conditions (Bracho *et al.*, 1998). However, this problem could be overcome by using the relatively error-free *Pfu*-polymerase instead. Data obtained for vesicular stomatitits virus (VSV) and hepatitis C virus (HCV) using *Pfu*-polymerase and optimized PCR amplification conditions revealed a reproducible degree of nucleotide diversity which was consistent with the virologists' concept of quasispecies (Bracho *et al.*, 1998; Torres-Puente *et al.*, 2003). Even more important, it has to be pointed out here that the quasispecies nature of RNA-based viruses was documented before *in vitro* amplification procedures were available (Batschelet *et al.*, 1976; Domingo *et al.*, 1978). Therefore, even if some studies based on sequence data obtained via inappropriate DNA amplification procedures do overestimate sequence variability in certain cases this does not violate the basic principle of unique sequence heterogeneity in RNA-based virus populations as a result of an unusually error-prone virus replicase combined with a high replication rate.

Another problem for the reliability of sequence data is posed by the sequencing template. The analysis of cloned sequences is less reliable than the analysis of directly sequenced PCR products derived by amplification of a single target molecule. When PCR products are directly sequenced an error produced during PCR amplification will represent a minority in the population of amplification products unless the PCR reaction is performed at limiting target concentration and the error occurs very early in the amplification process. Consequently most cycle errors will not be detected against the background of products amplified from the majority of molecules that have no mutation at this position (Simmonds *et al.*, 1990). In contrast, a sequenced clone represents a single amplified product; if this happens to harbour a misincorporation it will appear as the definitive base at that position and the majority of correctly amplified PCR products will remain unsequenced.

Consequently in any study of diversity it is essential to determine the suitability of the methods proposed. To address these issues a preliminary evaluation of the state-of-the-art procedures for their suitability to study virus diversity (e.g. deformed wing virus, DWV) was performed (E. Genersch, unpublished data). RNA was extracted from six worker bees from each of 2 different colonies (designated #357 and WaV in Table II) and a 355 bp-fragment from the 5'-region of the DWV-genome (pos. 3722 – pos. 4076) amplified. These PCR products were directly sequenced, each by five independent sequencing reactions. After optimisation of the

Table II. Sequence homologies obtained for a 355 bp-fragment of DWV (pos. 3722 – 4076) from six worker bees from hive #357 and as many from hive WaV.

#357	*#2*	*#3*	*#4*	*#5*	*#6*
#1	100	99.7	100	100	100
#2		99.7	100	100	100
#3			99.7	99.7	99.7
#4				100	100
#5					100

WaV	*#2*	*#3*	*#4*	*#5*	*#6*
#1	100	100	99.4	99.1	100
#2		100	99.4	99.1	100
#3			99.4	99.1	100
#4				99.7	99.4
#5					99.4

PCR- and sequencing-conditions the five independent sequencing reactions performed on one template were always 100% identical. The sequences (316 nucleotides) derived for the six individual bees of hive #357 were 99.7 to 100% identical (one A – G transition), whereas for hive WaV sequence homology ranged from 99.1 to 100% (two G – A transitions and one C – U transition). Since the observed sequence variant of hive #357 did not overlap with those of hive WaV an overall sequence variation of 1.3% was determined. These initial data suggest that reproducibility and reliability of the method is suitable for the analysis but the findings are insufficient in themselves to demonstrate the quasispecies nature of these viruses. More detailed studies will be required to address this question.

5. Taxonomy and phylogeny of honey bee viruses

With two exceptions (filamentous virus and *Apis* iridescent virus) all viruses identified in the honey bee (*Apis mellifera*) so far are positive stranded RNA-viruses. It is possible and reasonable to use sequence data to interpret the taxonomy of bee viruses and the relationship (phylogeny)

between different isolates. Quasispecies variation simply generates a cluster of closely related sequences in each taxonomic position rather than a single entity. Indeed sequence based classification is increasingly taking over from more traditional biologically based classification methods in all virus families.

One major family among the positive-stranded RNA-viruses infecting mammals was termed the *Picornaviridae* (small (pico) RNA viruses) and originally described as small (28nm), naked and featureless icosahedral particles. Their capsids were composed of 3-4 types of protein which enclosed an RNA genome of positive sense. The genome is organized into a single open reading frame (ORF) with the specification of structural proteins at the 5' terminus and non-structural proteins at the 3'. A specialised RNA structure at the 5' end of the genome, termed an internal ribosome entry site (IRES), permits the RNA to be translated directly by ribosomes bypassing the normal requirement for a methylated cap.

It was quickly established that the honey bee RNA-viruses resembled the better studied mammalian viruses in size, genome composition, biophysical properties and possession of more than one capsid protein. Consequently these viruses joined a large and heterogenous group of unassigned and poorly characterised viruses of insects, vertebrates and plants referred to as "picornavirus-like". The chronic bee paralysis virus possesses an RNA genome but is not picorna-like. Instead this virus has an unusual ellipsoid structure and possesses 5 molecules of RNA (Bailey 1976; Overton *et al.*, 1978). However, this may be complicated by the presence of satellite viruses which are dependent on the chronic bee paralysis virus for their replication and which themselves appear to contain at least 3 species of RNA (Overton *et al.*, 1982). Despite more recent examination of its polypeptide composition (Ribière *et al.*, 2000) and the determination of some partial sequences from the RNA polymerase, it remains unclassified. It is only relatively recently that molecular investigations have provided greater insight into this designation and allowed the construction of new taxonomic groupings for these agents. The honeybee viruses have been grouped with other invertebrate viruses into two major classifications: a floating genus *Iflavirus* as yet unassigned to any virus family but with some of the features of *Picornaviridae* (Christian *et al.*, 2002a) and a new family, the *Dicistroviridae* (Christian *et al.*, 2002b). Each grouping is constructed around a type virus for the group against which features of both current and potential members may be compared: these are cricket paralysis virus in the case of the Dicistroviruses and Infectious flacherie virus on which the Iflavirus genus is based. Dicistroviridae and Iflavirus now fall into the Picornavirus superfamily along with other agents infect-

ing both higher animals and plants, and form a part of the proposed order of positive stranded RNA-viruses the *Picornavirales*.

5.1. Dicistroviridae

The family *Dicistroviridae* contains a single genus (*Cripavirus*) and shows a distinct genomic organisation. Table III lists the currently confirmed and suspected members of the family. The type virus is cricket paralysis virus (CrPV) (Wilson et al., 2000b) and bee-infecting members include black queen cell virus (BQCV) (Leat et al., 2000) and Acute bee paralysis virus (ABPV) (Govan et al., 2000). Other honey bee viruses, such as Kashmir bee virus (KBV) (de Miranda et al., 2004), are likely to be assigned here in future. Only one partial sequence (RNA polymerase) has been presented for cloudy wing virus (accession no AF034543) and this is identical to that of Kashmir bee virus in the same region. Such identity at the nucleotide level is unusual and may indicate that the virus sequenced was misidentified or it may indicate a close relationship between these two viruses.

Based on the designation of these viruses as "picornavirus-like" early investigations had anticipated a virus with a similar genomic organisation (i.e. structural proteins at the 5' end and non structural proteins at the 3' end) but it quickly became apparent that this was not so (Koonin and Gorbalenya, 1992; Sasaki et al., 1998). In contrast the structural genes were located at 3' end and non-structural at the 5'. Perhaps more significant than this reversal in gene order was the observation that these proteins were encoded in distinct open reading frames, or cistrons. This arrangement was fundamentally different from that of the picornaviruses and ultimately gives rise to the family name for these viruses. In effect this gene order resembled at first sight that of the caliciviruses and the comparative organisation of these virus genomes is illustrated in Figure 4. Separate open reading frames specify non-structural and structural proteins respectively in both cases. Sequence motifs indicative of an NTP binding protein (possible helicase), genome-linked protein, protease and RdRp were also apparent in these sequences; they were distributed in the same order along the polyprotein and the projected proteins also showed some relationship. However, the Dicistroviruses possess extended 5' non translated regions (NTR) preceding the open reading frame in comparison with the *Caliciviridae* and the intergenic region lying between ORFs is likewise extended from only a few bases in caliciviruses to approximately 160 to 200 nucleotides in the *Dicistrovidae*. Although the 5' NTR differs in sequence between Dicistroviruses the intergenic region is more conserved (VanMunster et al., 2002). These differences suggested that the Dicistro-

Table III – Members of the Dicistroviridae

Dicistroviridae – Single Genus: Cripavirus		Reference*
Type member	Cricket paralysis virus	Wilson et al., 2000b
Others	*Drosophila* C virus	Johnson and Christian, 1998
	Plautia stali intestinal virus	Sasaki et al., 1998
	Rhopalosiphum padi virus	Moon et al., 1998
	Himetobi virus	Nakashima et al., 1999
	Triatoma virus	Czibener et al., 2000
	Black queen cell virus	Leat et al., 2000
Probable	Acute bee paralysis virus	Govan et al., 2000
Tentative	Kashmir bee virus	de Miranda et al., 2002
	Cloudy wing virus	accession no AF034543
	Aphid lethal paralysis	van Munster et al., 2002
	Taura syndrome virus	Robles-Sikisaka et al., 2001
	Solenopsis invicta virus	Valles et al., 2004

* Reference reporting sequence determinations on which assignment is based

viruses were neither bonafide Picornaviruses nor Caliciviruses, and led to their recognition as a distinct family. They also suggested that there would be novel features in the manner in which these viruses performed protein synthesis.

The observation of calicivirus-like gene order in Dicistroviruses implies the existence of some mechanism for the regulation of gene expression. Caliciviruses for instance encode their non-structural proteins at the 5' end and these are translated immediately from the incoming genome. In contrast capsid proteins are expressed from a separate subgenomic (sg) RNA synthesised in turn by the non-structural protein (RdRp) of the virus and corresponding to the 3' ORFs (Fig. 3).

In contrast Dicistroviruses do not synthesise a sg mRNA (Wilson *et al.*, 2000b), instead regulation of these two classes of protein is thought to be achieved at the translation level by a refinement of the internal ribosome entry system first identified in picornaviruses (Martinez-Salas and Fernandez Miragall, 2004) Study of these had already shown that extended 5' NTR regions are often indicative of the presence of internal ribosome entry sites (IRES). These are regions of folded RNA that permit ribosome assembly onto the interior of an RNA molecule. This bypasses the normal

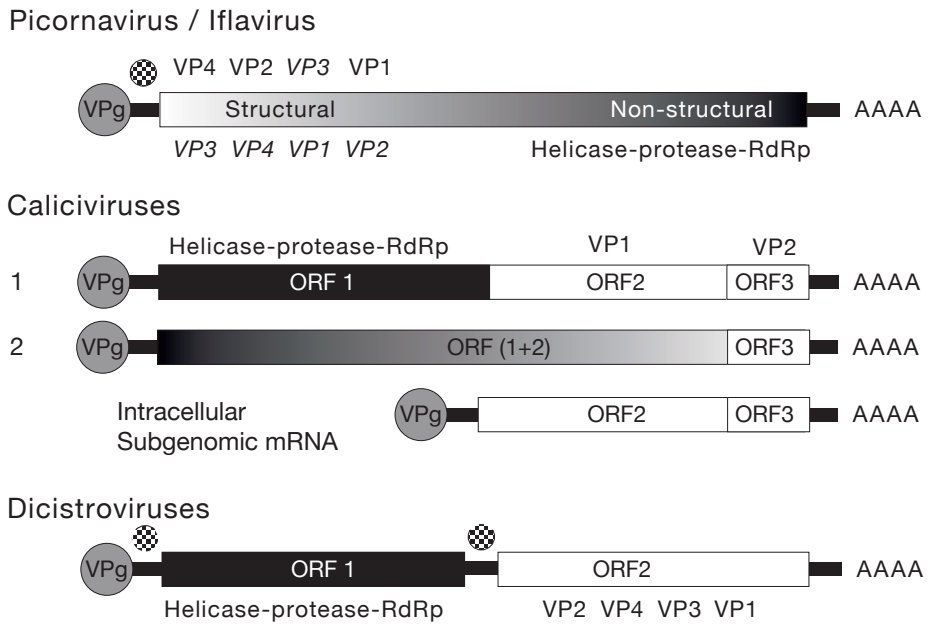


Figure 3. Comparative genome organisation of relevant positive strand RNA viruses: Picornavirus/Iflaviruses, Caliciviruses and Dicistroviruses.

All genomes are thought or known to commence with a covalently linked protein, termed VPg, at their 5' terminus and bear a poly-adenylic acid tail at their 3' terminus. Open reading frames (ORFs) are indicated, non-structural protein encoding ORFs are shown in black, and structural protein reading frames in white. Sharp transitions indicate a stop codon separating adjacent ORFs, shaded transition indicates readthrough translation of both. The presence of internal ribosome entry site (IRES) structures are indicated where applicable by the hatched circle preceding an open reading frame. The two variants of Calicivirus genome organisation are indicated (1) Vesivirus and Norovirus genera; (2) Lagovirus and Sapovirus genera. Subgenomic mRNA indicated is formed intracellularly to direct synthesis of protein from ORFs 2 and 3. Calicivirus VP1 matures by proteolytic cleavage of the ORF2-specified protein in Vesiviruses but not in Lagoviruses or Noroviruses. VP2 is a basic and minor protein constituent of the calicivirus capsid. The positions of structural motifs indicative of protein function are indicated. Non-structural proteins: Helicase, NTP binding protein (presumptive helicase activity); Protease (polyprotein maturation enzyme); RdRp, RNA dependent RNA polymerase. VP1, VP2, VP3 and VP4 are virus structural proteins indicated in the order they are encoded along the genome. (Not to scale).

means of translation initiation in the eukaryotic cell which depends on assembling a ribosome using the cap structure located at the 5' end of the mRNA to bind the 40S subunit. This is followed by downstream "scanning" until a favourable initiation codon is located and ribosome assembly is completed. Internal initiation may still require many host cell initiation factors but cap recognition is not needed and some viruses actively disrupt the cap recognition system to inhibit host cell translation and favour virus protein synthesis (Belsham et al., 2000). Internal assembly effectively assembles and directs a ribosome to the required initiator AUG (usually located close to the IRES) and avoids spurious AUG codons that may be situated upstream (i.e. more 5') of the IRES. IRES activity has been detected at both 5' and intergenic region (IGR) NTRs of CrPV (Wilson *et al.*, 2000b) and in the IGR of several Dicistroviridae (Wilson *et al.*, 2000b; Domier and McCoppin, 2003; Sasaki and Nakashima, 1999). Further, Wilson and colleagues (2000b) inserted each IRES element from cricket paralysis virus into artificial, uncapped mRNA molecules such that they could direct the translation of a reporter protein whose synthesis could be measured. They were then able to show that the IGR IRES (which would normally direct structural protein synthesis) led to an increased intracellular accumulation of reporter protein than did the 5' NTR IRES. This indicates that Dicistroviruses may be able to regulate the quantities of their proteins at translational level by differential IRES activity. However it is clear that both IRES elements have functional differences which render them distinct from the picornavirus IRES elements previously characterised.

Before the characterisation of the Dicistroviruses all IRES elements tested had functioned only in translation within animal cells; activity in rabbit reticulocyte lysates was stimulated by different cell factors (Dorner et al., 1984; Glass and Summers, 1993) and activity in cells varied with cell type (Roberts et al., 1998) but none had proved functional in plant cells. The first IRES demonstrated to function in this system was the IGR (but not the 5') IRES of CrPV. This was followed by the 5' IRES from *Rhopalosiphum padi* virus (RhPV) (Wilson, *et al.*, 2000b; Woolaway et al., 2001). The fact that such activity has not been found in any virus of vertebrates may well indicate something of the evolutionary relatedness between insect and plant viruses or of the similarities between insect and plant cells themselves. Although neither RhPV nor CrPV have been found to replicate in plants several insect-vectored plant viruses are able to replicate in both hosts, infection of the insect from the plant leading to lifelong transmission. Such viruses must presumably contain functional features applicable to both host cell systems.

However perhaps the most striking difference between Dicistroviral and other IRES elements is that the IGR IRES directs translational initiation at non-AUG codons; for instance capsid protein translation commences at CAA in the *Plautia stali* intestinal virus (PSIV; Sasaki and Nakashima, 1999), at CCU in RhPV (Domier et al., 2000) and in CrPV (Wilson, *et al.*, 2000b). Other viruses do possess an AUG codon at or near the start of ORF 2 (e.g. Taura syndrome virus) (Mari et al., 2002) but might nonetheless be able to use non-AUG codons. These IRES elements are believed to function through their tertiary structure which forms specific interactions with the ribosome, occupying the P-site (normally used at initiation by tRNA Met) with a pseudoknot structure and positioning the ribosome A-site over the non-AUG codon to be used to commence protein synthesis. This permits them to assemble complete functional 80S ribosomes without requirement for host cell initiation factors (RajBhandary, 2000; Wilson et al., 2000a). The ribosome is thought to initiate protein synthesis following an initial and unconventional translocation event in which no peptide bond is formed but the aminoacylated tRNA bound in the A site is moved to the P site. This step does require elongation factor 1A to deliver the first aminoacylated tRNA and elongation factor 2 which catalyses its translocation (Jan et al., 2003; Pestova and Hellen, 2003). The structure of the IRES is complex; secondary structure analysis predicted the existence of multiple stem-loops which could fold to form 3 pseudoknots. These were grouped into three domains, two of which were predicted to form a packed core structure (Kanamori and Nakashima, 2001). These structural features are conserved in PSIV, *Drosophila* C virus, RhPV, Himetobi virus (HiTV), *Triatoma* virus, and black queen cell virus (Kanamori and Nakashima, 2001). Functional analysis then showed that these were essential for the correct functioning of the IRES and revealed a complex pattern of flexible ribosome-IRES contacts that together functionally replace many of the usual cellular translational initiation factors (Jan and Sarnow, 2002). The structural features predicted were confirmed in a cryo-electron microscopy (cryo-EM study that also demonstrated ribosome-IRES contact right across the structure of the ribosome, involving both subunits and P, A and E sites. Furthermore these interactions appeared flexible allowing the IRES to manipulate the ribosome (Spahn et al., 2004). IRES structure is probably prefolded into a two part structure before binding to ribosome 40S subunits (Costantino and Kieft, 2005).

Van Munster and colleagues performed a phylogenetic analysis of the Dicistroviruses (Van Munster *et al.*, 2002). It is apparent from this data that the bee viruses do not cluster together. BQCV was grouped with PSIV, TrV and HiTV whereas ABPV was separated from the groupings on

its own. Including the recently sequenced honey bee virus KBV into such an analysis revealed that it clusters relatively closely together with ABPV (Fig. 4). The close relationship of these viruses has already been reported (Anderson, 1991; Allen and Ball, 1995). However, the two viruses are unambiguously distinguishable, both by serological (Stoltz *et al.*, 1995) and by molecular biological methods (Stoltz *et al.*, 1995; Evans, 2001; de Miranda, 2004). ABPV and KBV have been compared on the basis of genetic variability using BLAST analysis of open reading frames and in terms of protein similarity as presented in Figure 5 (non-structural) and Figure 6 (structural).These viruses (and BQCV as a member of the same group) are each aligned with the type virus of the group (CrPV) and also two by two against each other. Sequence divergence is indicated by a gap or deviation in the alignment. This is evident in the region between the helicase and 3C-like protease (Fig. 5) and also around 280 in the capsid proteins (Fig. 6). ABPV, KBV and BQCV are clearly related to CrPV across their sequence although identity scores are only around 20-25% in all cases. When aligned against each other, KBV and BQCV are related most closely in the helicase and RdRp areas. These are likely to be well conserved between RNA viruses since there is selection pressure to maintain enzyme function and thus to limit divergence in these genes. In contrast, KBV is closely related to ABPV with an identity score of over 70% in both open reading frames (Fig. 6).

5.2. Iflaviruses

The Iflaviruses are a floating genus, currently unassigned to any family (Christian *et al.*, 2002b). Iflaviruses have a genome order similar to that of the Picornaviruses, a single open reading frame encodes their structural proteins at the 5' end and non-structural proteins towards the 3'. However sequence analysis reveals they are distinct from this *Picornaviridae* and *Dicistroviridae* (Christian *et al.*, 2002a). The type virus for the genus is Infectious Flacherie virus (IFV) and current and probable members are listed in Table IV. These have been divided into 4 groups on the basis of sequence comparisons performed by N. Knowles (personal communication and http://www.iah.bbsrc.ac.uk/virus/Iflavirus – accessed 04-03-2005). Comparative sequence analysis between sacbrood virus and other members shows that similarity is relatively low and patchy (Fig. 7). Sacbrood has a fairly constant relation to IFV across the length of the genome but this is low at around 21% identity. Against the other members similarity is greater in the RdRp region (26-33%) although SBV has a region of greatest similarity to the structural proteins of *Perina nuda* virus (40%). However, analysis of the group D Iflaviruses (DWV, VDV-1 and KV)

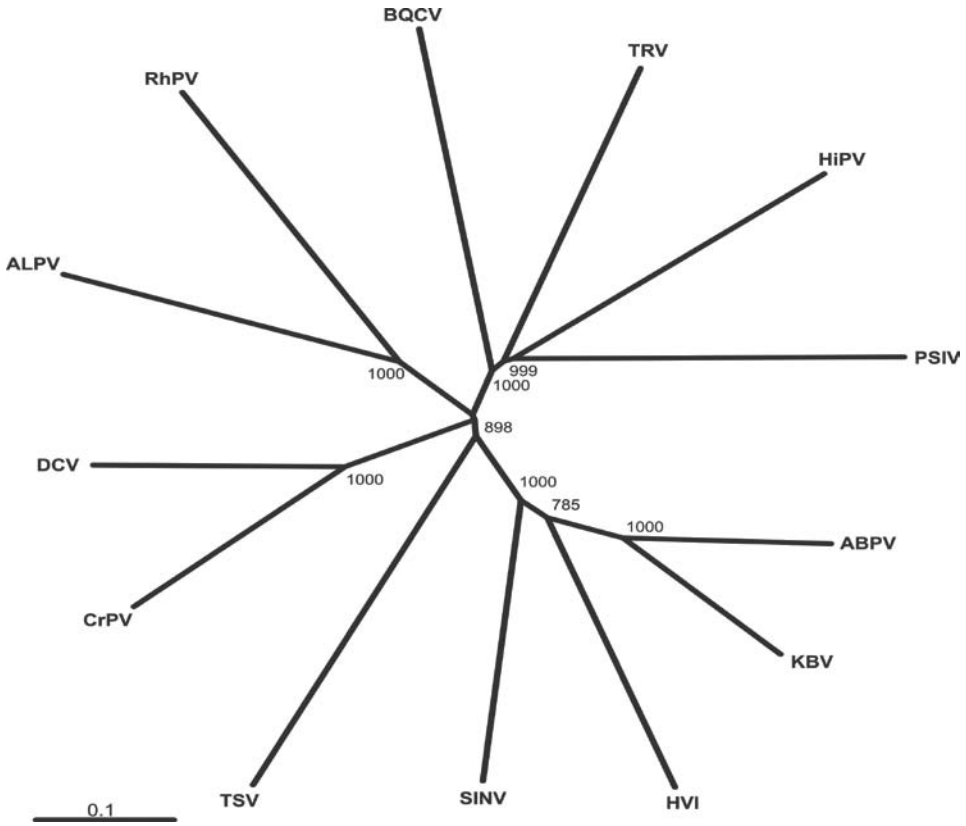


Figure 4. Unrooted Phylogenetic tree based on global nucleotide sequence alignments of the complete genomes of the known members of the Dicistroviridae Cripavirus genus. Abbreviations used for viruses are as follows: BQCV, black queen cell virus; TRV *Triatoma* virus; HiPV Himetobi P virus; PSIV, *Plautia stali* intestinal virus; ABPV, acute bee paralysis virus; KBV, Kashmir bee virus; HVI honey bee virus-Israel; SINV *Solenopsis invicta* virus; TSV Taura syndrome virus; CrPV, cricket paralysis virus; DCV, *Drosophila* C virus; ALPV, aphid lethal paralysis virus; RhPV, *Rhopalosiphum padi* virus. The tree was generated using the ClustalX software programme. Internal labels indicate the bootstrap values derived for 1000 replicates. The bar represents genetic distance. (NB For HVI, analysis has incorporated two partial sequences).

shows that these viruses have extremely high levels of identity (95-98% of amino acid residues identical) spread right across the genome (Tab. V). The closeness of this relationship is illustrated in the phylogenetic tree presented as Figure 8. This analysis includes some additional isolates of DWV. Such small differences cannot be resolved unless the scale is greatly expanded. These are clearly variants of the same virus but more work is needed to confirm whether these variants exhibit different tissue tropisms

or happen coincidentally to have been cloned from different locations or whether they are indicative of the quasispecies nature of DWV.

There is little information concerning the translation of the Iflavirus genomes. It is assumed that the 5' NCR will specify an IRES but attempts to demonstrate such function in sacbrood (Rothamsted) have found only weak activity (L.O. Roberts, personal communication). Further the 5'NCRs specified in each virus vary widely in size (Tab. VI). This is not unusual and size variation has been noted in picornaviruses both in terms of functional IRES length and in those sequences located at the 5' end but unnecessary for IRES function. However the 5'NCR given for Chinese sacbrood virus (accession no AF469603) aligns clearly with that for SBV Rothamsted, but is some 39 bases shorter. The authors do not claim to have reached the 5' end of their isolate and such heterogeneity seems common. The 5' end sequences for these viruses have mostly been derived by rapid amplification of cDNA ends (RACE) preparation. This induces a family of 5-end clones which extend to different lengths towards the real 5' terminus. Indeed Wu and colleagues noted a similar cluster of clones in their determination of the 5' end sequence of *Perina nuda* virus (Wu et al., 2002). In both this and the determination of SBV sequence (Ghosh et al., 1999) the longest clones were presented, but this variation could suggest that there is some condensed secondary structure towards the 5' end of the SBV NTR which inhibits 5'RACE and has yet to be determined. If so then the SBV 5'NCR may be incomplete and this could account for the weak activity observed.

Table IV. Iflaviruses; subdivisions as suggested by N. Knowles (http://www.iah.bbsrc.ac.uk/virus/Iflavirus)

Group	Members	Reference
Group A	Infectious Flacherie virus	Isawa et al., 1998
Group B	Sacbrood virus	Ghosh et al., 1999
Group C	*Perina nuda* virus	Wu et al., 2002
	Ectropis obliqua picorna-like virus	Wang et al., 2004
Group D	Deformed wing virus	Accession No NC_004830
	Varroa destructor virus	Ongus et al., 2004
	Kakugo virus	Fujiyuki et al., 2004
	Venturia canescens small RNA virus	Reineke et al., 2002, Reineke et al., 2003

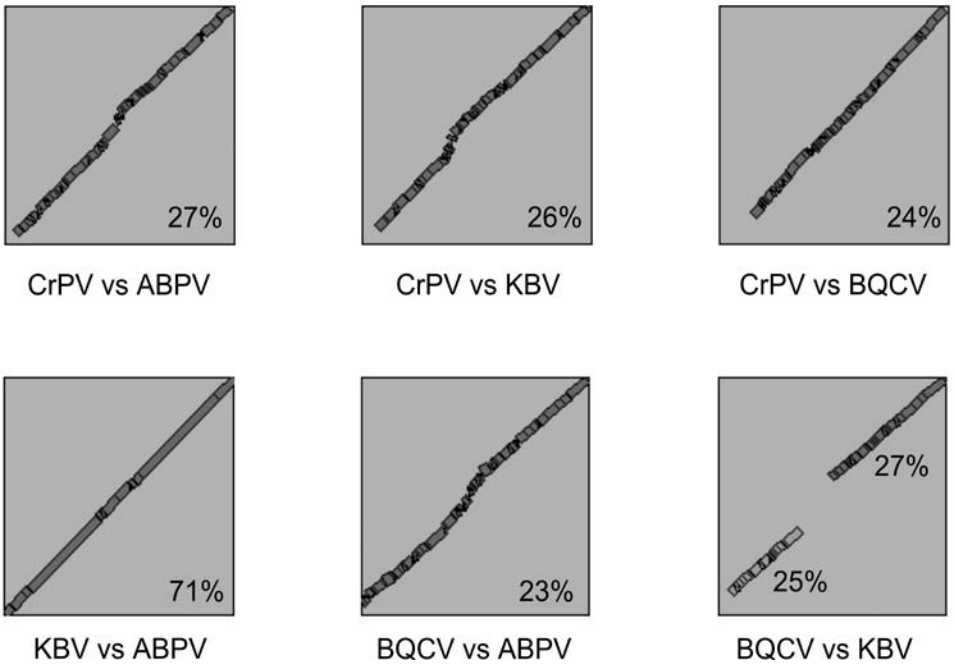


Figure 5. Comparison of non-structural proteins amongst members of the Dicistroviridae.

Virus abbreviations: BQCV, black queen cell virus; ABPV, acute bee paralysis virus; KBV, Kashmir bee virus; CrPV, cricket paralysis virus. BLAST alignments were prepared using the NCBI (http://www.ncbi.nlm.nih.gov/) Blink/Blast facility (program version BLASTP 2.2.10) to view pair-wise alignments selected from general BLink search. Alignments used the BLOSUM62 Matrix and an open gap cost of 11 (extension value 1). The four viruses are aligned two by two. In each graph the first sequence is plotted horizontally. Complete identity yields a continuous 45° line. Percent amino acid identities in the alignment are given in each panel. Non-structural protein sequences used in the alignment are given: CrPV NP_647481; ABPV AAU10100; KBV NP_851403; BQCV AAU10095. Honey bee viruses have low but widespread homology to CrPV but KBV and APBV are clearly the most closely related on this group with regions of high homology continuous throughout the gene. HVI (Honey bee virus - Israel) is not shown in this figure but has closest relationship to KBV at both nucleotide and protein alignments. It is not currently clear whether HVI represents a virus species in its own right or will be placed as a local variant of KBV. Note the break in continuity of relationship when BQCV is compared with either KBV and to a lesser extent (upward deviation of similarity plot) ABPV.

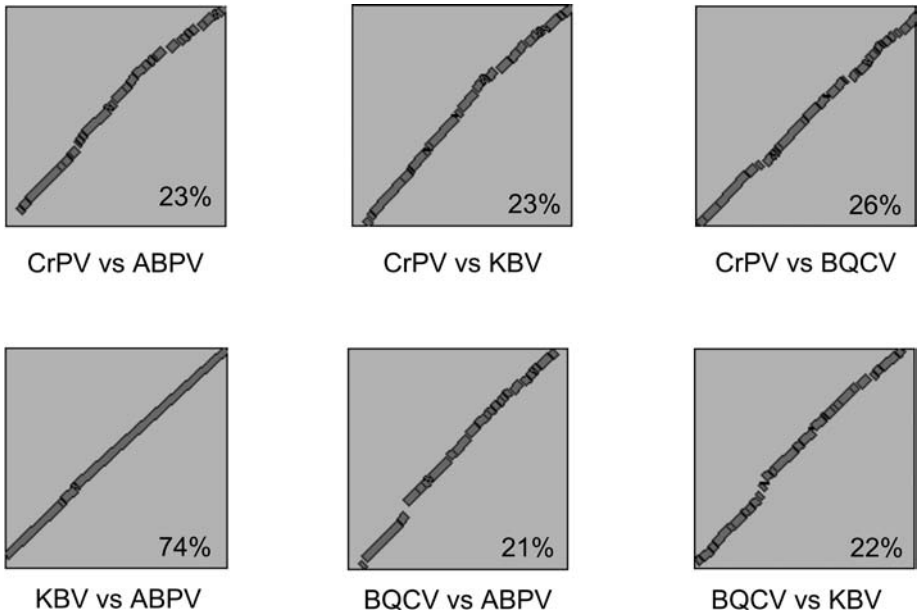


Figure 6. Comparison of structural proteins amongst members of the Dicistroviridae.
Virus abbreviations: BQCV, black queen cell virus; ABPV, acute bee paralysis virus; KBV, Kashmir bee virus; CrPV, cricket paralysis virus. BLAST alignments were prepared using the NCBI(http://www.ncbi.nlm.nih.gov/) Blink/Blast facility (program version BLASTP 2.2.10) to view pair-wise alignments selected from general BLink search. Alignments used the BLOSUM62 Matrix and an open gap cost of 11 (extension value 1). The four viruses are aligned two by two. In each graph the first sequence is plotted horizontally. Complete identity yields a continuous 45° line. Percent amino acid identities in the alignment are given in each panel. Structural protein sequences used in the alignment are given: CrPV NP_647482; BQCV NP_620565; KBV NP_851404; ABPV NP_066242. HVI (Honey bee virus - Israel) is not shown in this figure but has closest relationship to KBV in both nucleotide and protein alignments. It is not currently clear whether HVI represents a virus species in its own right or will be placed as a local variant of KBV.

Table V. Percentage nucleotide and amino acid sequence identity and similarity (in parenthesis) in an alignment between corresponding regions of VDV-1 and those of DWV and Kakugo virus (KV) (modified from Ongus et al., 2004).

VDV-1 sequence	Sequence identity (similarity) %					
	Nucleotide sequence		Amino acid sequence			
	DWV	KV	DWV		KV	
Genome w/o poly(A) tail	84	84				
Entire polyprotein			95	(98)	95	(98)
3' NTR	89	88				
RdRp	84	85	95	(98)	95	(98)
Protease	88	88	97	(98)	97	(98)
Helicase	85	85	95	(98)	95	(98)
VP1	84	84	97	(99)	96	(98)
VP2	84	84	98	(99)	98	(99)
First 1455 nt of ORF*	79	79				
First 485 aa of ORF*			90	(97)	89	(96)

* Including VP3 and VP4.

6. Genetic variability of selected honey bee viruses

Most honeybee viruses are thought to persist as inapparent infections in honey bee colonies. Such inapparent infections need not necessarily be associated with significant active replication. A high replication rate and short generation time are prerequisites for the unique complexity and molecular variation of RNA-based virus populations following the quasispecies theory. Therefore, for most honeybee viruses it will be difficult to prove the concept of quasispecies unless a virus population can be collected from bees undergoing an acute and productive infection, most likely in the case of bees exhibiting obvious signs of overt infection. This would easily be feasible for sacbrood virus and DWV because these two viruses cause characteristic visible symptoms during acute infection. The situation with KBV is different. Although no obvious symptoms are described it is the most virulent of all known honeybee viruses. It is potentially lethal to honeybee colonies and causes death of the individual within three days after experimental inoculation of a few particles into the haemolymph of adult bees or pupae (Allen and Ball, 1995). This is an excellent experimental model to verify the concept of the quasispecies

Table VI. 5'Non coding region (NCR) lengths reported for various Iflaviruses.

Virus	Presumptive 5' NCR	Reference
Sacbrood virus	179 (Rothamsted)	Ghosh et al., 1999
	138 (China)	Zhang, unpublished accession number AF469603
Infectious Flacherie virus	157	Isawa et al., 1998
Ectropis obliqua picorna-like virus	391	Wang et al., 2004
Perina nuda virus	474	Wu et al., 2002
DWV; VDV; Kakugo	1145; 1118; 1157	Accession No. NC_004830; Ongus et al., 2004; Fujiyuki et al., 2004

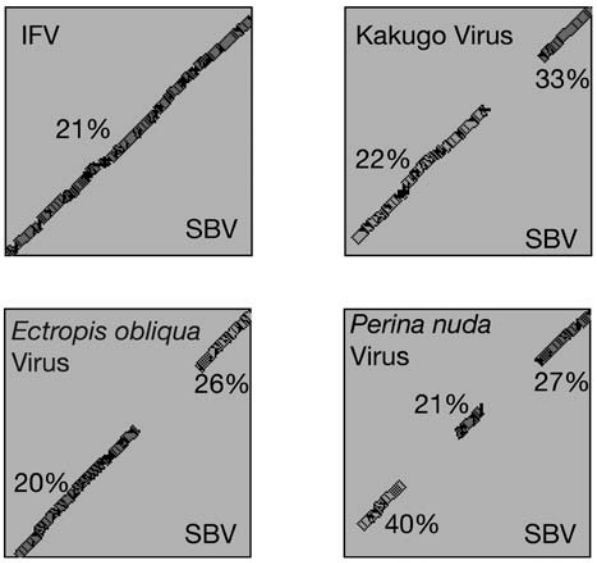


Figure 7. Protein alignments amongst the Iflaviruses.

Genome polyprotein protein alignments of sacbrood virus (horizontal axis) against other members of the Iflavirus genus were prepared using the NCBI (http://www.ncbi.nlm.nih.gov/) Blink/Blast facility (program version BLASTP 2.2.10) to view pair-wise alignments selected from general BLink search. Alignments used the BLOSUM62 Matrix and an open gap cost of 11 (extension value 1). Complete identity yields a continuous 45º line. Percent amino acid identities in the alignment are given in each panel. Polyprotein sequences used in the alignment are given: SBV NP_049374; IFV NP_620559; KakugoV YP_015696; *Ectropis obliqua* virus AAQ17044; Perina nuda virus NP_277061. Homology is continuous although low between SBV and IFV. Homology is discontinuous between SBV, Kakugo virus (equivalent DWV and VDV), *Ectropis obliqua* virus and *Perina nuda* virus.

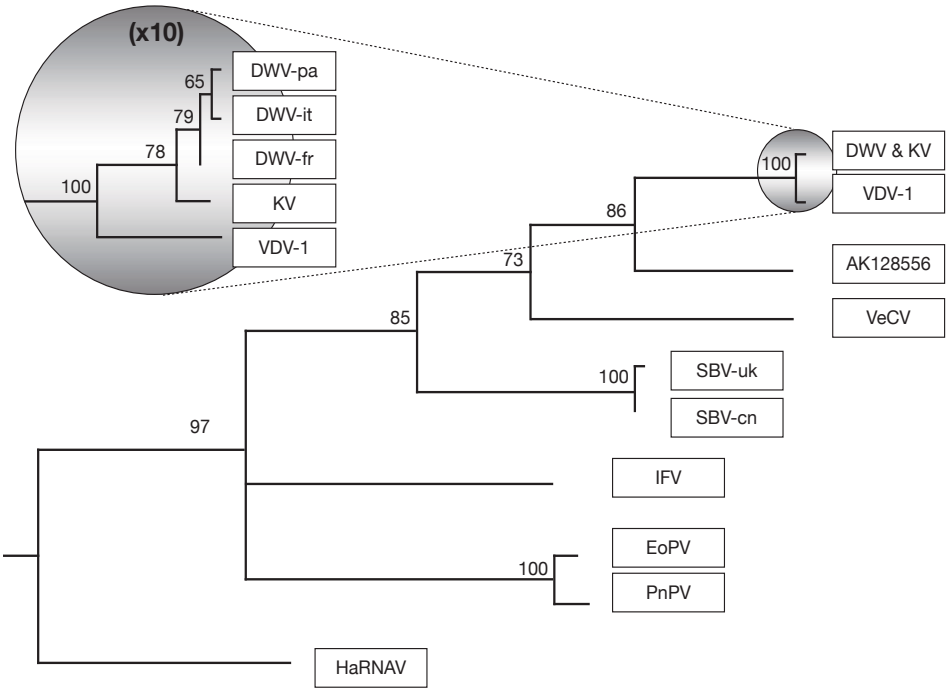


Figure 8. Iflavirus phylogeny including some recent sequence derivation.

Protein based phylogeny for iflaviruses abbreviations: DWV deformed wing virus (postscripts give geographical derivation of isolate, pa Pennsylvania; it, Italy; fr, France); KV, Kakugo virus; VDV-1, *Varroa destructor* virus 1; AK128556, human tracheal cDNA; VeCV, *Venturia canescens* virus; SBV, Sacbrood virus (postscripts give geographical isolation; uk, United Kingdom; cn, China); IFV, Infectious Flachrie virus; EoPV, *Ectropis obliqua* Picorna-like virus; PnPV, *Perina nuda* picorna-like virus. HaRNAV is a Marnavirus outgroup. Close relationship between *Ectropis obliqua* and *Perina nuda* viruses is shown; similarly sacbrood viruses from UK and China are revealed as close relatives. On this scale the extremely small divergence between Deformed wing virus, Kakugo virus and *Varroa destructor* virus cannot be depicted. Inset shows 10x greater resolution. Close relationships like these strongly imply that these viruses are variants of a single strain. This would be named from the first virus isolated and thus these should be regarded as forms of deformed wing virus. The identification of a cDNA related to these viruses from human tracheal cDNA library is unexpected and requires further investigation to see if it arises by chance, contamination or whether its observation should be regarded as significant.

Figure kindly supplied by Dr J.R. de Miranda.

nature of KBV. Given the virulence of KBV combined with the lack of diagnostic symptoms it is quite possible that colony collapse due to KBV is more common than previously recognized. If so, KBV might already form complex populations.

6.1. KBV

It has been known for a long time that different strains of Kashmir bee virus clearly differ serologically as well as in their capsid protein patterns (Bailey *et al.*, 1979; Allen and Ball, 1995). Both results are indicative of rather major differences at the protein and, hence, nucleotide level. However, only strains from different geographical origin were compared and no results are available on the analysis of virus isolates from a single hive or apiary which means that these data are not immediately applicable to the analysis of quasispecies occurrence. Indeed, one would not necessarily anticipate the nucleotide sequence diversity as described for RNA-based viruses to be evident at the level of protein profiles or serology as is the case for Kashmir bee virus. The fact that Kashmir bee virus has already acquired diversity at a level over and above that predicted by the quasi species occurrence implies that this virus is capable of significant variation and is thus a promising candidate in which to seek evidence of quasispecies occurrence. Indeed, analysis of the most recently determined entire genomic sequence (9506 nt) of Kashmir bee virus has revealed the high transition frequencies common for RNA viruses. 93 unique nucleotide variations were detected appearing uniformly across the genome at a frequency of 1.7×10^{-3} per nucleotide sequenced (de Miranda *et al.*, 2004). The vast majority (75%) of these changes were transitions (C – U and A – G changes). Only half of these transitions were silent, the other half resulted in amino acid changes (de Miranda *et al.*, 2004).

6.2. ABPV

Other bee viruses are known to show less serological variation but differ at the nucleotide level as shown by phylogenetic analysis (Grabensteiner *et al.*, 2001; Bakonyi *et al.*, 2002; de Miranda *et al.*, 2004).

Phylogenetic analysis of acute bee paralysis virus (ABPV; Bakonyi *et al.*, 2002) was carried out by directly sequencing PCR-products, thereby avoiding artifactual errors introduced by cloning and subsequent sequencing of PCR generated misincorporations. Observed genetic diversity between isolates can therefore be interpreted as real sequence variation. The authors constructed two phylogenetics trees, one based on a 3,071-nt fragment including the entire capsid protein region of eleven European ABPV samples and the other based on a 401-nt fragment from the partial capsid

protein region of 32 samples originating from Europe and the US For comparison, the ABPV-sequence available from GenBank (Govan *et al.*, 2000) was used as reference sequence. The phylogenetic tree constructed from the partial capsid protein region is the more meaningful in respect to sequence divergence since more isolates were included. At least two distinct genetic lineages of ABPV exist. One major branch is formed by the US and UK strains, while the other includes all continental-European isolates. Each branch again divides into several subclusters. The sequences exhibited between 89 and 96% similarity with the reference isolate. Since no analysis of the molecular variability within one population (infecting a single hive or apiary) was conducted it is difficult to answer the question of the quasispecies nature of ABPV. To expand the term quasispecies for the molecular variation of geographically distinct isolates seems inappropriate (Pinel *et al.*, 2000; Eigen, 1996). Nevertheless, looking for such variation within a virus population would be an interesting project.

6.3. SBV

Phylogenetic analysis of sacbrood virus (Grabensteiner *et al.*, 2001) was carried out by directly sequencing PCR-products, thereby avoiding artifactual errors introduced by cloning and subsequent sequencing of PCR created substitutions. The sequences obtained covered around one third of the entire genome. Three genetic lineages were identified: a European lineage (11 isolates including one from Nepal), a Far Eastern lineage (4 isolates from Nepal and India), and a South African lineage represented by a single isolate from South Africa. Sequence diversity within each lineage varied between 1 to 7% depending on the genomic region analysed. Inter-lineage similarities also differed between the regions analysed. The South African lineage was the most divergent one with only 78 to 83% similarity compared with the genomic SBV nucleotide sequence available through GenBank (acc. no. AF092924), originating from the UK and used as reference sequence. The Far Eastern lineage revealed between 90 and 96% similarity compared with the reference sequence. Since the European lineage included the reference sequence, the similarity of the European lineage with this sequence ranged from 93 to 100%.

Sacbrood virus infections are advantageous for study in that this virus causes clear symptoms in infected larvae and, therefore the disease can be diagnosed easily and accurately. Hence, we can assume that even the most divergent isolate from South Africa was classified correctly indicating that sacbrood virus populations from different geographical origin differ considerably.

For the two lineages/populations represented by more than one iso-

late intra-lineage variation was apparent, but rather low. Although these populations were not homogenous but consisted of minor variants that differed from the reference sequence at several positions this variation again does not justify describing them as forming quasispecies distribution (Domingo and Holland, 1994; Smith *et al.*, 1997; Eigen, 1996). Against the background of the highly divergent South African lineage a quasispecies nature of sacbrood virus populations is likely but needs to be confirmed by appropriate studies.

6.4. DWV

After the entire genomic sequence of DWV was made available many studies on the incidence and prevalence of DWV in bees and mites were conducted (Tentcheva *et al.*, 2004a; Tentcheva *et al.*, 2004b; Chen *et al.*, 2004; Genersch, 2005; Chen *et al.*, 2005), though no phylogenetic data have been published. In one study (Genersch, 2005) twenty crippled bees originating from one hive (E. Genersch, unpublished data) were analysed and the nucleotide sequences for PCR fragments covering one third of the entire genome were obtained and compared. Sequence variation was low and ranged from 0 to 1.1%, depending on the genomic region. The two PCR products showing no variation between the twenty isolates represented fragments of the genes coding for VP2 and the viral helicase. The other slightly divergent sequences derived from five different PCR products of the 3' non-structural region of the DWV genome. The non-structural region of the genome is expected to show the lowest variability and thus the high degree of similarity was not surprising. The sequences obtained showed around 99% homology to the two genomic DWV sequences published in GenBank (acc. nos. AJ489744, AY292384). Hence, based on these admittedly limited data we cannot deduce any high level of molecular variation for DWV.

Data indicating the possibility of a higher degree of complexity for the DWV species stem from two papers allegedly not dealing with DWV. In one study, the identification of an insect picorna-like virus isolated from the brains of aggressive worker bees is described (Fujiyuki *et al.*, 2004). The authors claim that they had found a novel virus and named it Kakugo virus. They reported that a database search revealed significant sequence similarities between Kakugo virus and polyproteins of various picorna-like viruses, especially sacbrood virus. Casually the authors mentioned that Kakugo virus showed 97 to 98% sequence similarity to the most recently published genomic sequence of DWV (AJ489744: 2002; AY292384: 2003). Nevertheless, the supposedly novel virus was not classified as DWV, based on two arguments: (i) 2 to 3% divergence translated into 201 nu-

cleotide substitutions, deletions, or insertions with 28 of them resulting in amino acid substitutions, (ii) the 5' UTR of Kakugo virus was 16 and 11 nt longer than that of the published DWV sequences. Actually, both arguments rather favour the view that Kakugo virus is in effect DWV. For RNA-based viruses a divergence of only 201 nucleotide changes resulting in as little as 28 amino acid substitutions over an entire genome of 10,152 nucleotides is unexpectedly low. The same is true for the varying length of the 5' UTR, which already differs between the two sequences published in GenBank. Assumed that Kakugo virus is an isolate of DWV the observed sequence differences can be explained most easily by the fact that three geographically diverse DWV isolates (Japan, US, Europe) were sequenced. Therefore, we propose to classify Kakugo virus as DWV.

In another recent study the complete sequence of a picorna-like virus replicating in the mite *Varroa destructor* was reported (Ongus *et al.*, 2004). Based on sequence divergence this virus was classified as novel and named VDV-1. VDV-1 had an overall RNA genome homology of 84% to DWV which translated into a polyprotein with 95% identical amino acids as compared to DWV. Considering also conservative amino acid exchanges, the VDV-1 polyprotein showed an overall similarity of 98%. Capsid proteins VP1 and VP2 even revealed 99% similarity to the respective DWV proteins (Tab. V). Therefore, despite a considerable divergence of the nucleotide sequence this sequence translates into a polyprotein nearly identical to the DWV polyprotein. Although at first sight nucleotide sequence divergence between VDV-1 and DWV seems high it is not so for RNA-based viruses which tend to form mutant spectra with a high degree of sequence variation.

Remarkably, the authors found both DWV and VDV-1 replicating in the mite population under study. Replication is a requirement for the proposed complexity of RNA virus populations infecting a single host. Against the background of the biology of bees, we should consider the bee colony as an individual host rather than a single bee. Since in a mite infested and virus infected colony bees and mites are in constant contact and exchange viruses the genetic variants of a virus population infecting a bee hive will be found equally distributed between bees and mites. Therefore, although the authors of the above mentioned study did not check whether or not both viruses coexisted in a single mite, since they were present in the same mite population their results are rather congruent with being the first example of a DWV population forming a quasispecies. It would have been interesting to know whether or not both or even more variants were also found replicating in the bee population infested by this mite population.

7. Outlook

Our understanding of insect viruses is advancing rapidly. The study of bee viruses is likewise advancing with it. More detailed knowledge is separating the traditional "picornavirus-like" agents into new groupings; it is unlikely that we have yet seen all the types of virus that occur in this host. Molecular taxonomy holds the promise of discovering more of the relationship between plant and animal viruses.

On the other hands, the concept of quasispecies conferred on RNA-based viruses allows us to describe and understand the unusual molecular heterogeneity of RNA virus populations. If bee virologists are interested in revealing the potential quasispecies nature of bee viruses they will have to look for genetic variants in individual infected organisms: in this case in single bees or colonies. Phylogenetic analysis of geographically diverse isolates although important does not answer this question. At the same time, by assigning a new species to a virus only differing at the nucleotide level – if at all – the fact that RNA viruses tend to form populations of phylogenetically related variants, so-called mutant spectra, in a single infected organism is neglected. We have to keep in mind that the concept of viral quasispecies invalidates the concept of wild type as a defined genome with a specific nucleotide sequence.

References

Allen, M.F. and Ball, B.V. (1995) Characterisation and serological relationships of strains of Kashmir bee virus, Ann. Appl. Biol. 126, 471-484.

Anderson, D.L. (1991) Kashmir bee virus – a relatively harmless virus of honey bee colonies, Am. Bee. J. 131, 767-770.

Bailey, L. (1976) Viruses attacking the honeybee. Adv. Virus. Res. 20, 271-304.

Bailey, L., Carpenter, J.M., Woods, R.D. (1979) Egypt bee virus and Australian isolates of Kashmir bee virus, J. Gen. Virol. 43, 641-647.

Bakonyi, T., Grabensteiner, E., Kolodziejekt, J., Rusvai, M., Topolska, G., Ritter, W., Nowotny, N. (2002) Phylogenetic analysis of acute bee paralysis virus strains, Appl. Environ. Microbiol. 68, 6446-6450.

Batschelet, E., Domingo, E., Weissmann, C. (1976) The proportion of revertant and mutant phage in a growing population, as a function of mutation and growth rate, Gene 1, 27-32.

Belsham, G. J., McInerney, G. M., Ross-Smith, N. (2000) Foot-and-mouth disease virus 3C protease induces cleavage of translation initiation factors eIF4A and eIF4G within infected cells, J. Virol. 74, 272-280.

Bracho, M.A., Moya, A., Barrio, E. (1998) Contribution of *Taq* polymerase-induced errors to the estimation of RNA virus diversity, J. Gen. Virol. 79, 2921-2928.

Chen, Y., Zhao, Y., Hammond, J., Hus, H., Evans, J., Feldlaufer, M. (2004) Multiple infections in the honey bee and genome divergence of honey bee viruses, J. Invertebr. Pathol. 87, 84-93.

Chen, Y.P., Higgins, J.A., Feldlaufer, M.F. (2005) Quantitative real-time reverse transcription-PCR analysis of deformed wing virus infection in the honeybee (*Apis mellifera* L.), Appl. Environ. Microbiol. 71, 436-441.

Christian, P., Carstens E., Domier, L., Johnson, K., Nakashima, N., Scotti, P., van de Wilk, F. (2002a) 00.105.0.01 The infectious Flachery-like viruses. ICTVdB Index of viruses. http:\//www.ncbi.nlm.nih.gov/ICTVdb/Ictv/fs_infec.htm accessed 03-03-2005

Christian, P., Carstens E., Domier, L., Johnson, K., Nakashima, N., Scotti, P., van de Wilk, F. (2002b) 00.101. Dicistroviridae ICTVdB Index of viruses. http:\//www.ncbi.nlm.nih.gov/ICTVdb/Ictv/fs__dicis.htm accessed 03-03-2005

Cline, J., Barman, J.C., Hogrefe, H.H. (1996) PCR fidelity of *Pfu* polymerase and other thermostable DNA polymerases, Nucl. Acids Res. 24, 3546-3551.

Costantino, D. and Kieft, J. S. (2005) A preformed compact ribosome-binding domain in the cricket paralysis-like virus IRES RNAs, RNA 11, 332-343.

Czibener, C., La Torre, J. L., Muscio, O. A., Ugalde, R. A. and Scodeller, E. A. (2000) Nucleotide sequence analysis of Triatoma virus shows that it is a member of a novel group of insect RNA viruses, J. Gen. Virol. 81, 1149-1154.

De Miranda, J.R., Drebot, M., Tyler, S., Shen, M., Cameron, C.E., Stoltz, D.B., Camazine, S.M. (2004) Complete nucleotide sequence of Kashmir bee virus and comparison with the acute bee paralysis virus, J. Gen. Virol. 85, 2263-2270.

Domier, L. L., McCoppin, N. K., D'Arcy, C. J. (2000) Sequence requirements for translation initiation of Rhopalosiphum padi virus ORF2, Virology 268, 264-271.

Domier, L. L., and McCoppin, N. K. (2003) In vivo activity of Rhopalosiphum padi virus internal ribosome entry sites, J. Gen. Virol. 84, 415-419.

Domingo, E., Sabo, D., Taniguchi, T., Weissmann, C. (1978) Nucleotide sequence heterogeneity of an RNA phage population, Cell 13, 735-744.

Domingo, E., Holland, J.J. (1994) Mutation rates and rapid evolution of RNA viruses, in: Morse, S.S. (ed) The evolutionary biology of viruses, Raven Press, New York, pp 161-174.

Domingo, E. (2002) Quasispecies theory in virology, J. Virol. 76, 463-465.

Dorner, A. J., Semler, B. L., Jackson, R. J., Hanecak, R., Duprey, E., Wimmer, E. (1984) In vitro translation of poliovirus RNA: utilisation of internal initiation sites in reticulocyte lysate, J. Virol. 50, 507-514.

Drake, J.W. (1993) Rates of spontaneous mutation among RNA viruses, Proc. Natl. Acad. Sci. 90, 4171-4175.

Drake, J.W., Charlesworth, B., Charlesworth, D., Crow, J.F. (1998) Rates of spontaneous mutation, Genetics 148, 1667-1686.

Drake, J.W. and Holland, J.J. (1999) Mutation rates among RNA viruses. Proc. Natl. Acad. Sci. 96, 13910-13913.

Eigen, M. (1971) Self-organisation of matter and the evolution of biological macromolecules, Naturwissenschaften 58, 465-523.

Eigen, M. (1996) On the nature of virus quasispecies, Trends Microbiol. 4: 216-217

Evans, J.D. and Hung, A.C. (2000) Molecular phylogenetics and the classification of honey bee viruses, Arch. Virol. 145, 2015-2026.

Evans, J.D. (2001) Genetic evidence for coinfection of honey bees by acute bee paralysis and Kashmir bee viruses, J. Invertebr. Pathol. 78, 189-193.

Fujiyuki, T., Takeuchi, H., Ono, M., Ohka, S., Sasaki, T., Nomoto, A., Kubo, T. (2004) Novel insect picorna-like virus identified in the brains of aggressive worker honeybees, J. Virol. 78: 1093-1100.

Genersch, E. (2005) Development of a rapid and sensitive RT-PCR method for the detection of deformed wing virus, a pathogen of the honeybee (*Apis mellifera*), Vet. J. 169: 121-123.

Ghosh, R. C., Ball, B. V., Willcocks, M. M., Carter, M. J. (1999) The nucleotide sequence of sacbrood virus of the honey bee: an insect picorna-like virus, J. Gen. Virol. 80, 1541-1549.

Glass, M. J. and Summers, D. F. (1993) Identification of a trans-acting activity from liver that stimulates hepatitis A virus translation in vitro, Virology 193, 1047-1050.

Govan, V.A., Leat, N., Allsop, M., Davison, S. (2000) Analysis of the complete genome sequence of acute bee paralysis virus shows that it belongs to the novel group of insect-infecting RNA viruses, Virology 277: 547-463.

Grabensteiner, E., Ritter, W., Carter, M.J., Davison, S., Pechhacker, H., Kolodziejek, J., Boecking, O., Derakhshifar, I., Moosbeckhofer, R., Licek, E., Nowotny, N. (2001) Sacbrood virus of the honey bee (*Apis mellifera*): rapid identification and phylogenetic analysis using reverse transcription-PCR, Clin. Diagn. Lab. Immunol. 8: 93-104.

Holland, J., Spindler, K., Horodyski, F., Grabau, E., Nichol, S., VandePol, S. (1982) Rapid evolution of RNA genomes, Science 215: 1577-1585.

Holmes, E.C. and Moya, A. (2002) Is the quasispecies concept relevant to RNA viruses? J. Virol. 76: 460-462.

Isawa, H., Asano, S., Sahara, K., Iizuka, T. and Bando, H. (1998) Analysis of genetic information of an insect picorna-like virus, infectious flacherie virus of silkworm: evidence for evolutionary relationships among insect, mammalian and plant picorna(-like) viruses, Arch. Virol. 143, 127-143.

Jan, E., Kinzy, T., G., Sarnow, P. (2003) Divergent tRNA-like element supports initiation, elongation, and termination of protein biosynthesis. *Proc Natl Acad Sci U S A* **100**, 15410-15415.

Ji, J.P. and Loeb, L.A. (1992) Fidelity of HIV-1 reverse transcriptase copying RNA in vitro, Biochem. 31: 954-958.

Johnson, K. N. and Christian, P. D. (1998) The novel genome organisation of the insect picorna-like virus Drosophila C virus suggests this virus belongs to a previously undescribed virus family, J. Gen. Virol. **7**9 (Pt 1), 191-203.

Kanamori, Y. and Nakashima, N. (2001) A tertiary structure model of the internal ribosome entry site (IRES) for methionine-independent initiation of translation, RNA 7, 266-274.

Keck, J.G., Matsushima, G.K., Makinko, S., Fleming, J.O., Vannier, D.M., Stohlman, S.A., Lai, M.M.C. (1988) In vivo RNA-RNA recombination of coronavirus in mouse brain, J. Virol. 62: 1810-1813.

Koonin, E. V., Gorbalenya, A. E. (1992) An insect picornavirus may have genome organisation similar to that of caliciviruses, FEBS Lett. 297, 81-86.

Lai, M.M.C. (1992) RNA recombination in animal and plant viruses, Microbiol. Rev. 56: 61-79.

Leat, N., Ball, B., Govan, V., Davison, S. (2000). Analysis of the complete genome sequence of black queen-cell virus, a picorna-like virus of honey bees, J. Gen. Virol. 81, 2111-2119.

Ledinko, N. (1963) Genetic recombination with poliovirus type I studies of crosses between a normal horse serum-resistant mutant and several guanidine-resistant mutants of the same strain, Virology 20: 107-119.

Lundberg, K.S., Shoemaker, D.D., Adams, M.W., Short, J.M., Sorge, J.A., Mathur, E.J. (1991) High-fidelity amplification using a thermostable DNA polymerase isolated from *Pirococcus furiosus*, Gene 108: 1-6.

Maas, S., Melcher, T., Seeburg, P.H. (1997) Mammalian RNA-dependent deaminases and edited RNAs, Curr. Opin. Cell Biol. 9: 343-349.

Mari, J., Poulos, B. T., Lightner, D. V., Bonami, J. R. (2002) Shrimp Taura syndrome virus: genomic characterisation and similarity with members of the genus Cricket paralysis-like viruses, J. Gen. Virol. 83, 915-926.

Martinez-Salas, E. and Fernandez-Miragall, O. (2004) Picornavirus IRES: Structure function relationship. Curr. Pharm. Des. 10, 3757-3767.

Meyers, G., Tautz, N., Dubovi, E.J., Thiel, H.-J. (1991) Viral cytopathogenicity correlated with integration of ubiquitin-coding sequences, Virology 180: 602-616.

Moon, J. S., Domier, L. L., McCoppin, N. K., D'Arcy, C. J. and Jin, H. (1998) Nucleotide sequence analysis shows that Rhopalosiphum padi virus is a member of a novel group of insect-infecting RNA viruses, Virology 243, 54-65.

Nakashima, N., Sasaki, J. and Toriyama, S. (1999) Determining the nucleotide sequence and capsid-coding region of himetobi P virus: a member of a novel group of RNA viruses that infect insects, Arch. Virol. 144, 2051-2058.

Ongus, J.R., Peters, D., Bonmatin, J.-M., Bengsch, E., Vlak, J.M., van Oers, M.M. (2004) Complete sequence of a picorna-like virus of the genus Iflavirus replicating in the mite *Varroa destructor*, J. Gen. Virol. 85: 3747-3755.

Overton, H.A., Buck, K.W., Bailey, L. and Ball, B.V (1982) Relationships between the RNA components of chronic bee paralysis virus and those of chronic bee paralysis virus associate. J. Gen. Virol. 63, 171-179.

Parvin, J.D., Moscona, A., Pan, W.T. (1986) Measurement of the mutation rates of animal viruses: Influenza A virus and poliovirus type I, J. Virol. 59: 377-383.

Pestova, T. V. and Hellen, C. U. (2003) Translation elongation after assembly of ribosomes on the Cricket paralysis virus internal ribosomal entry site without initiation factors or initiator tRNA, Genes Dev. 17, 181-186.

Pinel, A., Guessan, P.N., Bousalem, M., Fragette, D. (2000) Molecular variability of geographically distinct isolates of *Rice yellow mottle virus* in Africa, Arch. Virol. 145: 1621-1638.

Plyusnin, A., Kukkonen, S.K.J., Plyusnina, A., Vapalahti, O., Vaheri, A. (2002) Transfection-mediated generation of functionally competent Tula hantivirus with recombinant S RNA segment, EMBO J. 21: 1497-1503.

RajBhandary, U. L. (2000) More surprises in translation: initiation without the initiator tRNA, Proc. Natl. Acad. Sci. USA 97, 1325-1327.

Reineke, A., Asgari, S., Ma, G., Beck, M., Schmidt, O. (2002) Sequence analysis and expression of a virus-like particle protein, VLP2, from the parasitic wasp Venturia canescens, Insect Mol. Biol. 11, 233-239.

Reineke, A., Schmidt, O., Zebitz, C. P. (2003) Differential gene expression in two strains of the endoparasitic wasp Venturia canescens identified by cDNA-amplified fragment length polymorphism analysis, Mol. Ecol. 12, 3485-3492.

Ribiere, M., Faucon, J-P. and Pepin, M. (2000) Detection of chronic honeybee (Apis mellifera L.) paralysis virus infection: application to a field survey. Apidologie 31, 567-577.

Roberts, L. O., Seamons, R. A., Belsham, G. J. (1998) Recognition of picornavirus internal ribosome entry sites within cells; influence of cellular and viral proteins, RNA 4, 520-529.

Robles-Sikisaka, R., Garcia, D. K., Klimpel, K. R., Dhar, A. K. (2001) Nucleotide sequence of 3'-end of the genome of Taura syndrome virus of shrimp suggests that it is related to insect picornaviruses, Arch. Virol. 146, 941-952.

Sasaki, J. and Nakashima, N., Saito, H., Noda, H. (1998) An insect picorna-like virus, Plautia stali intestine virus, has genes of capsid proteins in the 3' part of the genome, Virology 244, 50-58.

Sasaki, J., Nakashima, N. (1999) Translation initiation at the CUU codon is mediated by the internal ribosome entry site of an insect picorna-like virus in vitro, J. Virol. 73, 1219-1226.

Simmonds, P., Balfe, P., Peutherer, J.F., Ludlam, C.A., Bishop, J.O., Brown, A.J. (1990) Human immunodeficiency virus-infected individuals contain provirus in small numbers of peripheral mononuclear cells and at low copy numbers, J. Virol. 64: 864-872.

Smith, D.B., and Inglis, S.C. (1987) The mutation rate and variability of eukaryotic viruses: An analytical review, J. Gen. Virol. 68: 2729-2740.

Smith, D.B., McAllister, J., Casino, C., Simmonds, P. (1997) Virus "quasispecies": making a mountain out of a molehill? J. Gen. Virol. 78: 1511-1519.

Smith, G.R., Borg, Z., Lockhart, B.E.L., Braithwaite, K.S., Gibbs, M.J. (2000) Sugarcane yellow leaf virus: a novel member of the Luteoviridae that probably arose by inter-species recombination, J. Gen. Virol. 81: 1865-1869.

Spahn, C. M., Jan, E., Mulder, A., Grassucci, R. A., Sarnow, P., Frank, J. (2004) Cryo-EM visualisation of a viral internal ribosome entry site bound to human ribosomes: the IRES functions as an RNA-based translation factor, Cell 118, 465-475.

Stoltz, D., Shen, X.R., Boggis, C., Sisson, G. (1995) Molecular diagnosis of Kashmir bee virus infection, J. Apic. Res. 34, 153-160.

Tentcheva, D., Gauthier, L., Zappulla, N., Dainat, B., Cousserans, F., Colin, M.E., Bergoin, M. (2004a) Prevalence and seasonal variations of six bee viruses in *Apis mellifera* L. and *Varooa destructor* mite populations in France, Appl. Environ. Microbiol. 70 (12): 7185-7191.

Tentcheva, D., Gauthier, L., Jouve, S., Canabady-Rochelle, L., Dainat, B., Cousserans, F., Colin, M.E., Ball, B.V., Bergoin, M. (2004b) Polymerase chain reaction detection of defromed wing virus (DWV) in *Apis mellifera* and *Varroa destructor*, Apidologie 35: 431-439.

Torres-Punete, M., Bracho, M.A., Jimenez, N., Garcia-Robles, I., Moya, A., Gonzales-Candelas, F. (2003) Sampling and repeatability in the evaluation of hepatitis C virus genetic variability, J. Gen. Virol. 84: 2343-2350.

Valles, S. M., Strong, C. A., Dang, P. M., Hunter, W. B., Pereira, R. M., Oi, D. H., Shapiro, A. M.,Williams, D. F. (2004) A picorna-like virus from the red imported fire ant, *Solenopsis invicta*: initial discovery, genome sequence, and characterisation, Virology 328, 151-157.

Van Munster, M., Dullemans, A. M., Verbeek, M., Van Den Heuvel, J. F., Clerivet, A., Van Der, W. F. (2002) Sequence analysis and genomic organisation of Aphid lethal paralysis virus: a new member of the family Dicistroviridae, J Gen Virol 83, 3131-3138.

Vartanian, J.P., Henry, M., Wain-Hobson, S. (1996) Hypermutagenic PCR involving all four transitions and a sizeable proportion of transversions, Nucleic Acids Res. 24: 2627-2631.

Williams, K.J., Loeb, L.A. (1992) Retroviral reverse transcriptases: error frequencies and mutagenesis, Curr. Top. Microbiol. Immunol. 176: 165-180.

Wilson, J. E., Pestova, T. V., Hellen, C. U., Sarnow, P. (2000a) Initiation of protein synthesis from the A site of the ribosome, Cell 102, 511-520.

Wilson, J. E., Powell, M. J., Hoover, S. E., Sarnow, P. (2000b) Naturally occurring dicistronic cricket paralysis virus RNA is regulated by two internal ribosome entry sites, Mol. Cell Biol. 20, 4990-4999.

Woolaway, K. E., Lazaridis, K., Belsham, G. J., Carter, M. J., Roberts, L. O. (2001) The 5' untranslated region of *Rhopalosiphum padi* virus contains an internal ribosome entry site which functions efficiently in mammalian, plant, and insect translation systems, J. Virol. 75, 10244-10249.

Wu, C. Y., Lo, C. F., Huang, C. J., Yu, H. T., Wang, C. H. (2002) The complete genome sequence of Perina nuda picorna-like virus, an insect-infecting RNA virus with a genome organisation similar to that of the mammalian picornaviruses, Virology 294, 312-323.

Chapter 3

DIAGNOSTIC TECHNIQUES FOR VIRUS DETECTION IN HONEY BEES

Joachim R. DE MIRANDA

Table of Contents

1. Introduction

Disease diagnosis has made enormous strides since the development of molecular biological techniques, both in the diagnostic options available and in the type of data generated. There is now a large variety of technologies and tools available for the accurate detection and quantification of specific protein and nucleic acid sequences, and even for the qualitative and quantitative diagnosis of microvariation within those sequences. To these can be added the classical disease diagnosis methods, consisting of symptomatology, microscopy, bioassays and early serological methods. Each has its strengths and weaknesses and choosing the technology that best matches the diagnostic requirements and the nature of the biomolecules is therefore the first step in designing a diagnostic strategy. Several topics relevant to making these choices are covered here, with particular reference to (honey bee) virus diagnosis. These include labelling-detection options, technologies used in diagnosis, diagnosing variation, quantitation options and errors in diagnosis. Another major topic in diagnosis is how data are generated, analysed and used. Classical diagnosis (symptoms, microscopy, early serology) is essentially qualitative in nature; symptoms, particles or precipitation lines either are or are not present. This type of diagnosis adapts well to current disease management practices, which also revolve around essentially qualitative acts (to treat, quarantine etc., or not). Most modern diagnostic techniques on the other hand are essentially quantitative in nature, currently rendered qualitative by artificial or calculated threshold values above which virus is deemed to be present and (economically) damaging and below which it is not, to facilitate the current management style. However, this treatment of the primary data de-emphasises the dynamic nature of disease epidemics, ignores the value of subliminal damage (below threshold level) and moreover makes the data very susceptible to changes in protocol, if such changes cause a large proportion of samples to shift across the threshold value. It will be interesting to see if disease management can adapt to possibilities offered by quantitative disease diagnosis, through a graded set of alternative treatment options, or integrated pest management. The ideal diagnostic protocol should be:

Sensitive	detecting the pathogen prior to damage, allowing effective treatment
Accurate	detecting only the disease agent, reducing false positives and negatives
Reliable (Robust)	insensitive to variations in the protocol, reducing variability

Universal	allowing detection at all life stages and disease states
Simple	adaptable to on-site testing
Fast	allowing immediate preventive or curative measures to be taken
Cheap	cost-effective alternative to prophylactic treatment of colonies

Of equally great importance in designing a diagnostic strategy is to determine which molecule, activity or characteristic is the best predictor for disease development, which may not necessarily be viral load (Marozsan *et al.,* 2004). Infectious virus particles consist of a functional genome wrapped in a protein coat but most molecular assays will detect all viral nucleic acid or capsid proteins, whether packaged and infectious or freely distributed and innocuous. Even different assays of the same molecule type can have different predictive qualities (Diaz-Mitoma *et al.,* 2003). A similar consideration with direct relevance for many mite-transmitted bee viruses is that pathogenicity or transmission may be a function of the type of tissue infected, such that a large viral load in one tissue may be entirely inconsequential while a miniscule load in another tissue is fatal. In this context the predictive quality of an early test for future disease development, rather than of mere pathogen presence, is the most important criterium for a diagnostic strategy (Ferreccio *et al.,* 2003).

2. Sample collection, storage and processing

Although expense and technological novelty in new detection methods makes researchers concentrate optimization efforts on the detection side of surveying it is often forgotten that much if not most of the error derives from the collection, storage and primary sample processing, *i.e.* the cheap end of surveying (Chernesky *et al.,* 2003). This becomes especially evident if thresholds are used to convert quantitative detection signal into qualitative data, as is generally the case, and when data from different studies or laboratories are compared (Birch *et al.,* 2004). The only solution to this is standardization of the collection, storage and primary processing protocol, taking into consideration the proportion of virus recovered from the sample, the variability of this recovery process between samples, and the speed, robustness and overall cost if such surveys are to be conducted on large scale. As is often the case, cue can be taken from other areas of biology where such practices have already been adopted, notably the medical field, where surveying for diseases is an industry unto itself

(Valentine-Thon *et al.,* 2001; Apfalter *et al.,* 2002; Verkooyen *et al.,* 2003). The overriding objective in collection, storage and processing is that the (virus) status and condition of the live bee is preserved as close as possible, since ultimately the data interpretation, conclusions and recommendations will again refer to live bees. A secondary consideration is that the sample often is used for several different analyses; proteins, nucleic acids, fats and lipids, metabolites *etc.*, requiring a collection and processing protocol suitable for all compounds analysed. Usually this means that the protocol follows the requirements for the least stable of the compounds. There are several means for collecting and processing bee samples.

2.1. Collection

The options here are live bees, frozen bees, storage on ice, dried whole bees, squashed bees or bee tissues dried on collection cards, dead bees, storage in salt solution, storage in alcohol or other solvents and storage at ambient temperature. The ideal sample of course is live bees, but unless these are processed and analysed instantly on site, most of these samples end up in the freezer until analysis. If so, it is better to freeze them on site, eliminating the effects of stress induced by transporting samples of lives bees, especially if through the post. Since freezing on site is often impractical, requiring containers of liquid nitrogen or dry ice, most samples are collected either live or on ice and it can be argued whether angry live bees suffocating in an (airtight) container or comatose bees on ice presents the greater stress. Dried bees present a convenient way of storage and transport at room temperature. Such bees should be frozen first to preserve as near as possible the live state and then either freeze-dried, preserving the activity of most compounds, or at high temperature in a drying oven, which will have greater effect on biomolecules. It should be noted here that reconstituted dried tissue is fundamentally different from frozen wet tissue, with different recovery efficiency and variability. Storage of bees in salt solution, alcohol, formaldehyde or other solvents, popular with many beekeepers sending in samples, is not recommended. These chemicals affect the integrity of the virus particle, leading to decay of the target, and often interact chemically with the proteins or nucleic acids altering the sites required for detection by the molecular probes (Whittier *et al.,* 2004). The exceptions to this are storage chemicals specifically designed to preserve a particular type of bio-molecule. An example of this are high concentrations of certain chaotropic salts (guanidine hydrochloride, guanidine isothiocyanate *etc.*), which are used to preserve nucleic acids by denaturing degrading enzymes. A similar commercial solution is RNA-later® (Qiagen). However, for these to function correctly requires full

penetration of the bee tissues which may be difficult in the case of adult bees, due to the exoskeleton. Most of such technologies were designed for the soft tissues analyzed in the medical field. Dead bee samples should not be used, since the dying process, subsequent decomposition and drying (all of unknown duration) will have differentially affected the live bee virus titre, as well as the recovery efficiency. Since only positive results out of such samples have validity (negative results could be due to lack of virus or decay), data-sets generated with dead bee samples are essentially non-interpretable.

A fundamentally different way of collecting samples is as squashed or spotted tissue, dried on collection cards. Not only virus particle integrity but also viability and function can be retained upon drying on filter paper (de Miranda *et al.,* 1996b). The method is ideal for liquid samples (blood, urine, sputum *etc*.) but has also been used for insect samples (Harvey, 2005) including honey bees and mites (M.W. Budura, B.A. Kahkonen and J.R. de-Miranda, unpublished results). Such filter-dried samples can be analysed for all manner of compounds (Jansson *et al.,* 2003; van Kuilenburg *et al.,* 2004; Chamoles *et al.,* 2004; Li *et al.,* 2005; Zurfluh *et al.,* 2005) including RNA (Karlson *et al.,* 2003; Kiatpathomchai *et al.,* 2004; Alvarez-Muñoz *et al.,* 2005; Prado *et al.,* 2005). Whatman FTA® cards are furthermore impregnated with chemicals to prevent bacterial or enzymatic degradation of nucleic acids (Becker *et al.,* 2004; Rensen *et al.,* 2005). The major advantages are the ease and reduced costs of collection (Fischer *et al.,* 2004; Kiatpathomchai *et al.,* 2004; Harvey, 2005) stability during long-term storage (Karlson *et al.,* 2003; Rensen *et al.,* 2005; Prado *et al.,* 2005), and the greatly reduced processing prior to detection, since usually a single core of dried tissue is analysed directly. The major drawbacks are a reduced recovery efficiency and increased variability, partly due to uneven virus distribution across the spotted/squashed sample, partly due to variability in the drying and recovery process and gradual loss of target during prolonged storage (Chaisomchit *et al.,* 2005). These drawbacks have to be measured against the recovery losses and variability incurred during sample processing of other collection methods. If beekeepers are used in sample collection for a survey it is best to have them freeze the samples, to be collected later, or use collection filter cards which can be sent in.

Perhaps the best sample type is not bee tissue at all. Many bee viruses shed large amounts of virus into the alimentary canal, presumably for a fecal-oral transmission route. These include DWV (Fievet *et al.,* 2006), KBV (Dall, 1987), ABPV (Bailey and Gibbs, 1964), BQCV (Bailey *et al.,* 1983), SBV (Mussen and Furgala, 1977), SPV (Denholm, 1999), CBPV, AIV, BVY and BVX (Bailey and Ball, 1991; Ribière *et al.,* 2000). This means that

the amount of virus detected in whole bee samples is dependent on the defecation status of the bee, leading to variability. One way around this conundrum is to exclude the abdomen from analysis (Ribière *et al.,* 2000; 2002). However, the virus loads in the fecal material may well be an excellent marker for the virus loads in the bee tissues proper (or symptoms), as well as for transmission and epidemiological potential. Fecal material is furthermore also a major repository for bacterial and protozoan pathogens (Shimanuki, 1997; Fries, 1997). The amount of fecal material submitted for an assay can be easily controlled by volume if fresh (Hung, 2000), or by area if dried on collection cards. KBV can be readily amplified directly from fecal material without purification (Hung, 2000), saving cost and variability. Finally, sampling fecal material means that the same bees can be sampled repetitively, providing more sensitive measures of disease progression in individual bees. It may prove particularly useful for determining the virus status of queens (Hung, 2000), especially if these are a major source of infection of the worker population (Chen *et al.,* 2005b; Fievet *et al.,* 2006).

2.2. Processing

The primary processing of a sample is the most crucial step in ensuring the uniformity and reliability of the entire test. Nevertheless, generally little attention is paid to optimizing this part of the protocol for both maximum recovery of the target molecule(s) and for reducing variability (Bustin, 2002). As a general principle, the shorter and faster the protocol the better, since each additional step logically will contribute to the overall error. This is to be balanced against the other general principle of sample processing, which is that higher recovery efficiencies tend to reduce the overall detection error, compared to lower efficiencies. For example, a 10% ± 2% recovery efficiency will lead to greater variability at the detection stage than a 90% ± 2% recovery efficiency.

One approach is to extract virus particles, or nucleo-proteins, which are appropriate for both protein and nucleic acid based detection methods. The sample is homogenised in a physiological buffer, usually either a TRIS-based or phosphate buffer, containing an antioxidant. An organic solvent is added and emulsified with the extract to solubilise membranes and other hydrophobic components and hence release trapped virus particles. The solid matter plus organic phase are then separated from the aqueous phase by centrifugation. The type, concentration and pH of the extraction buffer and the type of solvent all have significant influence over the proportion of virus recovered in the aqueous phase and the distribution of other host components that are co-purified (de Miranda *et al.,*

1996a). Addition of the solvent especially can be a double-edged sword, since viruses use hydrophobicity as one of the forces to maintain particle integrity. A proportion of virus protein will therefore end up anchored by its hydrophobic part in the organic phase, releasing naked nucleic acid into the aqueous phase at the mercy of nucleases. The advantages of releasing trapped virus in the solvent-buffer emulsion must be weighed against this loss. This extract is suitable for the detection of (virus specific) proteins. For nucleic acid detection a further purification step is recommended, to prevent their degradation by host enzymes in the extract. The quickest, least variable and most reliable way is purification on spin columns (Verheyden *et al.,* 2003), readily available commercially, which bind either RNA or DNA to a silica matrix in a disposable microcolumn. The nucleic acid is eluted after washing the column to remove all other host components. Phenol-chloroform extraction followed by precipitation of the nucleic acids with ethanol or isopropanol.

Another approach is to extract total nucleic acids directly from the sample by homogenizing in the presence of lysozyme and/or proteinases (Melt®), detergents, organic solvents, denaturing chaotropic salts, nucleic acid binding compounds such as CTAB (cetyl trimethylammonium bromide) or a combination of these (Trizol® is a guanidine isothiocyanate-phenol mix) followed by purification of the nucleic acids on spin columns, magnetic bead-linked nucleic acid binding agents or precipitation in alcohol. Despite the fact that most commercial kits are essentially the same (chaotropic salt extraction followed by silica spin column purification), their performances in comparative tests vary greatly, depending on the organism, tissue type and nucleic acid extracted (Konomi *et al.,* 2002; Burgener *et al.,* 2003; Tell *et al.,* 2003; Honore-Bouakline *et al.,* 2003; Salete de Paula *et al.,* 2003; Knepp *et al.,* 2003; Wilson *et al.,* 2004; Maaroufi *et al.,* 2004; Roos-vanGroningen *et al.,* 2004; Ribao *et al.,* 2004; Schuurman *et al.,* 2005; Hourfar *et al.,* 2005; Scansen *et al.,* 2005; Aldous *et al.,* 2005; Labayru *et al.,* 2005) further highlighting the importance of standardization and uniformity for primary processing prior to specific detection. NucliSens® (BioMerieux), RNAEasy® (Qiagen), NucleoSpin® (Clontech), Melt® (Ambion), HighPure® (Roche) and AccuPrep® (HyLab) are some of the better (known) kits. ViralXpress® (Chemicon) and Trizol® (InVitrogen) are cheaper and use precipitation to recover the nucleic acids, often using carrier tRNA to make recovery more efficient and less variable. The disadvantage of precipitation is that many undesirable compounds often co-precipitate with the nucleic acid, requiring further precipitations or washes to clean the sample.

The next step is to determine the condition of the RNA sample, prior

to any assay. The three critical parameters are quantity, quality and integrity (*i.e.* absence of degradation). The Ribogreen® system uses a RNA binding dye and standards for quantitation. NanoDrop markets a machine that provides a complete UV absorbance profile from 1 µl of sample, which determines both quantity and purity. The optimum solution however is provided by Agilent, who market a chip-based microelectrophoresis system that provides an electrophoretic trace identifying the two major rRNA peaks, which is used to quantify the integrity of the RNA, as well as the amount and purity (Bustin, 2002).

The most variable step in these approaches is the sample homogenization, not only between different homogenization options but also between different samples using the same protocol. Traditionally samples have been homogenized in mortar and pestle, usually in the presence of liquid nitrogen to ensure thorough pulverization of the tissues. However, this approach, while thorough, is not really suitable for individual bee samples since recovery of the powder into individual tubes is too variable. Disposable pestles shaped to fit microtubes containing bee samples do not macerate the tissues sufficiently to guarantee uniformity, especially for adult bee tissues. If objectivity and uniformity of homogenization are paramount, the best options for individual samples are bead mill homogenizers, where glass, ceramic or steel 1-3mm beads are mixed with extractions buffer and sample and shaken at high velocity. The TissueLyser (Qiagen), MagNA-Lyser (Roche) and FastPrep (qBiogene) are the best known bead mill systems. Another option is drill-based macerators with disposable blades, compatible with microtubes. Robotic automated extraction stations (BioRobot®, Qiagen; NucliSense®, BioMerieux; MagNa-Pure®, Roche) extend this objectivity in maceration, although this does not necessarily mean a reduction in cost, variability and time, or an increase in sensitivity (Schuurman *et al.,* 2005; Issa *et al.,* 2005; Wilson *et al.,* 2004; Hourfar *et al.,* 2005; Knepp *et al.,* 2003).

3. Symptoms

Symptoms are still one of the principal methods by which problems in the apiary are diagnosed. The advantages are that it is robust, simple, fast and cheap and for some diseases accurate. For these reasons alone beekeepers should known the symptoms of the major diseases. The major disadvantage is that for most diseases, diagnosis by symptoms is not specific and sensitive enough to allow preventive or even remedial treatment. By the time symptoms appear in a hive the pathogen has already spread

throughout the colony and often also to neighbouring hives in the apiary. Unfortunately this is especially the case for the most damaging diseases, such as American foulbrood, deformed wing virus and the paralysis viruses. The other disadvantages are that many virus diseases do not present visible symptoms at all, that not all life stages present symptoms (lack of universality) and that often different viruses produce similar symptoms (paralysis) or that a single virus present different symptoms (also paralysis). Colonies can furthermore suffer from more than one disease at the same time, confounding the symptoms. All viruses are asymptomatic at lower levels of infection and most shorten the life span of bees to varying degrees. The diagnostic symptoms for the major virus diseases have been described in detail in many prior publications (Bailey and Ball, 1991; Ball and Bailey, 1997). They are synthetised in Chapter 1 and can be summarized as follows:

3.1. Sacbrood virus

The clearest symptoms of sacbrood virus (SBV) appear a few days after capping, and consist of non-pupated pale yellow larvae, stretched on their backs with heads lifted up towards the cell opening, emersed in the unshed, saclike larval skin containing a clear, yellow-brown liquid. The virus is also present in adult bees, but without symptoms (Lee and Furgula, 1967; Bailey, 1968).

3.2. Chronic bee paralysis virus

Chronic bee paralysis virus (CBPV) manifests itself in adult bees through two distinct set of symptoms. One set consists of trembling of the wings and bodies and a failure to fly, causing them to crawl in front of the hive in large masses. They often have partly spread, dislocated wings and bloated bodies as well. The other set of symptoms consists of hairless, greasy black bees caused by nibbling attacks from healthy bees in the colony. They soon also become flightless, tremble and die (Bailey, 1965; Bailey and Ball, 1991). The virus also infects the larval and pupal stages and can be detected in fecal material.

3.3. Slow paralysis virus

Slow paralysis virus (SPV) is characterised by the paralysis of the front two pairs of legs of adult bees, a few days before dying. The virus can be detected in larvae and pupae, but produces no symptoms in these.

3.4. Acute bee paralysis virus

Acute bee paralysis virus (ABPV) is largely symptomless in individual adults, pupae and larvae, but can be lethal at colony level, particularly in association with *Varroa destructor* (Ball, 1985; 1987; Allen *et al.,* 1986).

3.5. Kashmir bee virus

Kashmir bee virus (KBV) is genetically closely related to ABPV (Allen and Ball, 1995; de Miranda *et al.,* 2004) and is similarly symptomless in individual adults, pupae and larvae (Bailey *et al.,* 1976; 1979; Dall, 1987; Anderson, 1991) and lethal at colony level (Allen and Ball, 1995; Hung *et al.,* 1996; Todd *et al.,* 2007).

3.6. Deformed wing virus

The symptoms for deformed wing virus (DWV) consist of crumpled and/or vestigial wings and bloated abdomen and die soon after emergence. Asymptomatic bees can also be heavily infected, though with lower titres than symptomatic bees (Bowen-Walker *et al.,* 1999; Lanzi *et al.,* 2006; Tentcheva *et al.,* 2006). The virus is detected in all other life stages as well, but without obvious symptoms (Chen *et al.,* 2005a; 2005b; Yue and Genersch, 2005; Lanzi *et al.,* 2006; Tentcheva *et al.,* 2006; Fievet *et al.,* 2006; Yue *et al.,* 2006).

3.7. *Varroa destructor* virus

Varroa destructor virus (VaDV-1) is genetically closely related to DWV and, like DWV, replicates in both honey bees and varroa mites (Ongus *et al.,* 2004). DWV-like symptoms have not been observed in association with VaDV-1 infection in bees, which appear much like asymptomatic DWV infections.

3.8. Egypt bee virus

Egypt bee virus (EBV) is serologically related to DWV, but has no symptoms in adults, pupae or larvae (Bailey *et al.,* 1979).

3.9. Black queen cell virus

The main symptoms for black queen cell virus (BQCV) consist of blackened cell walls of sealed queen cells, containing dead propupae (Bailey and Ball, 1991; Leat *et al.,* 2000). Diseased larvae have a pale yellow appearance and tough sac-like skin, much like sacbrood. The virus is present in adult bees but without obvious symptoms.

3.10. Cloudy wing virus

The symptoms for cloudy wing virus (CWV) consist of opaque wings of severely infected adult bees, with lower titres resulting in asymptomatic infected bees. It cannot be propagated in larvae or pupae.

3.11. Filamentous virus

Filamentous virus (FV) is a DNA virus has no physical symptoms. It renders the hemolymph of adult bees milky white with rod-shaped viral particles, when examined by microscopy (Clark, 1978).

3.12. Bee virus X

Bee virus X (BVX) is largely symptomless in adult bees and does not multiply in larvae or pupae. It is associated with the protozoa *Malphighamoeba mellificae* that causes dysentery in winter bees (Bailey *et al.,* 1983a).

3.13. Bee virus Y

Bee virus Y (BVY) is closely related to BVX and similarly symptomless in adult bees, larvae or pupae. It is associated in adult bees with the dysentery inducing protozoa *Nosema apis* (Bailey *et al.,* 1983a).

3.14. Apis iridescent virus

The symptoms for *Apis* iridescent virus (AIV) are similar to the adult flightless clustering symptoms of CBPV (Bailey *et al.,* 1976; Bailey and Ball, 1978). It is only known to occur in adult bees.

3.15. Arkansas bee virus

Arkansas bee virus, also known as Berkeley bee virus, has no known symptoms in adult bees.

4. Bioassays

Bioassays, or infectivity tests, were used before sensitive molecular techniques were developed to detect low levels of virus in surveys (Bailey, 1976; Bailey *et al.,* 1981; 1983b). These tests take advantage of the fact that most bee viruses when injected into adult bees or pupae multiply rapidly to high titres that can subsequently be detected by serology (Dall, 1987). Dilution series of the extracts provide a measure of quantitation. Different viruses develop titre and kill pupae at different rates, which can be detect-

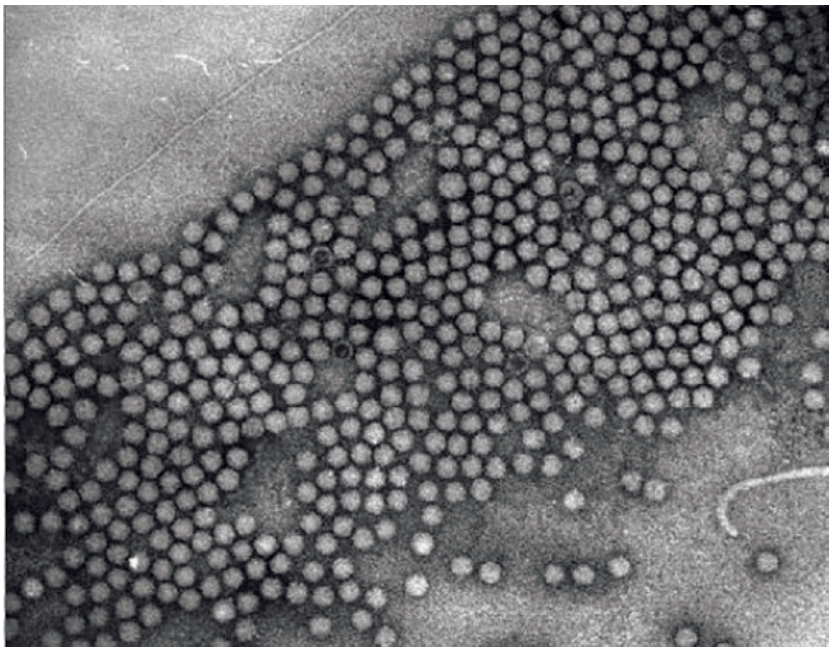


Figure 1. Electronmicrograph of honey bee virus particles. Most honey bee viruses have this morphology with a 30 nm diameter. © B. Ball, Rothamsted Research.

ed by the 'breaking' of the eye-colour development in white-eyed pupae (Anderson and Gibbs, 1988; 1989). This can provide an early indication of which virus is being multiplied. Although labour intensive, bioassays can rival the most sensitive molecular tests available (Denholm, 1999; Quinlivan *et al.*, 2004). One serious drawback of honey bee bioassays is that often inapparent viruses present at very low levels in the assay pupae can also be amplified, sometimes by the mere injection of buffer (Bailey, 1967; Anderson and Gibbs, 1988; 1989). Several important bee viruses (ABPV, KBV and SPV) were discovered this way, as a by product of the propagation of CBPV, AIV and BVX respectively (Bailey *et al.*, 1963; Bailey and Milne, 1969; Bailey and Woods, 1977; Ball and Bailey, 1997), and the technique may yet prove useful for the discovery of other harmful but symptomless bee viruses.

5. Microscopy

Microscopy is a very useful tool to catagorise viruses according to particle morphology (Fig. 1), tissue distribution, inclusion bodies or crystallization properties (Brcak and Kralik, 1965; Thomson and Smirk, 1966; Furgala and Lee, 1966; Bailey, 1968; Bailey and Milne, 1969; Mussen and

Furgala, 1977; Du and Zhang, 1985; Dall, 1987). Its primary value lies in the initial characterisation of novel viruses rather than the routine detection of known viruses. Microscopy can be combined with stains (histology), serology (immunohistochemistry, immuno-electron microscopy) or nucleic acid technologies (*in situ* hybridisation, *in situ* RT-PCR) to distinguish similar-shaped viruses (Fievet *et al.,* 2006; sections 7.7, 7.8, 8.4).

6. Molecular detection: tags, markers, dyes and probes

Since viruses are microscopic agents with often similar morphology, most molecular detection methods use a set of molecular probes, each specific for a virus. The probe-virus interaction is visualised using tags, markers or dyes that can be detected by eye or increasingly by automated instruments. There is now an enormous variety of such visualization technologies available each with their strengths and weaknesses. Matching these to the diagnostic requirements is an important early step in developing a diagnostic strategy. This section is therefore dedicated to several important considerations relevant to choosing a suitable marker-detection technology.

6.1. Types of tags and markers

6.1.1. Radioactivity

Radioactive isotopes of common elements in biomolecules (hydrogen, oxygen, nitrogen, phosphate, sulphur) are one of the most sensitive and precise means of labelling molecules for detection (Fig. 2b). The most commonly used are P^{32}, $P^{33}$and S^{35} which emit β- particles of high energy (especially P^{32}) and have a short half-life, ideal characteristics for sensitive detection (Sambrook and Russell, 2001). The obvious drawback is that they require special facilities, training, permits, transport and disposal and for these reasons they are increasingly being replaced by equally sensitive non-radioactive alternatives. Radioactive label is also rather expensive and its short half-life places time constraints on experiments and decrees that probes have to be made fresh each time, limiting their usefulness in repetitive protocols.

6.1.2. Particles

Several types of physical particles can be attached to biomolecules. Gold particles of various sizes are used primarily for electron microscopy localisation studies (Fig. 2a). Other particles include agarose, cellulose

or magnetic beads, used for purification and enrichment (section 8.6.2-8.6.4).

6.1.3. Enzymes

Molecular probes can be tagged with enzymes that process colourimetric or (chemi) luminescent substrates (Fig. 2c). The most commonly used enzymes are alkaline phosphatase and horse radish peroxidase, which are robust enough to withstand the chemical treatment that attaches them to the probe without losing activity. The reaction products can be either soluble, for liquid assays, or insoluble, for localization on solid supports (slides, membranes *etc.*). The enzymatic reaction proceeds until stopped by killing the enzyme. The usefulness of this is that it provides a form of signal amplification (see section 6.3) enhancing the sensitivity of the reaction. The drawback is that the enzymatic reactions are only partly linear requiring extra care during the colour development stage to maintain linearity between molecular detection and signal strength (colour intensity). Furthermore, the enzymes lose activity with storage, disrupting the relationship between molecular detection and signal strength, requiring extensive use of standards to provide consistency between assays. Another important consideration is that there may very well be native background enzyme activity present in the sample which will have to be taken into account.

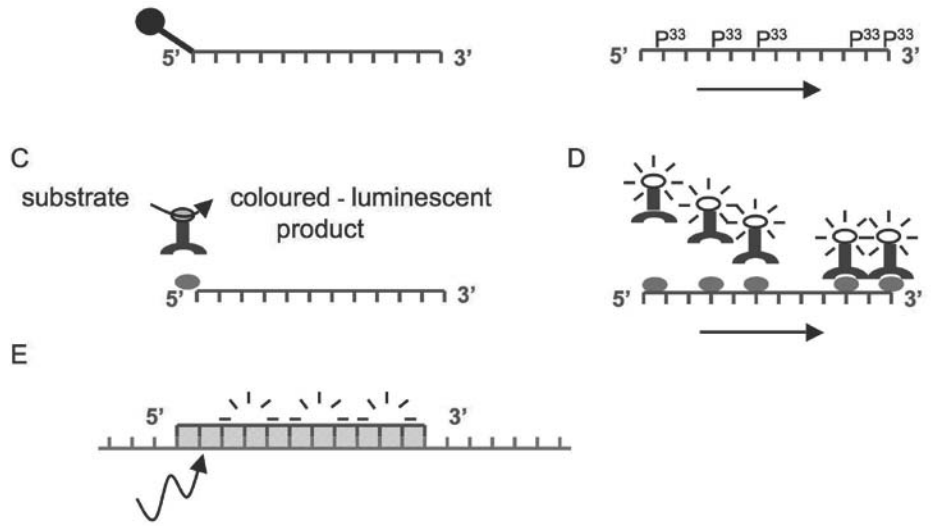


Figure 2. Illustration of different labelling options and techniques. Probes can be labelled directly (a, b, e) or indirectly (c, d) with particles (a), radioactivity (b), enzymes (c), fluorophores (d) or dyes (e), either through chemical modification of the 5' end (a,c) or through incorporation of modified nucleotides by a polymerase (b, d).

6.1.4. Dyes

There are many colourful dyes for detecting nucleic acids (Haugland, 2000). The classic ones include ethidium bromide, propidium iodide, actinomycin-D and the acridine dyes (which fluoresce differently with DNA than with RNA), while the newer ones include the cyanine family of dyes (POPO, TOTO, YOYO, BOBO and their derivatives, each with different colour) and the SYBR dyes (SYBR-green, SYBR-gold). Most of these fluoresce upon binding to nucleic acid and their sensitivity is determined by the binding density of the dye, the quantum yield upon excitation and the increase in fluorescence of the bound form compared to the unbound form. The best can attain sensitivities that rival radiolabeled probes (Sherwood *et al.,* 1995). The most useful ones for detecting hybridisation events are those that preferentially bind/fluoresce with double stranded DNA, *i.e.* the intercalating dyes that wedge between the two strands (Fig. 2e). Ethidium bromide and some of the SYBR dyes fall in this category. Dyes are also relatively cheap, simple to use, universal and often fluorescence is linearly related to the amount of nucleic acid over a large range of concentrations. The other main advantage is that the hybridisation probes do not have to be chemically or enzymatically altered and that hybridisation and detection can be achieved simultaneously, allowing real-time detection and eliminating the additional purification steps needed with labelled probes to remove unbound probe. The disadvantages of dyes are that the signal strength is related to the length of the probe, and for some dyes also on the AT/GC ratio, making quantitative comparisons between probes bothersome (Giglio *et al.,* 2003), and multiplex detection of several targets is only possible if the different target-probe complexes are separated (microarrays, electrophoresis, chromatography *etc.*).

6.1.5. (Electro)chemiluminescence

In chemiluminescence, light is emitted as a result of chemical degradation of certain organic peroxides. In most diagnostic applications the reaction is driven by an enzyme (peroxidase, phosphatase, luciferase) attached to the probe which provides one of the components of the reaction (Fini *et al.,* 1999). Many of the principles therefore are the same as for enzymatic tags. Chemiluminescence is a convenient alternative to radioactivity for sensitive autoradiographic detection in Southern/Northern/Western blots and *in situ* microscopic detection (Akhavan-Tafti *et al.,* 1998; Suzuki, 1998). Self-reporting chemiluminescent hybridisation probes have been developed, that rival FRET probes for sensitivity (Fig. 3; Arnold *et al.,* 1989; Wolcott, 1992). In electrochemiluminescence (ECL) the degradation is triggered by an electrical pulse (Wilson *et al.,* 2001). This version has

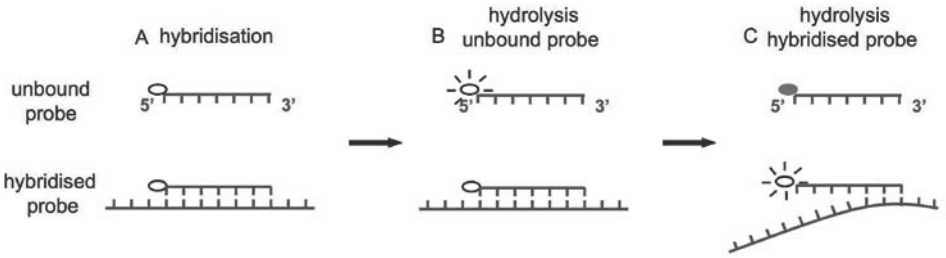


Figure 3. Self-reporting chemiluminescent probes. A chemiluminescent probe is hybridised to the target (a), excess probe is hydrolysed while hybridised probe is protected from hydrolysis (b) and the amount of hybridised probe is quantified through denaturation of the probe-target complex, exposing the probe to chemiluminescent hydrolysis (c).

been developed by OrganonTeknika and BioMerieux into a rather complex detection technology designed to concentrate the target-probe complex at an electromagnetic probe where an electrical pulse triggers luminescence which is then detected (Fig. 4; Baeumner *et al.,* 2001; Deiman *et al.,* 2002; Landry *et al.,* 2005). The advantage of chemiluminescence is that it does not require an external light excitation source, avoiding some of the background problems of fluorophores (section 6.1.6; Akhavan-Tafti *et al.,* 1994; Nieman, 1995). The disadvantage is that it is a non-regenerative reaction and can therefore only be used in end-point analysis, while fluorescence can also be used in real-time applications. Luminol, luciferin, peroxyoxalate, ruthedium chelate and acridinium-ester are a few common luminescent substrates (www.lumigen.com).

6.1.6. Fluorescence

Fluorophores are compounds that absorb and emit light. There are many such compounds, each with its own absorption and emission spec-

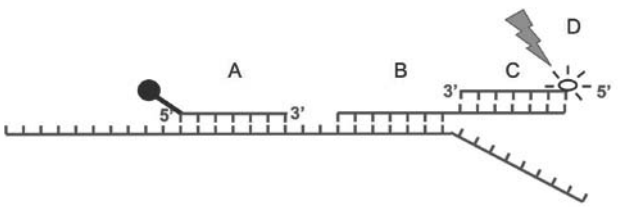


Figure 4. Illustration of the automated NucliSens® electrochemiluminescence (ECL) detection system. The target is captured and drawn to an electromagnet by a paramagnetic bead-linked probe (a), marked with a target-specific probe (b) containing sequences complementary to a generic ECL-probe (c) which is activated by an electric pulse from the electromagnet. Only ECL probes close to the electromagnet, i.e. those attached to the target, are thus activated.

trum. This makes them useful for detecting several different targets at the same time, and the wavelength specific excitation and simultaneous quantitative detection of fluorophores is very well suited to automation making them currently the label of choice for many routine detection procedures. Fluorophores are also very stable, with little or no decay over time, and the emitted light is largely linearly related to fluorophore density and hence to the molecular detection event (Haugland, 2002). They can also be remarkably sensitive and can be used in quantitative, competitive solution hybridisation in combination with background reducing, quencher-linked antisense primers (Nolan *et al.,* 2003, see also section 6.1.7; Fig. 5). Green fluorescent protein (GFP), isolated from the jellyfish *Aequorea corulescens*, is a unique protein capable of fluorescence upon excitation. The fact that it is a protein gives it unique advantages, since it can be cloned into proteins or behind promoters whose movement, localization or activity can thus be traced with great facility. Bioprospecting and genetic engineering has produced a range of fluorescent proteins each with slightly different colours, allowing multiplexing (www.clontech.com). GFP technology is used primarily in microscopic studies. The main drawback of many fluorophores, as with enzymes, is that biological samples naturally contain compounds that absorb and/or emit light, interfering with detection. Again, the best solution is to remove these compounds from the sample prior to analysis, or avoid their effect by using alternative tags. Another disadvantage is the small difference (Stokes shift) between the excitation and emission wavelengths of many fluorophores. Not all of the background excitation light can be filtered out of the fluorescing light, reducing the signal/noise ratio and hence sensitivity.

6.1.7. Fluorescence resonance energy transfer (FRET)

Fluorescence (or Förster) resonance energy transfer (FRET; Stryer and Haugland, 1967; Yaron *et al.,* 1979) is a very powerful technology that solves the real-time detection and quantitation problems of labelled

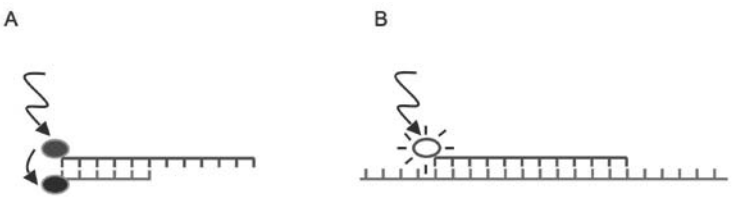


Figure 5. Illustration of the use of generic quencher-linked antisense primers to neutralize background fluorescence from excess fluorophore-linked primers (a) while these are displaced for target-probe hybridisations (b), enabling fluorescence.

probes and the multiplexing and specificity problems of nucleic acid dyes. In an experimental setting it is also very useful to investigate the interactions between nucleic acids, proteins, lipids and other biomolecules (Hofkens *et al.,* 2003; Eggeling *et al.,* 2005). This versatility has made molecular beacons immensely popular. The basic principle of FRET is outlined in Figure 6. Two compatible dye molecules (donor/fluorophore and acceptor/quencher) are attached to a biomolecule in such a way as to

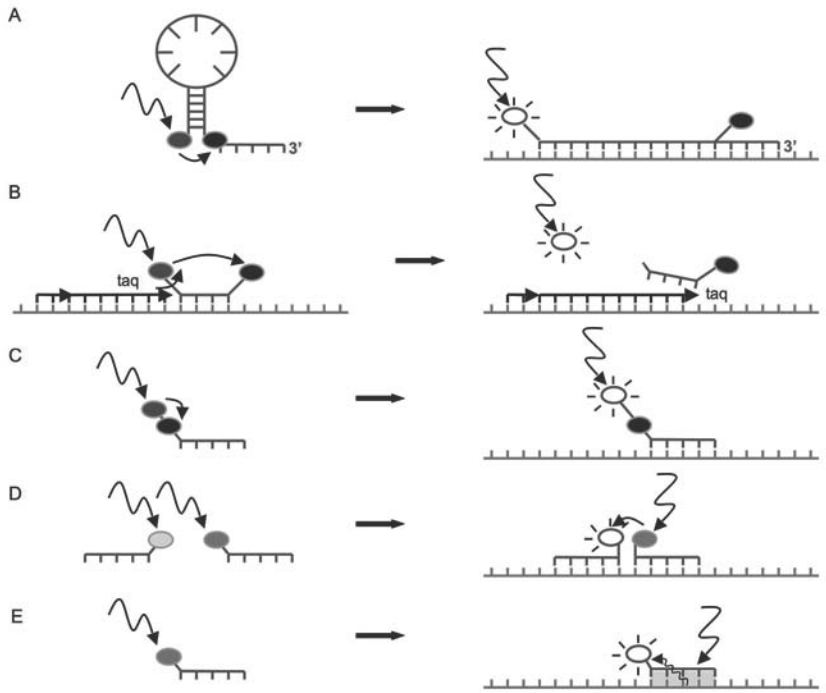


Figure 6. Illustration of the principles of Fluorescence resonance energy transfer, where the photon energy donor fluorophore is transferred to and quenched by the acceptor fluorophore in close proximity. For molecular beacons and Scorpions® this proximity is achieved by internal hybridisation between the 5' and 3' ends of the probe (a). Scorpions have a 3' extension for polymerisation from the scorpion primer, while molecular beacons cannot be extended at the 3' end. TaqMan® primers (b) are molecular beacons whose 5' fluorophore gets hydrolysed by Taq polymerase during PCR, enabling real-time fluorescence detection during the extension phase. SimpleProbes (c) have the fluorophore and quencher in tandem, with the hybridisation forcing sufficient distance between them to abolish quenching. In the two primer approach (d) the energy from specific excitation of one of the fluorophores is passed to a second fluorophore on an adjacent primer, whose emission is captured. Without co-hybridisation of both primers on the same target there is no fluorescence. iFRET achieves the same by combining a single fluorophore-linked primer with a general fluorescent dye (e).

be in close proximity (10-100Å) in the free configuration of the molecule, and approximately parallel to each other. In Figure 6a this is achieved by complementarity between the 5' and 3' ends of a nucleic acid probe. In this scenario excitation energy is transferred from the donor to the acceptor dye without emission of a photon. A change in the conformation of the molecule (for example through molecular interactions or hybridisation) or hydrolysis of the probe-fluorophore bond (TaqMan®) disrupts the energy transfer and the fluorophore emits light upon excitation (Fig. 6a). The quenching is highly dependent on the intermolecular distance between donor and acceptor. It is therefore sensitive to small changes in hybridisation intensity, such as single nucleotide differences between nucleic acid probe and target (Thelwell *et al.,* 2000; Solinas *et al.,* 2001), making it useful for population-level screening of genetic polymorphisms. FRET has been translated into several related technologies, such as molecular beacons (Fig. 6a excluding 3' extension; Tyagi and Kramer, 1996), Scorpions® (Fig. 6a including 3' extension; Whitcombe *et al.,* 1999), TaqMan® probes (Fig. 6b; Holland *et al.,* 1991) and SimpleProbes® (Fig. 6c; Roche). Generally the fluorophore is attached to the 3' end of the probe with the quencher as close as possible, either internal or at the 5' end. An alternative is to use two adjacent primers, with the resonance energy transfer resulting in light emission at a particular wavelength (Fig. 6d). A range of fluorophore-quencher combinations are available (see www.probes.com), making multiplexing possible. Recent improvements include the Locked Nucleic Acid™ (LNA®; www.sigma-aldrich.com) and peptide nucleic acid (PNA) technologies (Nielsen and Haaima, 1997; Fiandaca *et al.,* 2001) which involve modified nucleotide analogues that confer enhanced binding specificity and stability to the detecting FRET probe. This increases the Tm and the signal-noise ratio of the hybridisation, making it especially useful for multiplex qPCR, NASBA or SDA reactions (sections 8.5.5-8.5.7). In general diagnostics the technology can be used for real-time, one-tube, multiplex and probe-specific quantitation of several targets, each detected by probes with different donor/acceptor fluorophores. It is even possible, in theory, to simultaneously detect and quantify different biomolecules, *i.e.* nucleic acids, proteins, lipids *etc.*. Other advantages are that signal is independent of the size of the probe (one probe, one tag) and that the technology is well suited to automation. The disadvantages are expense, a complex optimization process, strict criteria for the condition of the probe (melting temperature and stem-loop length), probe instability during storage (Bustin, 2002) and a much weaker relative fluorescence compared to other fluorescence methods (Howell *et al.,* 2002; Nazarenko *et al.,* 2002). All of these disadvantages can be overcome by using a dsDNA-specific

dye (SYBR-green) as energy donor for an acceptor emitting at a different wavelength, attached to a target-specific probe (iFRET; Fig. 6e) or self-quenching monolabeled probes/primers (Nazarenko *et al.,* 2002). Different acceptor fluorophores allow multiplexing and real-time detection (Howell *et al.,* 2002). Another advantage of iFRET is increased flexibility, since the acceptor can be linked to one of the PCR primers (in which case quantitation is after the synthesis phase and care has to be taken to exclude non-specific amplifications) or to a separate detecting probe (in which case quantitation is during the annealing phase and target-specific; see sections 8.5 and 9.3 on PCR and quantitative PCR).

6.2. Direct or indirect labelling and detection

The reporting tags can be attached directly to the detecting probe (Fig. 2a), or indirectly, as the final component of a cascade of molecular interactions (Fig. 2c, 2d). The choice is largely a matter of economy, convenience and sensitivity. The advantages of direct labelling are simplicity, speed and reduced variability (fewer steps). The advantages of indirect labelling are enhanced sensitivity (signal amplification) and flexibility (the primary probe can be detected by generic secondary probes linked to a range of reporting tags). Although both approaches are common in commercial diagnostic systems, direct labelling is preferable whenever possible. For protein detection systems, which are mostly based on antibodies, indirect labelling-detection is popular due to the high cost of the primary detecting (monoclonal) antibodies, compared to the generic tagged secondary antibodies. For nucleic acid detection systems direct labelling is much more popular due to the relatively low cost of synthetic oligonucleotide probes. For such probes the reporting tag is usually attached to the 5' terminus, leaving the 3' end accessible to polymerases (Fig. 2a, 2c). Nucleic acid probes can also be synthesized by a DNA or RNA polymerase from a nucleic acid template, in which case the tag (for direct or indirect detection) is incorporated as part of a modified nucleotide (Fig. 2b, 2d). The advantages of this approach are increased sensitivity, since each probe molecule contains multiple tags, and universality, since these probes are much larger than oligonucleotide probes, covering more of the target. The disadvantages are that the amount of signal is related to the length of the probe, complicating quantitative comparisons between probes of different lengths (see section 6.2) and that DNA polymerases have difficulty processing nucleotides with bulky attachments, which means that only a few labelling options are readily available (radioactivity and some fluorophores for direct detection and biotin-strepavidin, fluorescein or digoxigenin for indirect detection). For end-labelled probes the signal is independent of

probe length (each molecule, regardless of length, has a single tag) and many more tagging options are available making it easier to multiplex. Biotin (vitamin H) is also a common compound in many animal tissues, leading to background problems. Digoxygenin, found naturally only in *Digitalis* plants, is less problematic in this respect (Sambrook and Russell, 2001). Most commercial non-radiactive detection kits use indirect labelling, with biotin, digoxigenin or fluorescein as the primary label and an enzyme-conjugated secondary label (Fig. 2c).

6.3. Signal amplification

This refers to a set of procedures used to amplify the signal obtained from the hybridised tag. For radiolabeled probes this means scintillation fluid for liquid processes or intensifying screens during autoradiography. For non-radioactive detection methods this generally refers to the attached enzymes that churn substrate in a colorimetric or luminescent assay, or complexing multiple layers of detecting molecules each with a multiplicity of reporter tags. The main problem with signal amplification in general is that it converts a linear relationship between reporter signal and the amount of target detected to an exponential one, making it difficult to keep the quantitation within the linear range of the curves (see also section 9). This becomes especially problematic if there are large signal differences between different samples processed together.

6.4. Label-free detection

Thus far detection and quantitation of hybridisation events have involved chemical or enzymatic modification/amplification of either probe or target nucleic acid in order to visualize the event. However, nucleic acids have several intrinsic physical properties that by themselves are enough to allow sensitive detection of hybridisation events. This is called label-free detection and is in many ways the Hoil Grail of detection methodologies, since neither probe not target needs to be modified, saving time, expense and variability. Most of these do not yet have the sensitivities expected from labelled probes, although this gap is closing fast (Gabl *et al.,* 2004; Koehne *et al.,* 2004). The most useful properties for diagnostic applications are mass, shape (3D-structure), electrical charge and optical properties. In each case the root principle for detection is very simple.

6.4.1. Mass: molecular cantilevers and quartz crystal microbalances

Upon specific hybridisation between target and probe, the combined mass of complex increases and this can be detected by molecular cantilevers by which the probe is attached to a fixed substrate (Fig. 7). The change

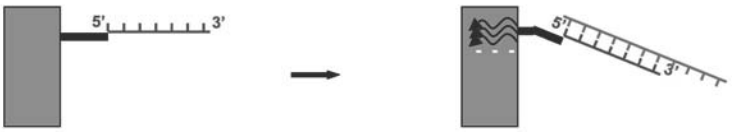


Figure 7. Gravimetric detection of hybridisation complexes. Microcantilevers or quartz-crystal microbalances translate the increased mass through piezoelectric effects into detectable current or voltage.

in stress in these cantilevers is translated through the piezoelectric effect into an electrical signal, which can be amplified using conventional electronic procedures (Alvarez *et al.,* 2004; Mukhopadhyay *et al.,* 2005). Piezoelectric effects are also the principle behind quartz-crystal microbalances (QCM), (Fawcett *et al.,* 1988; Okahata *et al.,* 1992; 1998; Caruso *et al.,* 1997; Towery *et al.,* 2001) and similar gravimetric devices. The sensitivity of these devices is moderate, and they do not have the capacity to detect genetic variations.

6.4.2. Mass-Volume: acoustic network analysis and Love-wave sensors

Acoustic network analysis (Su and Thompson, 1995), Love-wave biosensors (Tamarin *et al.,* 2004) and bulk acoustic wave devices (Gabl *et al.,* 2004; Lange *et al.,* 2003) work on the principle that the increased bulk (mass and/or volume) due to hybridisation or other biomolecular interactions effect changes in high frequency acoustic waves passed through or along the sensor surface, which is detected at the reference surface (Fig. 8) and converted to electrical signal (Stevenson *et al.,* 2004). Such sensors are sensitive to the characteristics of the medium (gas, liquid, viscosity, flow-rates *etc.*) where the molecular interactions take place (Hu *et al.,* 2004; Bender *et al.,* 2004) which may limit their universality. However, they are considerably more sensitive than quartz crystal microbalances, and have greater resolution (Gabl *et al.,* 2004; Tamarin *et al.,* 2004).

Figure 8. Acoustic detection of increased mass after hybridisation. The frequency of an acoustic wave is altered by increasd mass, detected and translated into electrical signal.

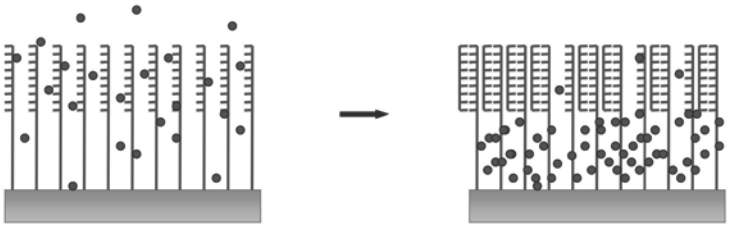


Figure 9. Detection of hybridisation by increased volume in nano-compartments. The hybridised complex creates a molecular 'membrane', whose permeability to detectable chemicals is a function of the amount of hybridised target.

6.4.3. Volume: nanocompartments

Double stranded nucleic acid also has much greater volume than single stranded nucleic acid. By controlling the density of the single-stranded detecting probes on a solid support, a semi-permeable barrier or 'membrane' of double-stranded nucleic acid can be created upon hybridisation, with the permeability of this 'membrane' a function of the ratio of hybridised and non-hybridised probe, *i.e.* the amount of target. This permeability is monitored by the flow of detecting molecules (dyes, electrolytes) in and out of the space between the solid support and this dsDNA membrane (DNA nanocompartment; Fig. 9), which again is translated into an electrical signal (Mao *et al.,* 2004).

6.4.4. Shape: surface plasmon resonance

Another way of measuring the increased bulk of a hybridised sample is through surface plasmon resonance imaging where the added bulk of a hybridised complex is visualised through a form of *in situ* optical shadowing. At a certain angle of incident light, photons are neither reflected nor refracted through the underlying surface and instead transfer their resonance energy to electrons (surface plasmons) in a thin metal film covering the solid support surface (Fig. 10). The angle at which this event occurs is influenced quantitatively by the mass of molecules close to the metal surface (Pollard-Knight *et al.,* 1990; Fagerstam *et al.,* 1990; Jost *et al.,* 1991). The technology has been developed primarily by BIAcore and is detailed enough to detect single nucleotide polymorphisms, or SNPs (Persson *et al.,* 1997; Nilsson *et al.,* 1997) which are of supreme importance in medical genetics. As a result it is being combined with targeted electrostatic control of hybridisation kinetics for quantitative analysis of nucleic acid microarrays (Heaton *et al.,* 2001; sections 6.4.5, 8.8). However, the primary application of surface plasmon resonance is in the study bio-

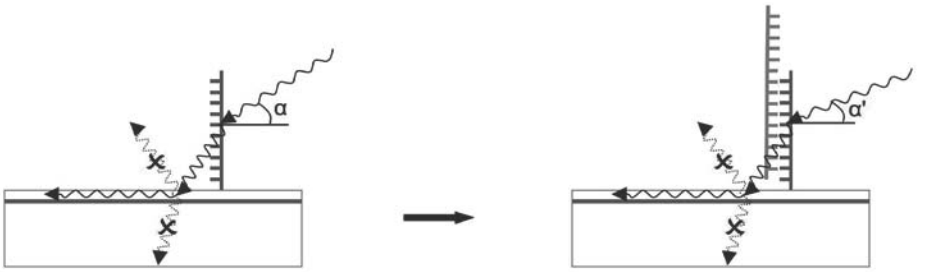


Figure 10. Surface plasmon resonance-based detection of increased mass-volume upon hybridisation. The angle of incidence that determines whether incident light is reflected, refracted or converted to detectable surface plasmons is quantitatively related to the total mass close to the surface.

molecular interaction kinetics and conformations (Fagerstam *et al.,* 1990; Jost *et al.,* 1991; Peterson *et al.,* 2001) including those of proteins and whole virus particles (Boltovets *et al.,* 2004). Quantitation is of the order of fmols of molecules (Pollard-Knight *et al.,* 1990; Nelson *et al.,* 2002).

6.4.5. Charge: electronic and electrochemical detection

Nucleic acids are also electrically charged molecules, courtesy of an ionized phosphate-sugar backbone. Hybridised DNA therefore has twice the charge of single stranded DNA. This property furthermore provides the most direct translation of genetic information into microelectronic signal. The electronics industry is also highly developed in the detection and amplification of weak electrical signals, providing an ideal platform for the development of sensitive electronic biosensors. The principles of the technology are outlined in Figure 11. Probe molecules are deposited on a surface, much as on a microarray and the added electrical charge from the target molecule after hybridisation is detected electronically. In field-effect sensors (Fritz *et al.,* 2002; Kim *et al.,* 2004; Uslu *et al.,* 2004; Macanovic *et al.,* 2004; Archer *et al.,* 2004) the hybridisation event in the electrolytic solution is separated from a detecting silicon layer by an insulator (an EIS structure). The added negative charge of the hybridised target molecule induces an electron depletion zone in the silicon layer, which can be detected and quantified by a change in conductance, capacitance or impedance in the silicon layer (Fig. 11a).

In electrochemical sensors the added charge of the hybridised target directly or indirectly affects the redox status of an electrochemical amplifier, such as a ferrocene layer (Umek *et al.,* 2001) which generates a Faradic micro current that is passed through molecular nano-wires to ultrasensitive electronic sensors (Fig. 11b). Other electrochemical amplifiers

include oxidation of the guanidine bases of the captured target (Koehne *et al.,* 2004), ferrocene labelled oligonucleotides (Liepold *et al.,* 2005) or positive-charged dsDNA intercalating dyes (Sakata and Miyahara, 2005). By incorporating multiwalled carbon nano-tube technology for both the hybridisation event and the semiconducting molecular nano-wires, the detection sensitivities can be considerably enhanced (Cui *et al.,* 2001; Koehne *et al.,* 2004; Uslu *et al.,* 2004), such that single virus particles can now be detected electrically (Patolsky *et al.,* 2004).

Other innovations include using peptide nucleic acid (PNA), a charge-neutral DNA mimic (Nielsen and Haaima, 1997; Weiler *et al.,* 1997; Macanovic *et al.,* 2004) as the bound probe and using electricity to move the target nucleic acid to specific microarray spots and to control the stringency of the hybridisation process (Heaton *et al.,* 2001; Sosnowski *et al.,* 2002; Weidenhammer *et al.,* 2002; Estrela *et al.,* 2005). These electrical detection events occur at molecular distances where even a single mismatch between probe and target results in a measurable drop in signal (Fritz *et al.,* 2002; Umek *et al.,* 2001) which can be controlled by temperature (Mao *et al.,* 2003). This means that SNPs can be detected; that non-specific hybridisations are not detected resulting in low backgrounds, and that the technology operates under a wide range of conditions, temperatures and the presence of other biomolecules (crude samples) since only near-perfect matches are detected (Fritz *et al.,* 2002, Umek *et al.,* 2001).

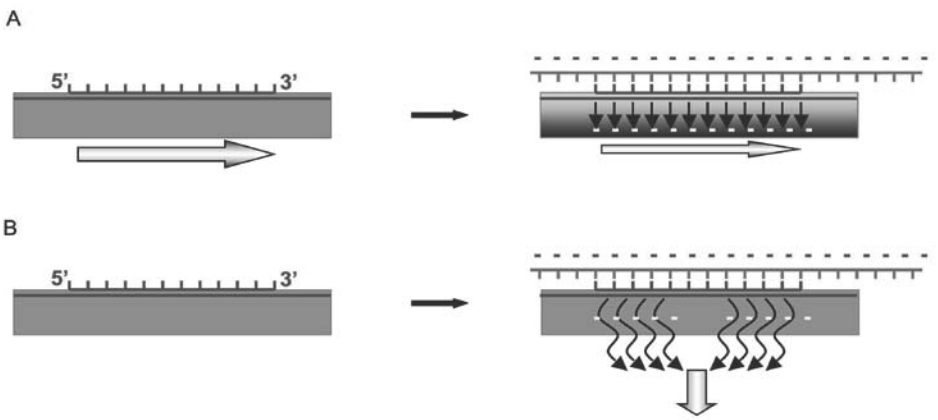


Figure 11. Illustration of the principles of electronic label-free hybridisation detection. Figure 11a shows a field-effect sensor, where the negative charge of the hybridised target displaces electrons in the silicon layer, detected as a change in capacitance or conductance. Figure 11b shows an electrochemical microcurrent system, where the hybridisation stimulates a redox reaction in a ferocene layer, whose charge is passed through molecular wires to ultrasensitive voltaic detectors. Other electrochemical transducers include target-derived guanidine.

The technology is very fast, *i.e.* detection occurs in minutes (Umek *et al.,* 2001; Weidenhammer *et al.,* 2002) and is nearing the sensitivities attained by fluorogenic processes (Koehne *et al.,* 2004). These strengths are also its weaknesses, in that naturally occurring pathogen variants may not be detected if sufficiently divergent from the probe sequence and that quantitation may be influenced by polymorphisms. The technology is also still experimental and therefore expensive, although it is only a matter of time before cost-effective devices based on these principles will become available (Mir and Katakis, 2005). Similar devices are also being developed for detecting proteins (Sosnowski *et al.,* 2002; Zheng *et al.,* 2005).

7. Proteins and serology

Proteins are the functional units derived from the genetic code and as such represent the variety of biological processes and structures. Some are extremely resilient to degradation, others very sensitive, especially when assayed for their function. Fortunately, in diagnostics it is often irrelevant whether a protein is functional or not, allowing for more universal sample preparation procedures. Most of the routine protein diagnostics is through serology, which provides further homogenisation of procedures and protocols. Protein and serological techniques are also usually the first molecular tools developed for the characterisation of new viruses, providing an indispensable link between the symptomatic and physical characteristics of a virus and its genetic identity (Leat *et al.,* 2000; Ribiere *et al.,* 2000; 2002; de Miranda *et al.,* 2004; Ongus *et al.,* 2004; Lanzi *et al.,* 2006; Todd *et al.,* 2007).

7.1. Protein profiling

Most known viruses are simple organisms, consisting of a nucleic acid genome encased in a protein shell. Even more complex viruses, whose infective particles have one or more layers of membranes, usually have their genetic material wrapped in protective nucleoprotein. The number and sizes of these particle or nucleoproteins are characteristic for each virus and a useful means for identification (Ball and Bailey, 1991; 1994; 1997; Ribiere *et al.,* 2000; Leat *et al.,* 2000; Govan *et al.,* 2000; Lanzi *et al.,* 2006; Todd *et al.,* 2007), global classification (Allen and Ball, 1996) and differentiation between species or even strains of a virus (Madriz *et al.,* 1996; 2000; Allen and Ball, 1995). The most precise way to separate these proteins is by polyacrylamide gel electrophoresis (PAGE), usually in the presence of the ionic detergent sodium dodecyl sulphate (SDS-PAGE)

which denatures the proteins and envelops them uniformly, such that the charge/size ratio is constant for all proteins (Sambrook and Russell, 2001). This ensures an even separation by size of the proteins in the gel, and the resulting pattern constitutes the protein profile of the virus (Fig. 12a, 12b). The proteins are visualized by staining, usually with Coomasie blue. Non-denaturing PAGE, where electrophoretic separation is dependent on the intrinsic charge of the protein in the buffers used, can be used to distinguish between minor virus variants with similar-sized proteins, but with different overall charge. One such procedure is isoelectric focusing (IF) where the proteins migrate along an immobilized pH gradient in the gel until they reach a point where their net charge is zero (the pI of the protein) and migration stops (Sambrook and Russell, 2001), resulting in very sharp bands. The advantage, and disadvantage, of IF is that it is independent of protein size. It is possible after IF to also separate the proteins by size, in a second dimension (2D-gel electrophoresis). Although useful for

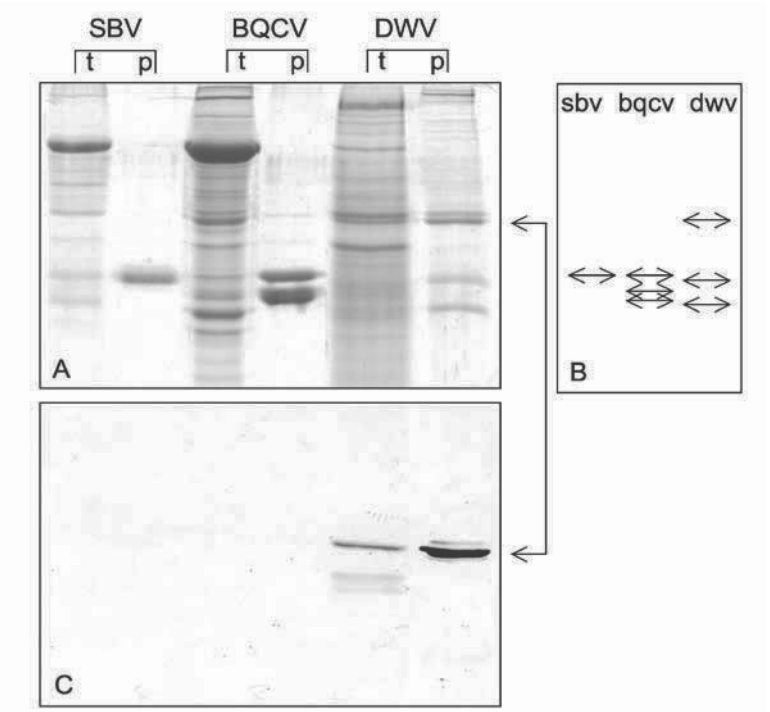


Figure 12. SDS-PAGE (a), schematic capsid protein profile (b) and Western blot with anti-DWV-VP1 serum (c) of crude (t) and purified (p) extracts of SBV, BQCV and DWV. Note that the antiserum only reacts against the VP1 protein of purified DWV, while in crude extracts it also reacts weakly with two smaller proteins. © J.R. de Miranda, Penn State.

precise identification and initial characterisation, protein profiling is too laborious and qualitative for routine diagnostics.

7.2. N-terminal and C-terminal sequencing

Another procedure more useful in characterisation and identification, rather than routine detection, is protein sequencing. N-terminal sequencing is more common and proceeds through a series of degradation steps of a protein (Edman degradation) starting at the N-terminal end (LeGendre *et al.,* 1993; Matsudaira, 1993) and the derivatized amino acid released at each step is identified by high performance liquid chromatography (HPLC). C-terminal sequencing works similarly, through one of several thiocyanate-based derivatization protocols (Bailey *et al.,* 1992; Tang *et al.,* 1999). 20-30 amino acids are usually sequenced, enough for identification and for the design of degenerate primers. Very small amounts (about 5 nmol) of protein are required these days for accurate sequencing, which can be purified from a single gel (Govan *et al.,* 2000; Leat *et al.,* 2000; Ongus *et al.,* 2004; Lanzi *et al.,* 2006). Many commercial laboratories exist that provide the service at reasonable cost. Its use is limited to identifying a virus, rather than routine and quantitative detection.

7.3. Mass spectrometry

In mass spectrometry, a molecule is fragmented using an electron beam. The process of fragmentation follows simple and predictable chemical pathways and the resulting (radical) cations reflect the most stable ones the molecule can form. These fragments are accelerated in a vacuum through a magnetic field and are sorted on the basis of mass-to-charge ratio. The analysis of mass spectroscopy data results in the re-assembly of fragments, working backwards to generate the original molecule (Suizdak, 1998). The technique is immensely powerful in the chemical analysis of complex biomolecules, and even interactions between molecules (Suizdak *et al.,* 1996). In virus diagnostics its use is limited to virus identification, through analysis of its capsid proteins, often in conjunction with prior protease digestion and fragment isolation (Thomas *et al.,* 2000).

7.4. Polyclonal antibodies

Polyclonal antibodies are produced when a suitable host is subjected to a series of injections with a purified antigen. The antigen is generally emulsified in an adjuvant, a mixture of compounds that boost the general immune response. The antibody titres are tested throughout the injection series with test bleeds and once a suitable titre is reached larger volumes are drawn. After coagulation and centrifugation the antiserum is recov-

ered, from which specific classes of immunoglobulins can be purified and concentrated with affinity chromatography, usually involving protein-A or protein-G (Harlow and Lane, 1988). The most common host is rabbit, which provides enough serum for most antibody uses. Occasionally goats, dogs, chickens, horses or humans are used. Polyclonal antibodies as diagnostic probes have several unique advantages, and disadvantages. The advantages are that they are cheap, sensitive, and robust to strain variation and can be quickly produced without requiring sequence information, as is the case with nucleic acid probes. They have many different uses and are indispensable during the early stages of pathogen discovery and characterisation (Anderson, 1984). Their main disadvantage is that they are essentially a soup of many different individual antibodies, which include those that are a natural part of the immune system of the animal used to raise the antiserum. This means that the specificity of polyclonal antibodies in diagnosis is largely dependent on the purity of the antigen. In the honey bee virus world, where multiple and correlated pathogen infections are common (Anderson and Gibbs, 1988; Bailey and Ball, 1991; Ball and Bailey, 1997; Nordström et al., 1999; Varis et al., 1992; Chen et al., 2004c; 2005b; Tentcheva et al., 2004b; Berenyi et al., 2006; Todd et al., 2007) and many viruses have a similar shape and purification characteristics, this can result in antisera with antibodies against a range of antigens, with no way of knowing the proportion or distribution of such 'contaminant' antibodies. Post-production analysis of an antiserum can determine exclude or quantify known pathogens as a source of contamination, but contamination from uncharacterised sources (antibody production host, unknown bee pathogens) cannot be accounted for. A popular solution is to incubate the antiserum with a series of 'healthy' bee or antiserum-host extracts to precipitate out undesired antibodies (Harlow and Lane, 1988). For many quantitative applications a small proportion of contaminant antibodies presents no problem: any effect these have will be lost in the general background noise of the assay. For other applications however, especially in qualitative situations where individual antibody-antigen interactions are visualised (such as electron and in situ microscopy) there is no way of knowing whether the visualised events are due to the contaminant or target antibodies, or both. For such experiments, monoclonal antibodies are preferable. The other disadvantage of both poly- and monoclonal antibodies is that it is difficult in quantitative assays to account for signal reduction due to qualitative differences between antibody and antigen, i.e. pathogen strain differences affecting the affinity of the antibody-antigen interaction (Allen and Ball, 1995). Polyclonal antibodies, with its range of alternative antibody options, may be less sensitive to such quantitative ef-

fects of qualitative differences than monoclonal antibodies. For polyclonal antibodies this antigenic variation can be assessed quickly with agarose gel immuno diffusion (AGID) tests (Fig. 13; sections 7.6, 10.2). For both poly- and monoclonal antibodies this can also be done with a far more laborious end-point dilution analysis (Harlow and Lane1988; Allen and Ball, 1995).

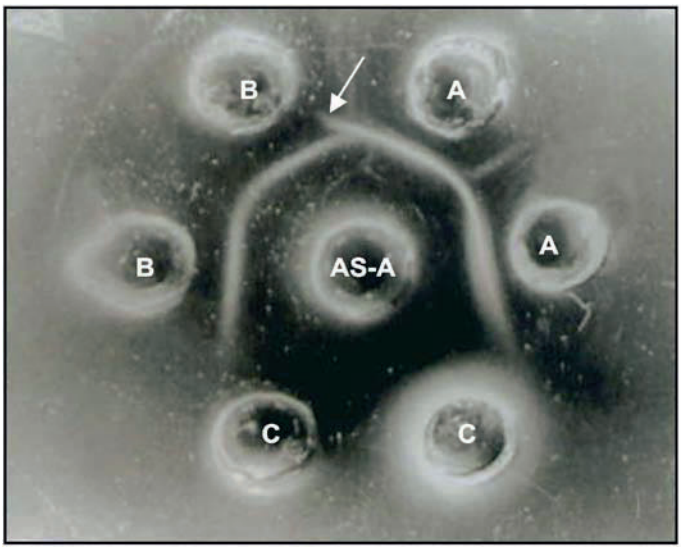


Figure 13. Agarose gel immuno diffusion analysis of two serologically related viruses. Both virus A and virus B react with antiserum against virus A, and the antigenic differences between the viruses is recorded through a precipitation 'spur' between wells A and B. Wells C contain a negative control.
© J. Madriz, University of Costa Rica.

7.5. Monoclonal antibodies and phage display

Monoclonal antibodies (MAbs) are the result of the fusion of primary antibody producing cells with HeLa cancer cells, resulting in a population of immortal cell lines, each producing a single, unique antibody (Harlow and Lane, 1988). These cell lines can be individually isolated and screened for affinity to the target. Although considerably more expensive, laborious and time consuming than polyclonal antibody production, MAbs have several important advantages. The end product is a battery of target-specific antibodies, circumventing the contaminating antibody problems of polyclonal antisera. This makes MAbs ideal for immunomicroscopy work. The epitope for each MAb can furthermore be analysed in detail, including sensitivity to strain variation in the epitope. Finally, each MAb cell line is uniform and immortal and can be produced in unlimited quantities, which is indispensable for diagnostic consistency in commercial situations. The initial immunization with antigen is in mice, whose spleen is recovered and homogenized for fusion with the HeLa cells (Harlow and Lane, 1988). Fused cell lines are isolated on solid media, screened for target-specific antibody production in microtitre plates (the most laborious

part) and mass produced in liquid media.

An alternative method for selecting genetically homogeneous antibodies is called 'phage display'. Here, instead of fusing the antibody producing cells with HeLa cells to create immortal cell lines, the genes for the antibodies are isolated (amplified) from the primary cells. These are then inserted into a bacteriophage genome in such a way that the antibodies will be 'displayed' on the surface of the bacteriophages, and this library of phages is then screened with the original antigen to identify the best antibodies (Padao and Crowther, 2006). This also allowed antibodies to be genetically manipulated, to enhance different properties or tailor them for specific uses, most commonly by grafting specific mouse-derived binding regions onto otherwise human antibodies, for medical antibody therapy (Lazer *et al.,* 2006a; 2006b; Padao and Crowther, 2006). The latest trend in antibody engineering is to produce the antibodies in yeast cells (Gasser and Mattanovich, 2006), which are easier to cultivate and can express genes at much higher levels than human cell lines, while retaining many of the post-translational modification functions particular to eukaryotic systems, that are lacking in bacteriophage-based expression systems.

7.6. Agarose gel immuno diffusion (AGID)

When an antibody encounters its homologous antigen, the molecules coagulate to form an insoluble precipitate. This property is used in agarose gel immuno-diffusion (AGID) for virus identification and strain differentiation (Anderson, 1984; Madriz *et al.,* 2000; Ribiere, 2000; Todd *et al.,* 2007). The antibody is placed in a central well made in a semi-solid agarose matrix, surrounded by peripheral wells containing various sample extracts. Both the antibody and antigens diffuse from their respective wells and where they meet a white precipitation line is formed (Harlow and Lane, 1988). Individual antibodies against epitopes not present in a particular test sample will diffuse through this precipitin line and react with the next sample, if this sample does have these epitopes, forming a 'spur' (Fig. 13). These spurs are therefore indicative of epitope differences between samples, and is the simplest way antibodies can be used to detect antigenic variation (Madriz *et al.,* 2000; section 10.2). AGID is very simple, fast, robust and relatively cheap, but is uneconomical with the antiserum and yields only qualitative data (Anderson, 1984).

7.7. Immunomicroscopy

Serology can be combined with microscopy to distinguish between similar shaped virus particles in tissue sections, and thus record the (co)distribution of each within the organism (Fievet *et al.,* 2006). The

availability of many different (fluorescent) tags and confocal microscopes makes such combinatorial studies possible, and similar effects can be obtained using different sized linked gold particles for intracellular electron microscopy studies. Such studies can be sensitive, accurate, and relatively cheap and fast, especially if the organ specificities of the viruses are known, but are subjective and susceptible to protocol derived variability (Habib-Bein *et al.,* 2003).

7.8. Squash blot and tissue prints

A much faster, cruder but more accessible alternative to immunomicroscopy is the tissue print or squashblot, where a cut section of an organism is dabbed (tissue print), or the entire organism/body part is smeared (squashblot) onto a solid support (slides or membranes) before being interrogated by specific probes. Direct immunofluorescence antibody staining (DFA) is a commercial name for tissue printing, done on slides and stained with any number of fluorophore-linked (monoclonal) antibodies for a range of diseases (Landry and Ferguson, 2000). Analysis is by visual estimation and therefore largely subjective (Habib-Bein *et al.,* 2003). DFA is also used in connection with protein dotblots (section 7.11). The usefulness of these procedures is that the virus distribution in specific tissues, whole organs or organisms can be assessed. Although none of these procedures are truly quantitative, their one great advantage is that the virus is accessed directly and with the absolute minimum of sample processing, saving time and expense.

7.9. Enzyme linked immuno sorbent assay (ELISA)

Enzyme linked immuno sorbent assay (ELISA) is possibly the most common diagnostic method currently in use. The basic protocol consists of adsorbing the target directly or indirectly onto the wells of a microtitre plate for subsequent detection by target-specific, enzyme-linked antibodies and soluble enzyme substrates. The absorbtion of light by the coloured reaction products, or the light emmitted by luminescent substrates, is related to the amount of target through standard curves (Allen *et al.,* 1986). There are two main formats: direct ELISA, where the sample is adsorbed directly onto the well walls (Fig. 14a, 14d) and double antibody sandwich (DAS) ELISA, where a target capture agent, usually the F^{ab} fragments of an antibody, is adsorbed onto the wells which then traps the target prior to detection (Fig. 14b, 14c). DAS-ELISA is about twice as sensitive as direct ELISA but requires an extra incubation (plus washes) and the preparation of F^{ab} fragments or directly labelled detecting antibodies. The quantitation of direct ELISA also has better linearity than that of DAS-ELISA

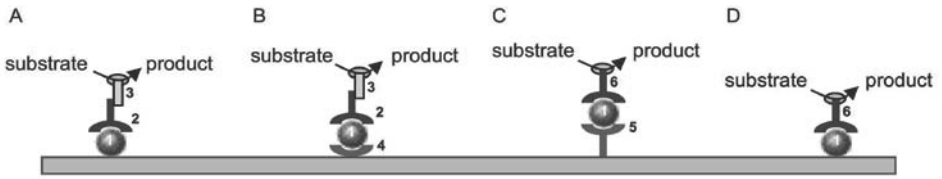


Figure 14. Different version of the enzyme immuno assay (EIA). The sandwich approach (b,c) of separate capture and detection stages is more sensitive than the direct detection (a,d), although the latter has better linearity between target and signal. The signalling enzyme can be linked directly to the detecting antibody (c,d) or to a secondary generic agent (a,b), usually either another antibody, protein-A or protein-G. EIAs are used in ELISA and immunochromatographic lateral flow devices, the two most common diagnostic methods currently in use, as well as in membrane EIA.

(Harlow and Lane, 1988). The main advantages of ELISA are low cost, acceptable quantitation, simple automation and high throughput. The main disadvantages are a propensity for serological artefacts, the disadvantages inherent in using antibodies and enzymatic reporters (sections 6.1.3, 7.4, 7.5), the need to wash away unbound probe (increasing variability and the chance of false positives) and the difficulty to multiplex, meaning that only single targets are detected and that all normalisation and quantitation is through external standards only (section 9.4). Until the late 1990s, ELISA was the principal means for diagnosing honey bee viruses (Anderson, 1984; 1985; Ball, 1985; Allen *et al.,* 1986; Ball and Allen, 1988; Anderson and Gibbs, 1988; 1989; Kulincevic *et al.,* 1990; Ball and Bailey, 1991; Varis *et al.,* 1992; Allen and Ball, 1995; Topolska *et al.,* 1995; Stoltz *et al.,* 1995; Bowen-Walker *et al.,* 1999; Nordström *et al.,* 1999). It is rapidly being replaced by RT-PCR for bulk screening of samples, which is more sensitive and accurate than ELISA (Stevens *et al.,* 1997; Mekuria *et al.,* 2003; Schneider *et al.,* 2004).

7.10. Immunochromatography and lateral flow devices

Immunochromatography is the principle behind lateral flow devices, well known from home pregnancy, drug, cholesterol and glucose detection kits and also much used in medical and veterinary diagnosis of metabolites and pathogens (Sharma *et al.,* 2005; Fernandez-Sanchez *et al.,* 2005; Garber *et al.,* 2005; Delmulle *et al.,* 2005; Arens *et al.,* 2005; Henderson and Eayrs, 2004; van Dam *et al.,* 2004; Cazacu *et al.,* 2004b). There are several variants, one of which is shown in Figure 15. An extract containing the target diffuses from the sample well across the chromatographic matrix, where it mobilises a labelled antibody, to a detection window containing a line of capturing antibody and a line of control antigen. The label

is usually colloidal gold, which shows as a red-brown line, but can also be enzymatic or fluorogenic, depending on the sensitivity requirements. These devices are popular for a good reason, since they unite nearly all the ideal characteristics of a diagnostic system. They can be calibrated to detect at specified sensitivities by manipulating the antibodies, the well size and chromatographic matrix control the amount of sample tested, they are portable, robust, accurate, simple, fast and relatively cheap, especially considering that they can be adapted for multiplexing, *i.e.* the simultaneous detection of multiple pathogens (Sharma *et al.,* 2005; Cazacu *et al.,* 2004b). Although the more advanced devices use disposable chromatographic strips and a quantitative electronic read-out (Fernandez-Sanchez *et al.,* 2004), most test kits are essentially qualitative. A lateral flow device for American foulbrood, a bacterial honey bee disease, is marketed by Vita Europe Ltd..

7.11. Western blots and dot blots

Western blot is a powerful adjunct to gel electrophoresis, where the separated proteins are transferred from the gel to a nitrocellulose or nylon-backed designer membrane, usually with the aid of an electric field (Harlow and Lane, 1988; Sambrook and Russell, 2001). The transferred

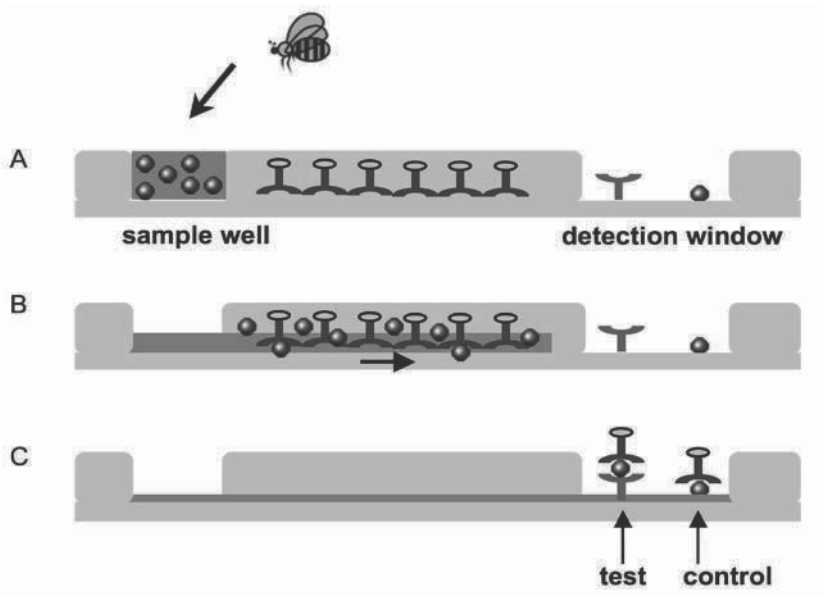


Figure 15. Principles of immunochromatography as used in lateral flow devices. Often internal blisters with washing solution are included to increase specificity and sensitivity by washing away excess reagents from the detecting window.

proteins are then interrogated with specific antibodies which in turn are detected with labelled secondary antibodies (Anderson, 1984; Allen and Ball, 1995; de Miranda *et al.,* 1996a; Ribiere *et al.,* 2000; Shen *et al.,* 2005a; Lanzi *et al.,* 2006). The advantage of the technology is that virus protein profiles can be investigated in crude samples (Fig. 12c). Multiplexing is limited by the number of different profiles that can be distinguished simultaneously and by the origins of the primary antibodies (indirect labelling) or the variety of compatible tags (direct labelling). A variation of the Western blot is the dot blot, also called membrane enzyme immunoassay (EIA) or direct fluorescence antigen detection (DFA) depending on the tag used. As the name implies, spots of samples are dotted in an array of the membrane or in a microtitre plate and interrogated directly with enzyme-linked or fluorophore-linked antibodies. This procedure is obviously much faster than electrophoresis and Western blot, but more limited since the information gained by separating the proteins is lost and extra care has to be taken to eliminate or account for background problems related to the tag used. Neither procedure is truly quantitative, although commercial membrane EIA kits are available for certain viruses (Chan *et al.,* 2002; Cattoli *et al.,* 2003; Cazacu *et al.,* 2004a; Butchko and Jordan, 2004; Reina *et al.,* 2004; Chiles *et al.,* 2004; Daniel *et al.,* 2005).

7.12. Protein microarrays

A new development in protein-based detection is the protein microarray, which is essentially an inverted dotblot: instead of sample dots, protein capture agents specific for a range of different proteins, generally (monoclonal) antibodies, are spotted at high densities in a fixed array on a solid support (Sage, 2004). The captured proteins are then detected with secondary antibodies coupled to fluorophores, much like for DAS-ELISA and lateral flow devices. It may even be possible to couple capture with detection directly, through FRET tags capable of detecting conformational changes attendant to the interaction between capture agent and target, or even label-free detection (Thompson *et al.,* 2005). Microarray technology is well developed for nucleic acid based detection (section 8.8). However, much of the protein microarray technology is still in the developmental stage since the unique nature of each protein makes it difficult to design a universal application, marking and detection strategy (Sage, 2004). The strength of the microarray technology lies in the enormous multiplexing capacity, making it possible to study the simultaneous changes in hundreds of different proteins. Another interesting use is in the study of interactions between different proteins. It is in these fields, rather than routine diagnostics, that their usefulness will be best realized.

8. Nucleic acids

At the root of nearly all nucleic acid based detection methods are two supreme qualities of nucleic acids; their chemical stability in aqueous solutions and the specificity and strength of the pairing of its nucleoside bases. This pairing, or hybridisation, is through hydrogen bonding and is dependent on the concentration of salts and temperature. Since temperature can be easily manipulated, it is used to control the specificity of the hybridisation and ultimately the accuracy of the diagnosis, with extreme sensitivity (Singh-Zocchi *et al.,* 2003). This has led to a number of techniques designed to detect such specific hybridisation events (Rudi *et al.,* 2002) and, increasingly, to do so in a high throughput automated setting, including completely autonomous, continuous monitoring systems (Hindson *et al.,* 2005; Service, 2006).

8.1. Hybridisation in solution

Solution hybridisation is a dynamic event infuenced by a range of factors, including absolute and relative target concentration and sample sequence complexity, as well as the usual physico-chemical parameters (temperature, salt, pH, electronic charge *etc.*), which can be manipulated to optimize the specificity and sensitivity of hybridisation (Sambrook and Russell, 2001; Heaton *et al.,* 2001; Estrela *et al.,* 2005). Target detection and quantitation requires self-reporting probes, such that only target-probe complexes are detected, or some means of separating hybridised from unhybridised probe prior to detection (Al-Mahrouki and Krylov, 2005). FRET, ECL, self-reporting chemiluminescence (Arnold *et al.,* 1989) quencher-linked antisense primers (Fiandaca *et al.,* 2001; Nolan *et al.,* 2003) are all suitable labels for self-reporting probes. Wolcott (1992) describes several hybridisation-based detection strategies. Multiplexing is limited, and sensitivity is moderate but quantitation can be very accurate since detection is direct.

8.2. Fragment separation

One useful way to drastically increase the information obtained is to separate your target nucleic acid, amplification products or hybridisation complexes by size. This makes it easy to multiplex, provides a means to disregard erroneous events (quality control; Fig. 16), analyze complex populations and polymorphisms (Fig. 17), and use both internal and external standards for quantitation (Fig. 18). Electrophoresis moves the charged nucleic acids through a gel matrix. The nucleic acid fragments can be visualised in the gel by staining, transferred to solid support for probing or

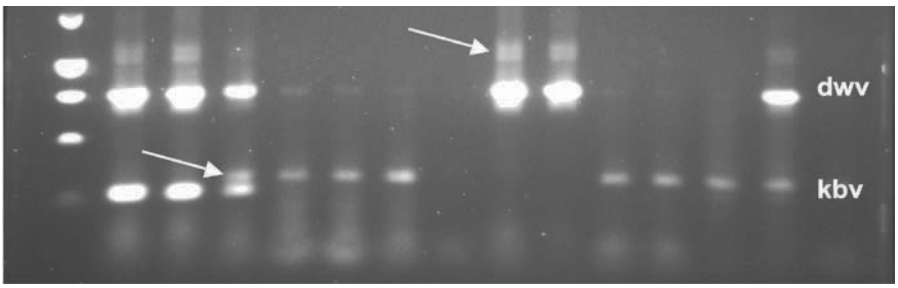
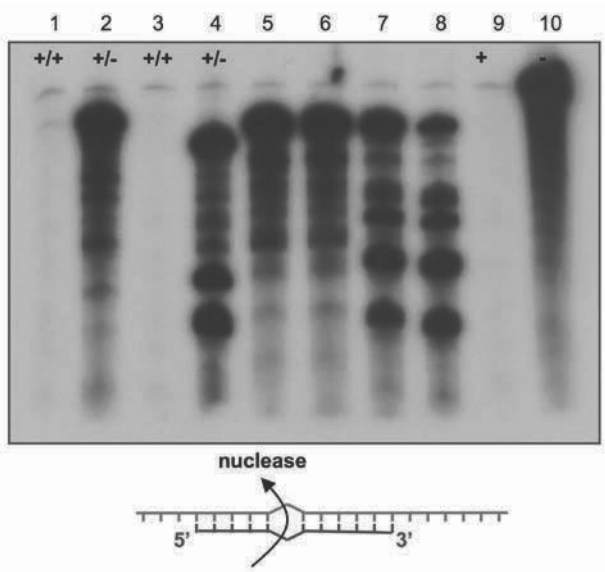


Figure 16. Appearance of qualitative RT-PCR errors (white arrows) in DWV-KBV multiplex RT-PCR of a series of fractions from an immunoaffinity purification of DWV. A molecular weight marker runs in lane 1.
© J.R. de Miranda, Penn State.



© J.R. de Miranda, S.R. Noble Foundation, Ardmore.

Figure 17. Example of an RNAse protection assay. Lanes 1 and 2 concern control reactions of the positive stranded probe with synthetic positive strand and negative strand of polymorph-1; lanes 3 and 4 control reactions with synthetic positive and negative strand of polymorph-2; lanes 5-8 are experimental samples; lanes 9 and 10 are positive and negative RNAse controls. A diagram of the nuclease digestion assay principle is also shown. The differences between the lanes are due to qualitative and quantitative differences in polymorphism.

their movement can be detected and quantified in real time while they migrate across a laser scanning window (capillary electrophoresis). Other separation techniques include various forms of chromatography (size, affinity, laminar, gas, liquid *etc*.) but these are rarely used in routine nucleic acid diagnosis. Another separation technique is the line probe assay (LiPA), where labelled nucleic acids (RT-PCR products) are hybridised to target-specific oligonucleotides fixed to a nylon membrane strip (Wilson *et al.,* 2000; Servais *et al.,* 2001; Eiros *et al.,* 2002; Labayru *et al.,* 2005). Something similar can also be achieved by stacking agarose bead-linked molecular probes in a capillary column in defined layers (Fig. 19b; Kohara *et al.,* 2001).

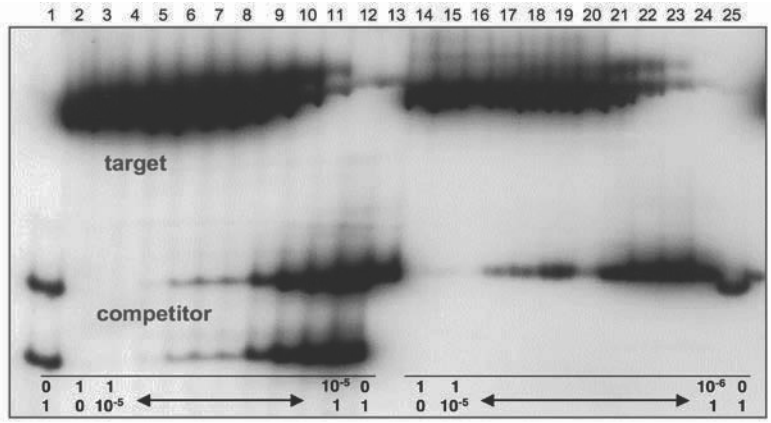


Figure 18. Autoradiograph of a series of control reactions for quantitative competitive RT-PCR/RFLP using two radiolabeling strategies. Lanes 2-12 concern radiolabeling by incorporation of dCTP32, lanes 14-25 concern P32 end-labeling of one of the PCR primers. Lanes 1 and 13 are qualitative controls for the PCR and RFLP reactions, using a DNA template for the PCR. In both experiments a logarithmic replacement series was set up at RNA level between the target and the competitor RNAs, where the competitor was distinguished from the target by a BamHI restriction site. Lanes 8 and 20 concern 1:1 ratios of target and competitor. Notice the heavier signal from incorporated label than from end-labeling, the relationship between band size and signal intensity, and the saturation of signal at the extremes of the replacement series. © J.R. de Miranda, S.R. Noble Foundation, Ardmore.

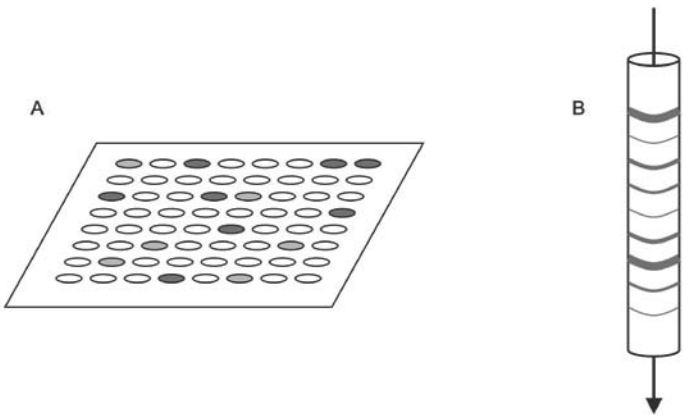


Figure 19. Principles of the microarray (a) and the capillary bead-array (b). In both cased probe molecules are arranged in a predetermined array for the capture of (direct or indirect) labelled target.

8.3. Southern and Northern blot

These are the DNA (Southern) and RNA (Northern) versions of a Western blot. Samples of DNA or RNA are fractionated through a gel matrix, usually agarose, transferred to a membrane and probed with a labelled nucleic acid probe. Detailed protocols can be found in Sambrook and Russell (2001). As with Western blot, this technique is semi-quantitative and useful for simultaneously checking the integrity of the RNA sample, size analysis, eliminating possible interfering compounds from the hybridisation reaction (through the gel separation and membrane transfer steps) and identifying possible background problems (Shen *et al.,* 2005b). The disadvantages are that the rate of transfer of RNA to the membrane varies for different sized fragments and may also be uneven across the gel and between gels, making quantitation, especially between gels, highly dependent on internal and external standards. It is also labour intensive and difficult to automate. Dot blots are simplified versions of Northern blots and very useful for parallel comparisons of many samples (Shen *et al.,* 2005b).

8.4. In situ hybridisation, squashblots and tissue prints

In situ hybridisation or *in situ* PCR is a technique specific to microscopic visualization of nucleic acids, much like immunohistochemistry and immuno(electron)microscopy for proteins (section 7.7). The development of light-based tags and confocal microscopy has simplified *in situ* technology enormously compared to the radioactive tags and photographic emulsions used earlier, such that it is now possible to detect hybridisation events in real-time, in living cells (Sokol *et al.,* 1998). The technique is very useful for detailed investigation of the nature of the pathogen (Fievet *et al.,* 2006) but unless the virus is located only in a very specific area, not generally useful for routine diagnosis. A much faster, cruder but more accessible alternative for routine diagnostics is the tissue print or squashblot (see section 7.8).

8.5. PCR and other target amplification methods

Without a doubt the most popular, universal and powerful method for nucleic acid detection and quantitation is with the polymerase chain reaction (PCR). There are several alternative target amplification methods (Lanciotti, 2003), each with their advantages and disadvantages, and relevance to virus detection. The main trick of PCR and related methods is the amplification of select target sequences through the use of a pair of primers, each complementary to one strand of the DNA, such that a

DNA polymerase can artificially replicate the target sequence between the primers. Repeated cycles of this process results in selective amplification of the target sequence (Fig. 20). The advantages of this technology are that very rare sequences can be accessed by current detection technologies, that less sensitive detection methods can be used (increasing detection options) and perhaps most importantly that PCR can be used as a purification process, relegating non-target sequences to a very low background through amplification of the target sequences. This has made it especially popular for isolating and cloning specific sequences. The use of thermostable polymerases has made automation of PCR possible and allows the primers to hybridise and be extended close to their melting temperature (Tm) thereby maximizing the specificity of the amplification. The enzymes used are generally very robust to varying conditions and the presence of other biomolecules in the sample, allowing even very crude samples to be amplified reliably. These considerable advantages, coupled with its universality (each new target only needs new primers), simplicity and popularity (ever reducing costs) have made PCR the method of choice for routine diagnostics, to the extent that portable thermocyclers for field use have been developed (Higgins *et al.,* 2003). In the context of quantita-

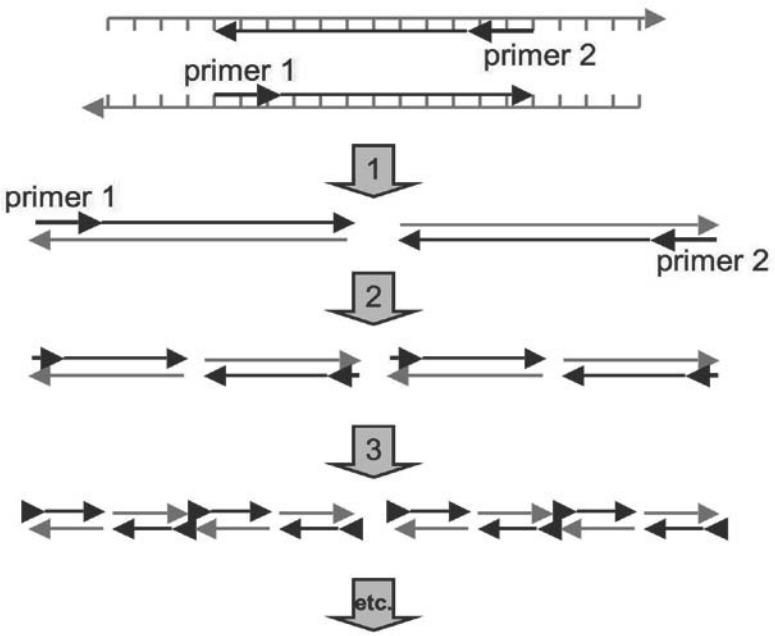


Figure 20. Illustration of the principles of the Polymerase Chain Reaction (PCR). The target sequence is selectively amplified through repeated cycles of denaturation, primer annealing and polymerisation between the primers.

Table I (part 1 of 9). RT-PCR protocols published for the detection of various honey bee viruses. The protocols are ordered by virus first, then by the genome location of the primers. The usual code for nucleotides is used: A=Adenine; C=Cytosine; G=Guanine; T=Thymidine; I=Inosine; U=Uracyl; R=A/G; Y=C/T; K=G/T; M=A/C; S=C/G; W=A/T; H=A/C/T; B=C/G/T; V=A/C/G; D=A/G/T; N=A/C/G/T. The specifics of the TaqMan probes are found in the original publications. Nucleotides in lower case letters are extensions not derived from virus sequence. All protocols use target-specific cDNA primers except those marked with * which use random-hexamer primed cDNA. The extension step of the RT-PCR protocols marked with ** increases by 5 seconds every cycle, starting cycle ten (Topley et al., 2005). #=codon position of the 3' nucleotide of the primer; denat.=denaturation; ann.=annealing; ext.=extension.

VIRUS		3'	#	PRIMER	SIZE	LOCATION	RT	denat.	cycles	denat.	ann.	ext.	REAL-TIME	REFERENCE
DWV	F	1183	2	CTTACTCTGCCGTCGCCCA	194	L-protein	48°C	95°C	40	95°C	55°C	68°C		Chen *et al.*, 2004b, 2004c
	R	1338	1	CCGTTAGGAACTCATTATCGAG			45min	2min		30sec	60sec	120sec		Chen *et al.*, 2005b; 2006
DWV	F	2360	2	ATTGTGCAAGATTGGACTAC	434	VP2/VP4/VP1	50°C	95°C	40	94°C	55°C	72°C		Berenyi *et al.*, 2006
	R	2755	2	AGATGCAATGGAGGATACAG			30min	15min		30sec	50sec	60sec		
DWV	F	2452	2	CAACTACCTGTAATGTCGTCGTGTT	206	VP4	42°C	95°C	40	95°C	59°C	72°C	SYBR-Green	Yang and Cox-Foster 2005
	R	2611	2	GACAAAATGACGAGGAGATTGTT			60min	10min		30sec	60sec	60sec		
DWV	F	2730	1	ACGACACAACATCCTGTAG	621	VP1	42°C	94°C	35	94°C	50°C	72°C		Shen *et al.*, 2005b
	R	3313	2	TAAACTAGGTTGGACTGGAA			60min	5min		20sec	20sec	60sec		
DWV	F	3739	2	CCTGCTAATCAACAAGGACCTGG	355	VP3	50°C	95°C	35	94°C	54.3°C	72°C		Genersch 2005
	R	4047	1	CAGAACCAATGTCTAACGCTAACCC			30min	15min		30sec	60sec	30sec		
DWV	F	3739	2	CCTGCTAATCAACAAGGACCTGG	355	VP3	50°C	95°C	35	94°C	52.0°C	72°C		Yue and Genersch 2006
	R	4047	1	CAGAACCAATGTCTAACGCTAACCC			30min	15min		30sec	30sec	30sec		
DWV	F	4244	3	ATCGTAGACTGGAAGGATGGTCC	568	pre-Helic.	50°C	95°C	35	94°C	54.3°C	72°C		Genersch 2005
	R	4766	3	GAGAAGACATTTGCTTGAACCTCC			30min	15min		30sec	60sec	30sec		
DWV	F	4800	1	GCAAATGTCTTCTCACTGGTGTCTC	516	Helicase	50°C	95°C	35	94°C	54.3°C	72°C		Genersch 2005
	R	5270	3	TGCTTTCAAAATCTCAGGCTCG			30min	15min		30sec	60sec	30sec		
DWV	F	5786	3	TTTCCAGGTCCATTCCCCTATC	393	Helicase	50°C	95°C	35	94°C	54.3°C	72°C		Genersch 2005
	R	6136	2	TCATTCGCCTTACGACGGTTAG			30min	15min		30sec	60sec	30sec		

Table I (part 2 of 9).

VIRUS		3'	#	PRIMER	SIZE	LOCATION	RT	denat.	cycles	denat.	ann.	ext.	REAL-TIME	REFERENCE
DWV	F	6269	3	ATCAGCGCTTAGTGGAGGAA	702	pre-VpG	48°C	95°C	40	95°C	55°C	68°C	TaqMan®	Chen *et al.*, 2005a
	R	6941	3	TCGACAATTTTCGGACATCA			45min	2min		30sec	60sec	120sec		
DWV	F	6785	3	TCATCTTCAACTCGGCTTTCTACG	479	VpG	50°C	95°C	35	94°C	54.3°C	72°C		Genersch 2005
	R	7221	1	CGAATCATTTTCACGGGACG			30min	15min		30sec	60sec	30sec		
DWV	F	6699	1	GTAAGCGTCGTGAACATACTG	1129	VpG 3C-protease	55°C	94°C	30	94°C	55°C	72°C		Ongus *et al.*, 2004
	R	7790	3	GACTCCTCTCCCGCGAGA			60min	5min		30sec	30sec	120sec		
DWV	F	7472	3	ggatCCTAGAATCCATAGATTG	495	3C-protease	42°C	94°C	30	94°C	59°C	72°C		Todd *et al.*, 2007
	R	7926	1	aagctTATGGTCGCCAGTTAC			30min	2min		30sec	30sec	30sec		
DWV	F	7495	2	TGCCACTTACTACTAAGCCTCAGGG	596	3C-protease	50°C	95°C	35	94°C	54.3°C	72°C		Genersch 2005
	R	8047	2	CGAACCACAAACACCATCGC			30min	15min		30sec	60sec	30sec		
DWV	F	8581	2	TTTGCAAGATGCTGTATGTGG	395	RdRp	50°C*	94°C	35	94°C	55°C	72°C		Tentcheva *et al.*, 2004b
	R	8936	3	GTCGTGCAGCTCGATAGGAT			60min	2min		30sec	30sec	60sec		
DWV	F	8581	2	TTTGCAAGATGCTGTATGTGG	395	RdRp	50°C*	94°C	35	94°C	55°C	72°C		Tentcheva *et al.*, 2004a
	R	8936	3	GTCGTGCAGCTCGATAGGAT			60min	2min		30sec	30sec	60sec		
DWV	F	8581	2	TTTGCAAGATGCTGTATGTGG	395	RdRp	50°C	95°C	40	94°C	55°C	72°C		Antunez *et al.*, 2006
	R	8936	3	GTCGTGCAGCTCGATAGGAT			30min	15min		60sec	60sec	60sec		
DWV	F	8730	1	GGATGTTATCTCCTGCGTGGAA	69	RdRp	50°C*	95°C	40	95°C	-	60°C	SYBR-Green	Tentcheva *et al.*, 2006
	R	8751	1	CCTCATTAACTGTGTCGTTGATAATTG			2min	10min		15sec	-	60sec		
DWV	F	9273	1	TCCATCAGGTTCTCCAATAACGG	451	RdRp	50°C	95°C	35	94°C	54.3°C	72°C		Genersch 2005
	R	9678	1	CCACCCAAATGCTAACTCTAACGC			30min	15min		30sec	60sec	30sec		
DWV	F	9608	3	CCTGGACAAGGTCTCGGTAGAA	125	RdRp	48°C	95°C	40	95°C	-	60°C	TaqMan®	Chantawanakul *et al.*, 2006
	R	9692	3	ATTCAGGACCCCACCCAAAT			30min	10min		15sec	-	60sec		

Table I (part 3 of 9).

VIRUS		3'	#	PRIMER	SIZE	LOCATION	RT	denat.	cycles	denat.	ann.	ext.	REAL-TIME	REFERENCE
VaDV-1	F	6677	1	CGAAACGAAGAGAGCATGTAT	1129	VpG 3C-	55°C	94°C	30	94°C	55°C	72°C		Ongus *et al.*, 2004
	R	7768	3	CGACTCTTCCCCAGCTAAG			60min	5min		30sec	30sec	120sec		
ABPV	F	5290	3	TGAGAACACCTGTAATGTGG	452	RdRp	50°C*	94°C	35	94°C	55°C	72°C		Tentcheva *et al.*, 2004b
	R	5703	2	ACCAGAGGGTTGACTGTGTG			60min	2min		30sec	30sec	60sec		
ABPV	F	6261	2	TATCAGAAGGCCACTGGAGA	722	intergenic &VP1	50°C	95°C	40	95°C	55°C	72°C		Bakonyi *et al.*, 2002b
	R	6995	1	TCCACTCGGTCATCATAAGG			30min	5min		20sec	20sec	60sec		
ABPV	F	6867	2	TCTTGGACATTGCCTTCAGT	778	VP1/VP4	50°C	95°C	40	95°C	55°C	72°C		Bakonyi *et al.*, 2002b
	R	7607	1	ATACCATTCGCCACCTTGTT			30min	5min		20sec	20sec	60sec		
ABPV	F	7466	1	TGCAGTTCCAGAAGTTAAGA	686	VP1/VP4/ VP2	50°C	95°C	40	95°C	55°C	72°C		Bakonyi *et al.*, 2002b
	R	8114	1	ATAGTRGCTCGCCAATATGA			30min	5min		20sec	20sec	60sec		
ABPV	F	7947	2	GTGCTATCTTGGAATACTAC	619	VP2	50°C	95°C	40	95°C	55°C	72°C		Bakonyi *et al.*, 2002b
	R	8527	3	AAGGYTTAGGTTCTACTACT			30min	5min		20sec	20sec	60sec		
ABPV	F	7947	2	GTGCTATCTTGGAATACTAC	619	VP2	50°C	95°C	40	94°C	55°C	72°C		Berenyi *et al.*, 2006
	R	8527	3	AAGGYTTAGGTTCTACTACT			30min	15min		30sec	50sec	60sec		
ABPV	F	8134	3	CATATTGGCGAGCCACTATG	398	VP2	42°C	94°C	40	94°C	55°C	72°C		Bakonyi *et al.*, 2002a
	R	8493	2	CCACTCCACACAACTATCG			60min	3min		60sec	60sec	60sec		
ABPV	F	8134	3	CATATTGGCGAGCCACTATG	398	VP2	50°C	95°C	35	94°C	49.5°C	72°C		Yue and Genersch 2006
	R	8493	2	CCACTCCACACAACTATCG			30min	15min		30sec	30sec	30sec		
ABPV	F	8134	3	CATATTGGCGAGCCACTATG	398	VP2	50°C	95°C	40	95°C	55°C	72°C		Siede and Büchler 2006
	R	8493	2	CCACTCCACACAACTATCG			30min	5min		20sec	20sec	60sec		
ABPV	F	8137	3	ATTGGCGAGCYACTATGTGC	858	VP3	50°C	95°C	40	95°C	55°C	72°C		Bakonyi *et al.*, 2002b
	R	8957	1	CGCGGTAYTAAGAAGCTACG			30min	5min		20sec	20sec	60sec		

Table I (part 4 of 9).

VIRUS		3'	#	PRIMER	SIZE	LOCATION	RT	denat.	cycles	denat.	ann.	ext.	REAL-TIME	REFERENCE
ABPV	F	8232	2	TCCTATATCGACGACGAAAGACAA	65	VP2	48°C	95°C	40	95°C	-	60°C	TaqMan®	Chantawanakul *et al.*, 2006
	R	8251	3	GCGCTTTAATTCCATCCAATTGA			30min	10min		15sec	-	60sec		
ABPV	F	8484	2	TTATGTGTCCAGAGACTGTATCCAI	900	VP3	50°C	94°C	35	94°C	60°C	72°C		Benjeddou *et al.*, 2001
	R	9336	-	GCTCCTATTGCTCGGTTTTTCGGTI			30min	2min		30sec	30sec	30sec		
ABPV	F	8484	2	TTATGTGTCCAGAGACTGTATCCAI	900	VP3	58°C	94°C	35	94°C	63°C	68°C**		Topley *et al.*, 2005
	R	9336	-	GCTCCTATTGCTCGGTTTTTCGGTI			30min	2min		30sec	30sec	60sec		
ABPV	F	8484	2	TTATGTGTCCAGAGACTGTATCCA	900	VP3	50°C	95°C	40	94°C	55°C	72°C		Antunez *et al.*, 2005; 2006
	R	9336	-	GCTCCTATTGCTCGGTTTTTCGGT			30min	15min		60sec	60sec	60sec		
ABPV	F	8713	3	GGAACATGGAAGCATTATTG	687	VP3	50°C	95°C	40	95°C	55°C	72°C		Bakonyi *et al.*, 2002b
	R	9362	-	AATGTCTTCTCGAACCATAG			30min	5min		20sec	20sec	60sec		
ABPV & KBV	F	7259	1	ggatcCAGTCTATATGTGGT	543	VP4	42°C	94°C	30	94°C	59°C	72°C		Todd *et al.*, 2007
	R	7763	1	aagctTCCAGGCACATTCTG			30min	2min		30sec	30sec	30sec		
ABPV & KBV	F	8140	3	GGCGAGCCACTATGTGCTAT	401	VP3	50°C	94°C	35	94°C	50°C	68°C**		Evans 2001
	R	8507	1	ATCTTCAGCCCACTT			30min	2min		30sec	30sec	45sec		
KBV	F	3029	3	ATGACGATGATGAGTTCAAG	290	pre-3C-protease	42°C	94°C	35	94°C	50°C	72°C		Shen *et al.*, 2005a
	R	3282	1	AATTGCAAGACGGCATC			60min	5min		20sec	20sec	60sec		
KBV	F	3029	3	ATGACGATGATGAGTTCAAG	290	pre-3C-protease	42°C	94°C	35	94°C	50°C	72°C		Shen *et al.*, 2005a
	R	3282	1	AATTGCAAGACGGCATC			60min	5min		20sec	20sec	60sec		
KBV	F	5425	2	GATGAACGTCGACCTATTGA	414	RdRp	37°C*	94°C	35	94°C	57°C	72°C		Stoltz *et al.*, 1995
	R	5800	2	TGTGGGTTGGCTATGAGTCA			60min	10min		60sec	60sec	60sec		
KBV	F	5425	2	GATGAACGTCGACCTATTGA	414	RdRp	50°C	94°C	35	94°C	57°C	68°C**		Hung *et al.*, 1996; 2000; Hung 2000
	R	5800	2	TGTGGGTTGGCTATGAGTCA			30min	2min		30sec	30sec	45sec		Hung & Shimanuki 1999; 2000

Table I (part 5 of 9).

VIRUS		3'	#	PRIMER	SIZE	LOCATION	RT	denat.	cycles	denat.	ann.	ext.	REAL-TIME	REFERENCE
KBV	F	5425	2	GATGAACGTCGACCTATTGA	414	RdRp	50°C	94°C	35	94°C	50°C	68°C**		Evans & Hung 2000; Evans 2001
	R	5800	2	TGTGGGTTGGCTATGAGTCA			30min	2min		30sec	30sec	45sec		
KBV	F	5425	2	GATGAACGTCGACCTATTGA	414	RdRp	50°C*	94°C	35	94°C	55°C	72°C		Tentcheva *et al.*, 2004b
	R	5800	2	TGTGGGTTGGCTATGAGTCA			60min	2min		30sec	30sec	60sec		
KBV	F	5425	2	GATGAACGTCGACCTATTGA	414	RdRp	50°C	95°C	35	94°C	61°C	72°C		Siede and Büchler 2004
	R	5800	2	TGTGGGTTGGCTATGAGTCA			30min	5min		60sec	60sec	60sec		
KBV	F	5425	2	GATGAACGTCGACCTATTGA	414	RdRp	48°C	95°C	40	95°C	55°C	68°C		Chen *et al.*, 2004a; 2004c Chen *et al.*, 2005b; 2006
	R	5800	2	TGTGGGTTGGCTATGAGTCA			45min	2min		30sec	60sec	120sec		
KBV	F	5425	2	GATGAACGTCGACCTATTGA	414	RdRp	50°C	95°C	35	94°C	50.5°C	72°C		Yue and Genersch 2006
	R	5800	2	TGTGGGTTGGCTATGAGTCA			30min	15min		30sec	30sec	30sec		
KBV	F	5425	2	GATGAACGTCGACCTATTGA	414	RdRp	50°C	95°C	40	94°C	55°C	72°C		Berenyi *et al.*, 2006
	R	5800	2	TGTGGGTTGGCTATGAGTCA			30min	15min		30sec	50sec	60sec		
KBV	F	5425	2	GATGAACGTCGACCTATTGA	414	RdRp	50°C	95°C	40	94°C	55°C	72°C		Antunez *et al.*, 2006
	R	5800	2	TGTGGGTTGGCTATGAGTCA			30min	15min		60sec	60sec	60sec		
KBV	F	5561	3	ATGAAGTGTCTATTGGAACG	550	RdRp	50°C	94°C	35	94°C	57°C	68°C**		Evans & Hung 2000
	R	6069	1	ttcgaaCCCTCGCCTCCAACTCC			30min	2min		30sec	30sec	45sec		
KBV	F	7568	3	gggatccGTTTCTATGCAAATCGCA	282	VP4	42°C	94°C	30	94°C	59°C	72°C		Todd *et al.*, 2007
	R	7806	1	aagctTCCAGGCACATTCTG			30min	2min		30sec	30sec	30sec		
KBV	F	8269	2	ACCAGGAAGTATTCCCATGGTAAG	69	VP2	48°C	95°C	40	95°C	-	60°C	TaqMan®	Chantawanakul *et al.*, 2006
	R	8304	3	TGGAGCTATGGTTCCGTTCAG			30min	10min		15sec	-	60sec		
CBPV	F	?	3	TCTGGCTCTGTCTTCGCAAA	68	RdRp	48°C	95°C	40	95°C	-	60°C	TaqMan®	Chantawanakul *et al.*, 2006
	R	?	1	GATACCGTCGTCACCCTCATG			30min	10min		15sec	-	60sec		
CBPV	F	?	-	AGTTGTCATGGTTAACAGGATACGAG	455	RdRp	42°C*	95°C	30	95°C	55°C	72°C		Ribiere *et al.*, 2002
	R	?	1	TCTAATCTTAGCACGAAAGCCGAG			60min	2min		60sec	30sec	60sec		

Table I (part 6 of 9).

VIRUS		3'	#	PRIMER	SIZE	LOCATION	RT	denat.	cycles	denat.	ann.	ext.	REAL-TIME	REFERENCE
CBPV	F	?	-	AGTTGTCATGGTTAACAGGATACGAG	455	RdRp	50°C*	94°C	35	94°C	55°C	72°C		Tentcheva *et al.*, 2004b
	R	?	1	TCTAATCTTAGCACGAAAGCCGAG			60min	2min		30sec	30sec	60sec		
CBPV	F	?	-	AGTTGTCATGGTTAACAGGATACGAG	455	RdRp	50°C	95°C	40	94°C	55°C	72°C		Antunez *et al.*, 2005; 2006
	R	?	1	TCTAATCTTAGCACGAAAGCCGAG			30min	15min		60sec	60sec	60sec		
CBPV	F	?	-	AGTTGTCATGGTTAACAGGATACGAG	455	RdRp	48°C	95°C	40	95°C	55°C	68°C		Chen *et al.*, 2005b; 2006
	R	?	1	TCTAATCTTAGCACGAAAGCCGAG			45min	2min		30sec	60sec	120sec		
CBPV	F	?	-	TGTCGAACTGAGGATCTTAC	315	RdRp	50°C	95°C	40	94°C	55°C	72°C		Berenyi *et al.*, 2006
	R	?	2	GACCTGATTAACGACGTTAG			30min	15min		30sec	50sec	60sec		
SBV	F	240	2	ACCAACCGATTCCTCAGTAG	469	L-protein	50°C	95°C	40	95°C	55°C	72°C		Grabensteiner *et al.*, 2001
	R	670	3	CCTTGGAACTCTGCTGTGTA			30min	5min		20sec	20sec	60sec		
SBV	F	240	2	ACCAACCGATTCCTCAGTAG	469	L-protein	50°C	95°C	40	94°C	55°C	72°C		Berenyi *et al.*, 2006
	R	670	3	CCTTGGAACTCTGCTGTGTA			30min	15min		30sec	50sec	60sec		
SBV	F	321	2	AAGTTGGAGGCGCGYATTTG	70	L-protein	48°C	95°C	40	95°C	-	60°C	TaqMan®	Chantawanakul *et al.*, 2006
	R	342	2	CAAATGTCTTCTTACDAGAAGYAAG-GATTG			30min	10min		15sec	-	60sec		
SBV	F	1932	2	CACTCAACTTACACAAAAAC	211	VP1	42°C	94°C	35	94°C	50°C	72°C		Shen *et al.*, 2005a
	R	2104	3	CATTAACTACTCTCACTTTC			60min	5min		20sec	20sec	60sec		
SBV	F	2370	2	GTGGCAGTGTCAGATAATCC	816	VP1	50°C	95°C	40	95°C	55°C	72°C		Grabensteiner *et al.*, 2001
	R	3147	2	GTCAGAGAATGCGTAGTTCC			30min	5min		20sec	20sec	60sec		
SBV	F	2370	2	GTGGCAGTGTCAGATAATCC	816	VP1	50°C	95°C	35	94°C	52.0°C	72°C		Yue and Genersch 2006
	R	3147	2	GTCAGAGAATGCGTAGTTCC			30min	15min		30sec	30sec	30sec		
SBV	F	3875	1	ACCGTTGTCTGGAGGTAGTT	249	Helicase	50°C	95°C	40	95°C	55°C	72°C		Grabensteiner *et al.*, 2001
	R	4085	1	GCCGCATTAGCTTCTGTAGT			30min	5min		20sec	20sec	60sec		

Table I (part 7 of 9).

VIRUS		3'	#	PRIMER	SIZE	LOCATION	RT	denat.	cycles	denat.	ann.	ext.	REAL-TIME	REFERENCE
SBV	F	4978	3	GCTGAGGTAGGATCTTTGCGT	824	pre-3C-protease	48°C	95°C	40	95°C	55°C	68°C		Chen *et al.*, 2004; 2005b; 2006
	R	5781	1	TCATCATCTTCACCATCCGA			45min	2min		30sec	60sec	120sec		
SBV	F	5726	1	ATATACGGTGCGAGAACTGC	879	pre-3C-protease	50°C	95°C	40	95°C	55°C	72°C		Grabensteiner *et al.*, 2001
	R	6566	1	CTCGGTAATAACGCCACTGT			30min	5min		20sec	20sec	60sec		
SBV	F	7766	1	GGATGAAAGGAAATTACCAG	426	RdRp	50°C*	94°C	35	94°C	55°C	72°C		Tentcheva *et al.*, 2004b
	R	8154	2	CCACTAGGTGATCCACACT			60min	2min		30sec	30sec	60sec		
SBV	F	7766	1	GGATGAAAGGAAATTACCAG	426	RdRp	50°C	95°C	40	94°C	55°C	72°C		Antunez *et al.*, 2006
	R	8154	2	CCACTAGGTGATCCACACT			30min	15min		60sec	60sec	60sec		
SBV	F	8057	1	AATGGTGCGGTGGACTATGG	579	RdRp	50°C	95°C	40	95°C	55°C	72°C		Grabensteiner *et al.*, 2001
	R	8597	1	TGATACAGAGCGGCTCGACA			30min	5min		20sec	20sec	60sec		
SBV	F	8192	1	GTGGCGCGCCCATTACTGTAGTGI	435	RdRp	58°C	94°C	35	94°C	63°C	68°C**		Topley *et al.*, 2005
	R	8580	1	CTCGACAATTCTCCCTAGTAGCCI			30min	2min		30sec	30sec	60sec		
BQCV	F	2578	2	AGTAGTTGCGATGTACTTCC	472	pre-3C-protease	50°C	95°C	40	94°C	55°C	72°C		Berenyi *et al.*, 2006
	R	3011	2	CTTAGTCTTACTCGCCACTT			30min	15min		30sec	50sec	60sec		
BQCV	F	4630	1	GGACGAAAGGAAGCCTAAAC	424	RdRp	50°C*	94°C	35	94°C	55°C	72°C		Tentcheva *et al.*, 2004b
	R	5015	2	ACTAGGAAGAGACTTGCACC			60min	2min		30sec	30sec	60sec		
BQCV	F	7873	3	TGGTCAGCTCCCACTACCTTAAAC	701	VP1	50°C	95°C	40	94°C	55°C	72°C		Antunez *et al.*, 2006
	R	8527	-	GCAACAAGAAGAAACGTAAACCAC			30min	15min		60sec	60sec	60sec		
BQCV	F	7873	3	TGGTCAGCTCCCACTACCTTAAAC	701	VP1	48°C	95°C	40	95°C	55°C	68°C		Chen *et al.*, 2004; 2005b; 2006
	R	8527	-	GCAACAAGAAGAAACGTAAACCAC			45min	2min		30sec	60sec	120sec		
BQCV	F	7873	3	TGGTCAGCTCCCACTACCTTAAAC	701	VP1	50°C	95°C	40	95°C	55°C	72°C		Siede and Büchler 2006
	R	8527	-	GCAACAAGAAGAAACGTAAACCAC			30min	5min		20sec	20sec	60sec		
BQCV	F	7873	3	TGGTCAGCTCCCACTACCTTAAAC	701	VP1	50°C	95°C	40	94°C	58°C	72°C		Siede and Büchler 2003
	R	8527	-	GCAACAAGAAGAAACGTAAACCAC			30min	15min		60sec	60sec	60sec		

Table I (part 8 of 9).

VIRUS		3'	#	PRIMER	SIZE	LOCATION	RT	denat.	cycles	denat.	ann.	ext.	REAL-TIME	REFERENCE
BQCV	F	7874	1	TGGTCAGCTCCCACTACCTTAAACI	701	VP1	50°C	94°C	35	94°C	60°C	72°C		Benjeddou *et al.*, 2001
	R	8526	-	GCAACAAGAAGAAACGTAAACCACI			30min	2min		30sec	30sec	30sec		
BQCV	F	7905	2	GGAGATGTATGCGCTTTATCGAGI	317	VP1	58°C	94°C	35	94°C	63°C	68°C**		Topley *et al.*, 2005
	R	8175	2	CACCAACCGCATAATAGCGATTGI			30min	2min		30sec	30sec	60sec		
BQCV	F	8214	2	GGTGCGGGAGATGATATGGA	71	VP1	48°C	95°C	40	95°C	-	60°C	TaqMan®	Chantawanakul *et al.*, 2006
	R	8244	2	GCCGTCTGAGTGCATGAATAC			30min	10min		15sec	-	60sec		
SPV	F	nonstr	2	GTTTGTCCTCCTATTTACATTGATTG	192	RdRp	50°C	95°C	40	95°C	-	58°C	SYBR-Green	de Miranda *et al.*, unpublished
	R	nonstr	1	AATACCTATGCCATGCTGACC			10min	5min		30sec	-	30sec		
AmMLV	F	?		GCTCGTATTCCTGCTACTCC	152	RdRp	50°C	95°C	40	95°C	-	58°C		de Miranda *et al.*, unpublished
	R	?		TTGACGGATTGAGGGATGC			10min	5min		30sec	-	30sec		
AcIV	F	?	3	GGCTAGTAAACGTAGTGGATATGACAAT	95	capsid	48°C	95°C	40	95°C	-	60°C	TaqMan®	Chantawanakul *et al.*, 2006
	R	?	2	CACCTGGTGGTCCAAGAGAAG			30min	10min		15sec	-	60sec		
β-actin	F	723	-	ATGAAGATCCTTACAGAAAG	515		42°C	94°C	35	94°C	50°C	72°C		Shen *et al.*, 2005a
	R	1199	-	TCTTGTTTAGAGATCCACAT			60min	5min		20sec	20sec	60sec		
β-actin	F	852	-	TCCTCAAGCTTGGAAAAGAG	357		48°C	95°C	40	95°C	55°C	68°C		Chen *et al.*, 2006
	R	1170	-	GGTGGACAAAGAAGCAAGAA			45min	2min		30sec	60sec	120sec		
β-actin	F	956	-	AGGAATGGAAGCTTGCGGTA	181		48°C	95°C	40	95°C	55°C	68°C	TaqMan®	Chen *et al.*, 2005a
	R	1097	-	AATTTTCATGGTGGATGGTGC			45min	2min		30sec	60sec	120sec		
β-actin	F	1038	-	ATGCCAACACTGTCCTTTCTGG	151		42°C	95°C	40	95°C	59°C	72°C		Shen *et al.*, 2005b
	R	1148	-	GACCCACCAATCCATACGGA			60min	10min		30sec	60sec	60sec		
β-actin	F	1038	-	ATGCCAACACTGTCCTTTCTGG	151		42°C	95°C	40	95°C	59°C	72°C	SYBR-Green	Yang and Cox-Foster 2005
	R	1148	-	GACCCACCAATCCATACGGA			60min	10min		30sec	60sec	60sec		
TMV	F	555	1	AAAAACAGTCCCCAACTTCC	601		50°C*	94°C	35	94°C	55°C	72°C		Tentcheva *et al.*, 2004b
	R	1117	2	AAGGAGGATTCTCTCGCTGT			60min	2min		30sec	30sec	60sec		

Table I (part 9 of 9).

VIRUS		3'	#	PRIMER	SIZE	LOCATION	RT	denat.	cycles	denat.	ann.	ext.	REAL-TIME	REFERENCE
TMV	F	641	3	CATGCGAACATCAGCCAATG	50		50°C*	95°C	40	95°C	-	60°C	SYBR-Green	Tentcheva *et al.*, 2006
	R	657	1	TGTAGCGCAATGGCGTACAC			2min	10min		15sec	-	60sec		
Varroa 16S- rRNA	F	12305	-	GACTTACGTCGGTCTGAACTCAAA	109		48°C	95°C	40	95°C	-	60°C	TaqMan®	Chantawanakul *et al.*, 2006
	R	12370	-	TTGCGACCTCGATGTTGAATT			30min	10min		15sec	-	60sec		
RP-S5	F	230	-	AATTATTTGGTCGCTGGAATTG	115		42°C	95°C	40	95°C	58°C	72°C		Evans 2004; 2006 Wheeler *et al.*, 2006
	R	302	-	TAACGTCCAGCAGAATGTGGTA			60min	3min		30sec	30sec	90sec		
RP-49	F	87	-	AAGTTCATTCGTCACCAGAG	205		50°C	95°C	40	95°C	-	58°C	SYBR-Green	de Miranda *et al.*, unpublished
	R	260	-	CTTCCAGTTCCTTGACATTATG			10min	5min		30sec	-	30sec		

tive diagnostics, PCR has the same drawbacks as signal amplification, *i.e.* that the exponential nature of the amplification process makes it difficult to keep control over quantitation. This has been largely resolved through real-time PCR and the use of internal amplification standards, which has returned a measure of linearity to quantitation. A more serious drawback is that there is a near absolute requirement for the complementarity of the 3' ends of both primers with the target sequence (at least the last two nucleotides) for the polymerase to polymerise. A mismatch in these positions usually results in failure to amplify, or at the very least severely underestimates the amount of target sequence. This property is used advantageously in many PCR-based SNP analyses. However, for viruses where there is considerable natural sequence variation, primer design becomes critical for correct amplification of all variants. Designing RT-PCR protocols for virus detection will be discussed in detail in section 9.6, and errors in detection in section 11.

8.5.1. RT-PCR

This process refers to the amplification of RNA by converting it first to cDNA with reverse transcriptase (RT) followed by PCR. A problem specific to RT-PCR is that reverse transcriptases are far more delicate and variable than the thermostable DNA polymerases and operate at lower temperatures. Hence many important parameters of reliability in virus detection by this method, such as specificity of primer binding, consistency of the enzymes over time and between samples *etc.* are defined by the reverse transcriptase part of RT-PCR. This has been partly solved by heat tolerant reverse transciptases (natural or engineered) and a single buffer system for both enzymes such that the entire RT-PCR reaction can take place in a single tube (Genersch, 2005) reducing variability and saving time and costs.

An alternative solution is to use three different primers; one for the reverse transcriptase and two different ones for the PCR reaction, such that only the target cDNA gets amplified. This approach is called nested, or semi-nested PCR, and a few such protocols have been developed for honey bee viruses (Grabensteiner *et al.,* 2001; Ongus *et al.,* 2004; Todd *et al.,* 2007). Their main purpose is to increase detection specificity in case of multiple or erroneous RT-PCR products, but it can reduce the reliability of the detection (see also section 11). There is one version of semi-nested RT-PCR with specific relevance to honey bee virus detection and quantitation. All honey bee viruses sequenced to date are naturally poly-adenylated (Gosh *et al.,* 1999; Govan *et al.,* 2000; Leat *et al.,* 2000; de Miranda *et al.,* 2004; Fujiyuki *et al.,* 2004; Ongus *et al.,* 2004; Lanzi *et al.,* 2006).

This means that the reverse transcription reaction can be primed with oligo-dT, followed by specific amplification of each virus with unique PCR primers (Jensen and Whitehead, 1998; Nie and Singh, 2000). Since reverse transcription is without doubt the most variable and sensitive part of the RT-PCR reaction, such an approach would normalise the reverse transcription step between all viruses (and potential control host mRNAs) in the sample. This would be particularly useful for multiplex amplifications and quantitative comparisons between viruses and (control) host genes (see section 9). In this approach, the virus-specific amplicons should ideally be equally distant from the start of the poly-A tail, to avoid quantitative differences between the virus amplifications due to RNA degradation or incomplete cDNA synthesis. Naturally, this approach is only suitable for poly-adenylated viruses and among the honey bee viruses still to be characterised several may well lack a poly-A tail.

Often there is a need to amplify several viruses or other target RNAs from a single sample. This can be done in several parallel uniplex reactions, or in a single multiplex reaction containing the primer pairs for all different targets (Williams *et al.,* 1999; Wetzl *et al.,* 2002; Syrmis *et al.,* 2004; Szemes *et al.,* 2002). Detection of the different amplicons is usually by size difference and electrophoresis, or by target-specific labelled probes (Mackay *et al.,* 2003). Multiplex RT-PCR is considerably less sensitive than uniplex RT-PCR (the reagents will be exhausted by several targets instead of just one), as much as several orders of magnitude depending on the number of targets (Herrmann *et al.,* 2004). However, it is extremely useful for absolute and relative quantitation and for direct comparison between several different targets (see sections 9.4 and 9.5). Numerous RT-PCR protocols have been published for the detection of the main honey bee viruses, shown in Table I. A couple of multiplex RT-PCR protocols for the simultaneous detection of several viruses have also been developed (Chen *et al.,* 2004b; Topley *et al.,* 2005).

8.5.2. Ligase chain reaction (LCR)

The ligase chain (LCR) reaction is an alternative target amplification protocol which employs two pairs of complementary primers, one pair per strand. The primers of each primer pair are adjacent to each other on the target sequence and can be ligated into a single molecule when the correct target is found. The complementary pair then repeats this using the newly ligated molecule as its target sequence (Fig. 21). A thermostable ligase is required (Saunders and Clewly, 1998) and the procedure has been commercialised (Birkenmeyer and Armstrong, 1992) although with mixed results (Buimer *et al.,* 1996; Puolakkainen *et al.,* 1998; Mahony *et al.,* 1998;

Gronowski *et al.,* 2000; Chernesky *et al.,* 2002; 2003). The advantage with respect to PCR is faster cycling times and a slightly reduced sensitivity to 3' mismatch problems, while the greatest drawback is that the amplification products can yield no information other than detection, with no possibility for analysing false positives and very limited ability to multiplex.

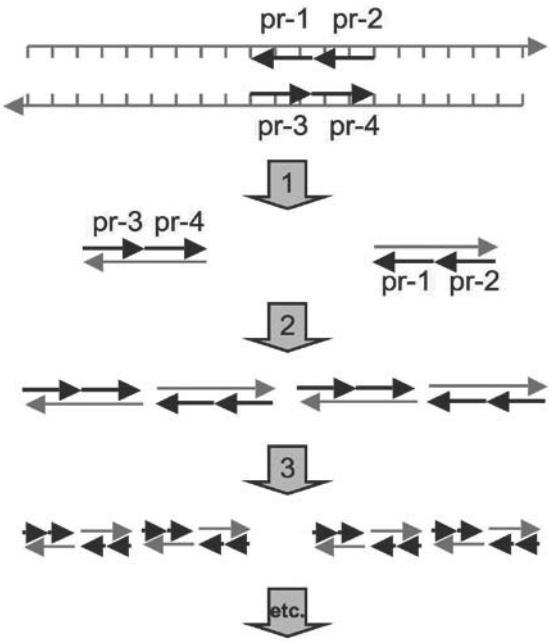


Figure 21. Illustration of the principles of the Ligase Chain Reaction (LCR). The target sequence is selectively amplified through repeated cycles of denaturation, primer annealing and ligation of the primer pairs.

8.5.3. Isothermal RNA amplification methods

Another set of procedures revolve around transcription-based amplification (Kwoh *et al.,* 1989), variously called nucleic acid sequence based amplification (NASBA; Compton, 1991; Borst *et al.,* 2001), self sustaining sequence replication (3SR; Guatelli *et al.,* 1990) or the Eberwine method (van Gelder *et al.,* 1990). These are designed specifically for RNA targets (Fig. 22) and as such are of particular interest for the detection of RNA and retroviruses (Romano *et al.,* 2000; Heim and Schumann, 2002). The RNA is converted by reverse transcriptase to 1st strand cDNA using a primer containing the T7 RNA polymerase promoter sequence. This is converted in a second reaction to double stranded cDNA by DNA polymerase using a specific 2nd strand primer. In a third reaction the T7 RNA polymerase then

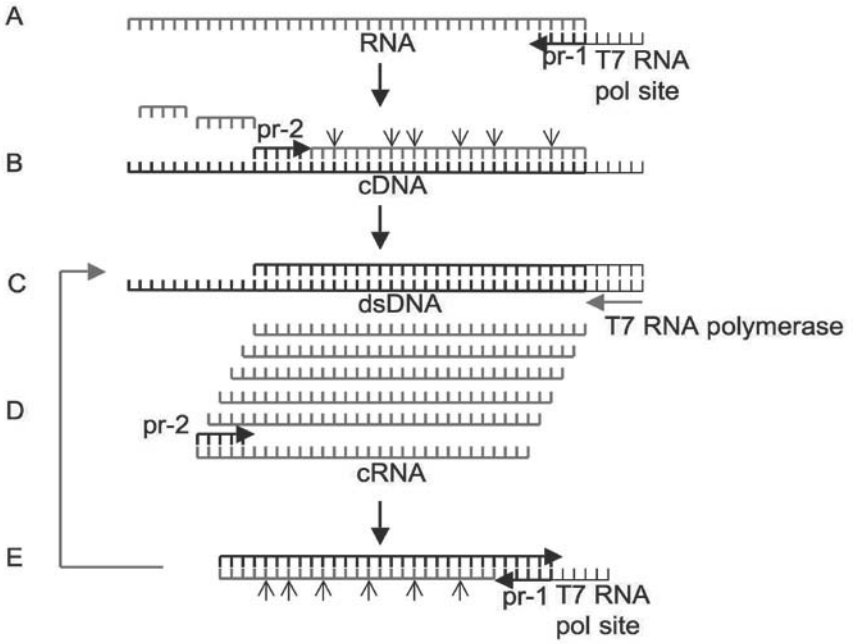


Figure 22. Illustration of the principles of T7 RNA polymerase-mediated amplification (NASBA, 3SR, Eberwine etc.). RNA is transcribed by reverse transcriptase into cDNA using a T7 promoter-linked primer (a), thence into dsDNA using a second strand primer, DNA polymerase and the RNAse H activity of reverse transcriptase (b), which completes the T7 promoter region for continuous cRNA synthesis (c) that serves as template for cDNA synthesis from the second strand primer (d) and the generation of further T7-promoter linked dsDNA template (e). RNA synthesis is the linear component and steps c, d and e are the exponential component of the amplification.

runs multiple RNA copies off the T7 transcription promoter attached to the cDNA. This RNA is complementary to the original RNA and is therefore often called cRNA, or aRNA (for antisense RNA). All reactions occur simultaneously in a single tube and exponential amplification is achieved when the two primers reverse roles using the cRNA as template. The cRNA can be detected in a variety of ways, most commonly by ECL for end point analysis (Fig. 4) or in real-time by molecular beacons (Fig. 6a), but also by gels, Northern and dot blots. Sense strand RNA can also be produced with the same principles (Marko *et al.,* 2005). NASBA is faster, and at least as sensitive as quantitative RT-PCR, with fewer background problems (Loeffler *et al.,* 2001; Schneider *et al.,* 2005; Jean *et al.,* 2001; Wacharapluesadee *et al.,* 2001; Simpkins *et al.,* 2000; Mahony *et al.,* 2001). The NASBA-ECL detection option is more sensitive, but slower than the NASBA-molecular

beacon option (Lanciotti and Kerst, 2001). One potentially useful variation is to use T7-promoter linked, anchored oligo-dT for the 1st strand primer and random hexamers for the second strand, thereby amplifying the entire mRNA population, *i.e.* the original Eberwine method (van Gelder *et al.,* 1990), which can be screened simultaneously with molecular beacons for any number of sequences, or end-labelled for microarray analysis ('t Hoen *et al.,* 2003). This becomes especially interesting when considering that most honey bee viruses are naturally poly-adenylated and therefore optimally suited to this variation. The procedure is also used to bulk-amplify minute amounts (single cell) of mRNA for subsequent manipulations (van Gelder *et al.,* 1990). The main advantages are that the process is isothermic, operating at a constant 41°C, so that no thermocycler is required. This means that amplification of RNA can occur in a DNA background, a considerable advantage for mRNA analyses and for discriminating between viable (mRNA) and non-viable (no mRNA) pathogens (Simpkins *et al.,* 2000; Schoone *et al.,* 2000; Birch *et al.,* 2001; Baeumner *et al.,* 2001; Min and Baeumner, 2002; Keer and Birch, 2003). It is furthermore well suited to multiplexing and internal controls (see section 8.7) in combination with various molecular beacons (Jean *et al.,* 2004; Ayele *et al.,* 2004) and is less affected by the 3' primer mismatch problem of RT-PCR, especially if oligo-dT is used as one of the primers. The disadvantages are the complex mixture of reagents (three enzymes, several primers, NTPs and dNTPs *etc.*), the lack of control over the three separate reactions and their interactions (adding variability), that the main product is single-stranded cRNA, which is susceptible to degradation (Spiess *et al.,* 2003), and the possibility of non-specific amplifications at 41°C which will compete for reagents but are not detected (see section 9.2 on real-time PCR). The method is also specific for (quantitative) detection only since the amplification products have little further use, unlike PCR products which can be analysed directly, cloned or sequenced.

8.5.4. Isothermal DNA amplification methods

Several DNA-specific isothermal amplification methods have also been designed. They are generally complex and prone to errors and, being designed for DNA detection, of less interest for RNA virus detection. Some of the better known methods are strand displacement amplification (SDA; Walker *et al.,* 1992a; 1992b; 1996; Nadeau *et al.,* 1999), loop-mediated isothermic amplification (LAMP; Notomi *et al.,* 2000; Nagamine *et al.,* 2001; Sugiyama *et al.,* 2005) and linear target isothermal multimerization and amplification (LIMA; Hafner *et al.,* 2001). Much like NASBA for total mRNA, SDA has been used to faithfully bulk amplify DNA samples prior

to subsequent analysis (Dean *et al.,* 2002; Luthra and Medeiros, 2004; Paez *et al.,* 2004), identify SNPs (Wang *et al.,* 2003) and differentiate viable from non-viable pathogens through the specific amplification of mRNA in a DNA background (Hellyer *et al.,* 1999).

8.6. Target enrichment and probe amplification methods

An alternative to amplifying the target prior to detection is to detect or enrich the target first and then amplify the probe. There are several such methods available.

8.6.1. Primer trees and branched DNA signal amplification

One technique specific to hybridisation involves constructing a primer 'tree' of overlapping oligonucleotides (Fig. 23a) each tagged with a reporter molecule (Saunders and Clewey, 1998) or branched DNA probes (Wolcott, 1992). The pay-off is several-fold increased sensitivity of detection, the liability is increased variability, especially for quantitation, and several more steps to optimize and control.

8.6.2. Hybrid capture PCR (HC-PCR)

In hybrid capture processes, target sequences are purified by hybridisation to oligonucleotides linked to agarose or paramagnetic beads, prior to detection with tagged probes or PCR (Fig. 23b). The process is relatively simple and fast and particularly useful when persistent background or inhibitory factors are encountered or a further enrichment procedure is required for successful detection. The disadvantage is that another step and variability is added which may affect the reliability of quantitation.

8.6.3. Immuno capture PCR (IC-PCR)

Immunocapture-PCR is a similar technique, where a pathogen is captured by antibodies prior to identification of its genome by PCR or hybridisation with tagged probes. This is especially popular for certain virus assays (Sefc *et al.,* 2000; Helguera *et al.,* 2001; Le-Gall-Reculé *et al.,* 2001; Lunello *et al.,* 2004) since on top of enriching it also distinguishes between infectious particles (captured) and non-infectious virus replication intermediates (not captured). This discrimination does require care and consistency during the washing stages, which may affect quantitation and variability (Fig. 23c).

8.6.4. Hybrid capture-2 (HC2)

This is a curious technology where RNA target is captured by a specific DNA probe (Fig. 23b). The RNA:DNA duplex is then detected by anti-

bodies specific for such hybrids and visualised using any of a range of tags attached to the antibody (Niesters *et al.,* 2000; Guyot *et al.,* 2003; Hesselink *et al.,* 2004; Konnick *et al.,* 2005).

8.6.5. Q-Beta replicase method

The final alternative, the Q-Beta replicase method, is entirely RNA based (Lizardi *et al.,* 1988; Shah *et al.,* 1994; 1995; Saunders and Clewey, 1998). An RNA probe to the target is engineered to contain the recognition sequences for Q-Beta RNA polymerase. The target RNA is immobilised on a solid support, hybridised to the probe and after the excess probe is washed away the hybridised probe is detected by amplification with the polymerase (Fig. 23d). Again this is an isothermic amplification but it is highly susceptible to overestimation of the target if the washing is inadequate and requires considerable probe engineering. However, it does not have the 3' primer mismatch problem of PCR as the amplification is entirely sequence based and not dependent on primer annealing.

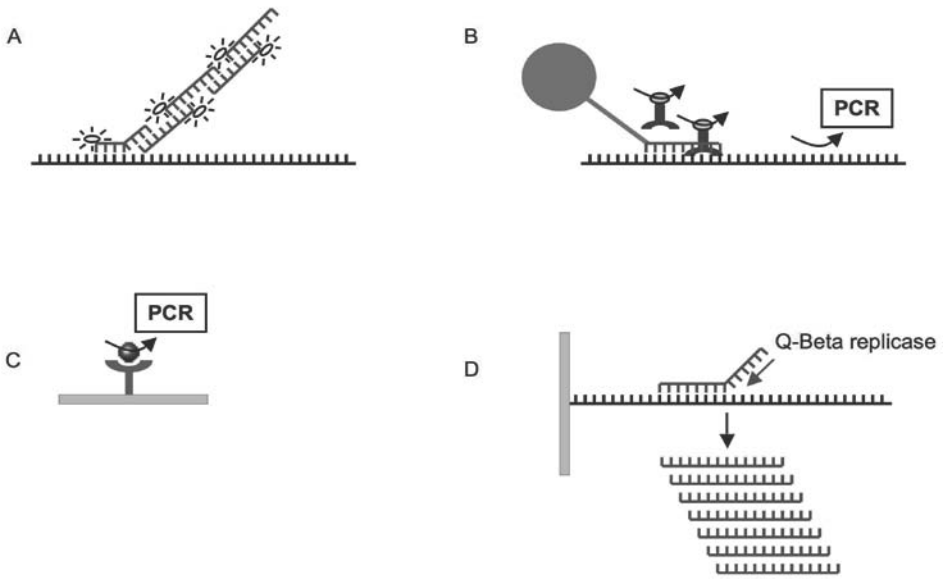


Figure 23. Four probe amplification and/or target enrichment strategies. Primer trees (a), hybrid capture-PCR and hybrid capture 2 (b), immuno capture-PCR (c) and the Q-Beta RNA polymerase method (d).

8.7. Sequencing

The most definitive way to identify a virus is by cloning and/or sequencing its nucleic acids, usually an RT-PCR product, and compare the

sequence to known viruses. This also identifies any new virus strains and micro variation, which may be informative for other aspects of the survey/sampling. With ever decreasing costs and increasing throughput (Service, 2006), sequencing may become a cost-effective way to add a large amount of information to a surveying/sampling project. Most current sequencing uses chain termination technology, where a population of different sized DNA fragments is polymerised from a target-primer complex. The fragments are terminated by one of four chain-terminating nucleotides (A, C, G or T) each labelled with a unique fluorophore. The population of fragments are resolved by capillary electrophoresis such that each nucleotide is identified by a different fluorophore. A different approach is sequencing by synthesis, or pyrosequencing, developed by Biotage and 454 Life Sciences. In this case a polymerase is presented with different nucleotides in turn, until it incorporates one. The pyrophosphate released in this reaction drives a chemiluminescent reaction that marks the incorporation of the nucleotide into the growing chain. Any unincorporated nucleotides are flushed out or neutralized by apyrase and the next nucleotide is tried. Stretches of identical nucleotides generate proportionally greater signal. The technology is designed for automation, by flushing different solutions past the growing chain-template complex, and recording the sequence as it is being generated. Without the capillary electrophoresis required for conventional sequencing and with much increased speed, pyrosequencing may become cheaper alternative for the genetic typing of large numbers of samples (Adelson *et al.,* 2005; Gharizadeh *et al.,* 2005). Pyrosequencing can also be multiplexed, by comparing the composite pyrogram obtained from several simultaneous sequencing reactions with the expected pyrogram, based on the known sequences of the different targets (Pourmand *et al.,* 2002; Palmieri *et al.,* 2003). The advantages of chain-termination sequencing are longer and more accurate sequence reads (1000 bases, compared to a 200 bases for pyrosequencing), while the advantages of pyrosequencing are automation, speed and cost, making the former ideal for new sequences and the latter ideal for screening and mutation analyses. In the race for ever faster and cheaper sequencing (a complete mammalian genome for $1000), sequencing is being combined with nanotechnology, microarrays and software to maximize multiplexing, miniaturization, speed and automation (Service, 2006).

8.8. Nucleic acid microarrays

Microarrays are a technology where hundreds of molecular probes are bound to a solid support, usually a slide, in a grid-like array. Hybridisation by the target sequences to their probes is assessed by examining all

the positions in the grid (Fig. 19a). The power of the technology lies in the massive multiplexing potential where the relative and absolute amounts of hundreds of different targets can be determined simultaneously (Cheadle *et al.,* 2003; Gentry *et al.,* 2006). As molecular biology, pathology and diagnostics moves away from single organism/gene effects to surveying interactions among pathogens and (host) genes this technology will become ever more prominent. With the sequencing of the *Apis mellifera* mitochondrial (Crozier and Crozier, 1993) and nuclear genomes (Honeybee Genome Sequencing Consortium, 2006), the *Varroa destructor* mitochondrial genome (Evans and Lopez, 2002), and the *Paenibacillus larvae* and *Ascosphaera apis* genomes (Qin *et al.,* 2006) as well as the (semi-commercial) availability of many honey bee cDNA/EST microarrays (Whitfield *et al.,* 2002; Evans and Wheeler, 2000; 2001; Robinson *et al.,* 2006; Wheeler *et al.,* 2006) including a bee pathogen array (Evans, 2006), the honey bee is at the forefront of these developments (Evans and Gundersen-Rindal, 2003). Detection can be made sensitive by high-density printing of the probe molecules into small dots, thereby concentrating all the target molecules into a small area that can be scanned by laser-based detection/quantification. The detection of probe-target hybridisation can be through FRET-based probes, SYBR-green-I dye, or labelling of the nucleic acid sample containing the target sequences. Microarray technology can also be combined with quantitative RT-PCR, multiplex (pyro)sequencing and label-free electronic or optical detection technologies to increase the speed, accuracy, specificity or information content of the diagnosis (Weidenhammer *et al.,* 2002; Erali *et al.,* 2003; Gharizadeh *et al.,* 2003; Fixe *et al.,* 2004) although at times apparently at the expense of simplicity and applicability. The major disadvantage of microarrays at the moment is their high cost and variable quality. Part of this cost is off-set by the multiplexing, where one microarray can replace many single-sequence tests for different targets, and by the limited possibility to strip and re-use microarrays. With increasing popularity, the microarray printing technology is becoming cheaper and more reliable, and single-use disposable microarrays for specific multi-target diagnosis are increasingly available (Yuen *et al.,* 2003; Lieberfarb *et al.,* 2003; Noerholm *et al.,* 2004; Lin *et al.,* 2004; Perreten *et al.,* 2005; Fiorini *et al.,* 2005). Uniformity of hybridisation across the microarray, important for reliability in quantitation, is a technical issue currently being solved with a range of nano-technological innovations (Yuen *et al.,* 2003; Noerholm *et al.,* 2004; Fiorini *et al.,* 2005) and improved oligonucleotide design (Rouillard *et al.,* 2003) and with replication of the spots or even whole arrays (an array of arrays) across the slide. Capillary bead-arrays are linear versions of microarrays where bead-linked molecular probes are stacked

in a predetermined order in a capillary tube and (labelled) target is driven through the capillary (Fig. 19b). This naturally increases the speed and uniformity of hybridisation (Kohara *et al.,* 2001). It also has a much simplified reading mechanism (a linear scanner) and may be especially suited for limited target disposable uses. Microarrays are also being developed for detecting proteins (see section 7.12).

9. Quantitation in amplification based detection

The exponential nature of any amplification process makes any quantative determination of the amount of virus originally present in the sample difficult without the use of external or internal controls. Despite optimization, chance events early in the amplification reaction can have a major influence over the final amount of product produced, as can substances and factors in the sample affecting the efficiency of the enzymatic reactions (Peterson *et al.,* 1997; Berg *et al.,* 1997; 2005; Mahony *et al.,* 1998; Verkooyen *et al.,* 2003). This applies both to target amplification (*i.e.* RT-PCR and similar techniques) and signal amplification (*i.e.* enzyme-substrate marker detection systems). Another factor obscuring the relation between the initial amount of target and the final amount of amplified product/signal is the gradual exhaustion of enzymes and reagents in the reaction mix. This makes the later cycles in PCR superfluous for reactions with abundant target, while reactions with less initial target use these later cycles to effectively catch up in product synthesis. This is also the reason why multiplexed RT-PCR reactions are about an order of magnitude less sensitive than separate uniplex reactions. A comparable situation in ELISA and other enzyme-substrate assays is the changing relative intensities of different wells as the enzymatic assay development proceeds: left long enough, all wells will reach saturation absorbance levels. For this reason ELISA protocols have a defined time for colour development of the substrate reaction and generally use thresholds for scoring samples into discreet categories, in a semi-quantitative manner. Several approaches have been used to overcome these obstacles.

9.1. Competitive PCR

Until the development of real-time RT-PCR, competitive PCR was the most reliable manner to quantify the amount of target in PCR reactions. A known amount of competitor RNA/DNA is added to the sample prior to amplification. The ratio of target to competitor post-amplification reflects their ratio pre-amplification, and the absolute amount of target

in the sample can be calculated through this ratio and the known absolute amount of competitor added to the reaction. The ideal competitor is a cloned DNA copy of the target (or in the case of RNA targets; RNA transcripts from such a clone) which uses the same PCR primers but is distinguished from the target through a small deletion, unique restriction enzyme site or other unique identifiable feature such as a signal sequence for hybridisation probe (Nygren *et al.,* 2001), that allows post-amplification identification and quantitation of target and competitor (Lim *et al.,* 2001; Achenbach *et al.,* 2004; Grove *et al.,* 2006). Deletion competitors are preferable, since restriction digestion is not 100% efficient, leading to over- or underestimation of the target and greatly increased variability in estimation (Bustin, 2000). If different primers are used for target and competitor, the reaction becomes essentially a multiplex PCR with an added exogenous internal control (Nie and Singh, 2000). Competition in this case is for nucleotides and enzymes only, and adjustments may have to be made in the calculations for the different amplification efficiencies of the different primer pairs. Target and competitor can be distinguished by uniquely labelled primers and/or probes and in this format competitive PCR principles have been included in real-time quantitative PCR protocols, through the co-amplification of internal controls, as a secondary means for target quantitation and normalisation between samples. The ideal partner for competitive RT-PCR is capillary electrophoresis, which automatically quantifies fragments during detection. Although less accurate than real-time quantitation, it is sufficient for many diagnostic applications, and combines automated quantitation with extensive multiplexing potential (Elvidge *et al.,* 2005). These considerable advantages make this a powerful and popular combination (Erdman *et al.,* 2003; Gómez-Llorente *et al.,* 2004; García-Cañas *et al.,* 2004; Lim *et al.,* 2001; Luyten *et al.,* 2006; Richards and Poch, 2002; Spyres *et al.,* 2005). Other approaches for the detection-quantitation of the different amplicons in the multiplex competitive RT-PCR are microtitre plate based hybridisation assays (Mavropoulou *et al.,* 2005; Poumannova *et al.,* 2006), mass-spectroscopy (Yang *et al.,* 2005), DNA microarrays (Rudi *et al.,* 2003) and microchip electrophoresis (Cantafora *et al.,* 2004).

9.2. Real-time quantitative PCR

Real-time PCR is a unique approach to quantifying PCR reactions. The accumulation of PCR products is monitored as the reaction progresses through the cycles, through a light-based detection system integrated into the thermocycler. The philosophy is that abundant target sequences will exhaust the primers and nucleotides at an earlier cycle than rare se-

quences, thus providing a means to quantify the original target sequences. The critical parameter is not the amount of final product, but the time when the increase in PCR product reaches a particular threshold value (Ct value). Internal and external controls provide the means for relative and absolute quantitation and for normalisation between different samples (Bustin, 2000; Pfaffl, 2000; Pfaffl and Hageleit, 2001; Livak and Schmittgen 2001; Pfaffl *et al.,* 2002). Although developed for PCR, the real-time approach to quantitation could also be applied to other amplification systems, such as NASBA and other isothermic or transcription based systems. Real-time monitoring of the PCR reaction is either by SYBR-green or FRET-based probes, of which TaqMan® probes are the most popular (Holland *et al.,* 1991; Thelwell *et al.,* 2000; Solinas *et al.,* 2001; Howell *et al.,* 2002; Bustin and Nolan, 2004). The advantages of SYBR-green are low cost and universality, since it can be applied to any target-primer combination. Most commercial real-time SYBR-green kits now have the dye included in the reagent mixtures. A critical disadvantage is that only a single target can be monitored per reaction. This means that relative quantitation through internal controls requires separate reactions for each control. This adds variability, although the significance of this added variability in the context of the many other sources of variability in the overall assay is unclear. A post-amplification melting curve analysis is required to identify and correct for signal derived from amplification artefacts, usually primer-dimers with a significantly reduced melting temperature (Ruiz *et al.,* 2005). TaqMan® primers and other self-reporting probes are specific for each target, which means that several targets can be competitively amplified in the same reaction. Current technologies (both the thermocyclers and unique FRET options) allow up to four separate targets to be monitored concurrently, in the same reaction (Candotti *et al.,* 2004; Stram *et al.,* 2004; Pugnale *et al.,* 2006). This offsets to some degree the larger cost of TaqMan® probes and, perhaps most importantly, allows one or more endogenous internal controls to be co-amplified with the target. The disadvantages of hybridisation probes are high initial cost, variable quality and instability during prolonged storage (Bustin, 2002). Whether in separate uniplex reactions or a single multiplex reaction, comparisons between different targets have to be corrected for the different amplification efficiencies of different target-primer set combinations. This may be more difficult to optimize in multiplex reactions due to deviations from linear predictions based on uniplex reactions for each of the targets (Candotti *et al.,* 2004) Further information concerning these issues can be found in several excellent reviews (Bustin , 2000; 2002; Pfaffl *et al.,* 2002; Wilhelm and Pingoud, 2003; Bustin and Nolan, 2004; Peters *et al.,* 2004) including

one specific for virus detection (Mackay *et al.,* 2002) and on the www.gene-quantification.info website. The major advantage of real-time quantitative PCR over competitive PCR quantitation is the full automation of amplification, quantitation and analysis in a single, close-tube system. The single major limitation is that in this approach, all the multiplexing benefits of post-amplification analysis through fragment size separation are also lost. In terms of quantitation accuracy and reproducibility, real-time quantitation compares favourably with competitive approaches (Bustin, 2000; Pagliarulo *et al.,* 2004; Pumannova *et al.,* 2006), mostly through reduction in the number of independent steps and by avoiding the tail-end of amplification where most of the irregularities and variability resides.

9.3. Multiplexing real-time RT-PCR

One way to enhance multiplexing in real-time detection is by integrating fragment analysis with real-time amplification-quantitation (Li *et al.,* 2002; García *et al.,* 2005; Kita-Matsuo *et al.,* 2005). In this procedure, ds cDNA is made with target-specific primers containing universal adaptor sequences. The cDNAs of many targets are then amplified competitively with PCR primers to the adaptor sequences, one of which is labelled with a fluorophore. Samples taken after each cycle are analysed by capillary electrophoresis to separate the different target amplifications by size and quantify each peak. The data are then re-assembled to determine the Ct value of each target. There are several advantages of this approach. Apart from the extensive multiplexing potential (capillary electrophoresis has reliable 1 base-pair resolution between 50 and 750 base-pairs), direct fluorophore labelling is significantly more sensitive than FRET probes (unincorporated label is removed by electrophoresis) and spurious amplifications can be identified (unexpected size fragments) and discounted. The extent of multiplexing is not unlimited however, and despite a single PCR primer pair for all targets, corrections for amplification efficiency differences of the different fragments will still be required for accurate relative and absolute quantitation. The savings in time and money by multiplexing are lost with the large number of capillary electrophoresis analyses required. By reducing the capillary electrophoresis to certain cycle intervals it may be possible to reduce the cost and labour without compromising the quantitation accuracy too much.

9.4. External standards and absolute quantitation

The classic way to relate indirect measurements to absolute amounts of target is through standard curves established by putting a dilution series of known amounts of target (external standards) through the detec-

tion procedure and converting all experimental data through this curve or equation (Bustin, 2000). Such curves are also extremely useful during optimization of the RT-PCR reaction conditions. The main drawback is that factors unique to each sample, such as RNA quality and quantity, enzyme inhibitors, sample degradation, internal fluorescence *etc.* cannot be accounted for. Internal standards circumvent many of these uncertainties and are therefore to be preferred where practical. One major decision with respect to external standards for absolute RNA quantitation is whether to use a DNA or RNA based curve. DNA curves tend to be more sensitive and reproducible but RNA curves are more realistic. The professional literature is divided on the issue, with good arguments for both approaches (Pfaffl and Hageleit, 2001). Both curves still require several positive control RNA samples per run, to normalize between runs for differences in reagent mixtures and, in the case of the DNA curve, to account for the reverse transcription step as well. An alternative, but less accurate, absolute quantitation method has been described that does not require an external curve, but instead uses the total amount of TaqMan® probe in the reaction (as estimate by the total fluorescence at the amplification plateau) to normalise and quantify the amount of cDNA in the sample (Swillens *et al.,* 2004).

9.5. Internal controls and relative quantitation

A second approach to quantify the original target in an RT-PCR reaction is with internal controls. Internal controls allow normalizing between samples for many samples-specific factors, such as the differences in the total amount of RNA extracted, the presence of inhibitors, stimulants, sample degradation *etc.*, since target and control are equally affected. There are two options available. The first option is an exogenous standard, *i.e.* one that is added in known amounts prior to RT-PCR. Exogenous standards can normalize for sample specific factors affecting the performance of the RT-PCR reaction, but not for differences in the quantity or quality (degradation) of the original RNA. As such, it is very useful for identifying inconsistencies and variability in the assay. Tentcheva *et al.*, (2006) used this approach to analyse assay variability when quantifying DWV from a range of bee samples. The second option is to use an endogenous control, *i.e.* a relatively invariant and abundant RNA species in the original sample. Such a control can normalise for all differences between samples and is therefore very well suited for comparisons between different types of samples (different tissues, life stages *etc.*) which may affect the assay in different ways. Chen *et al.,* (2005) used this approach for relative DWV quantitation between different bee samples. Generally one or more

well known 'housekeeping' genes or structural protein genes are used on the assumption that any variation in expression in these genes is minimal compared to changes in the expression of the target gene (Bustin, 2000; 2002; Pfaffl, 2001; Radonić *et al.,* 2004; Lund and Madsen, 2006). In the case of bee viruses an obvious candidate for endogenous internal control is ribosomal RNA, since ribosomes have similar abundance, shape and purification characteristics as the bee viruses in symptomatic infections. When properly considered, internal controls are little more than markers for the total amount of (amplifiable) RNA: the equivalent of total µg of RNA on Northern blots. The problem is that it is impossible to prove categorically that the expression of any candidate 'invariant' gene is not affected by the expression of the target gene. This is furthermore unique for each host and tissue, making selection of a single 'constant' reference gene suitable for all situations practically impossible (Radonić *et al.,* 2004). For this reason it is currently recommended to use a battery of 3 or 4 internal controls, from different classes of genes (metabolic enzymes, structural proteins, transcription factors, ribosomal proteins *etc.*) and construct a control-gene index, with which to normalise between samples (Bustin, 2000). Common internal controls used for honey bee mRNA and virus quantitation are β-actin (Chen *et al.,* 2005; Shen *et al.,* 2005a; 2005b), rRNA (Chantawannakul *et al.,* 2006), microsomal glutathione-S transferase (Evans and Wheeler, 2000; Gregory *et al.,* 2005); ribosomal proteins RP-S5 (Evans, 2004; 2006; Wheeler *et al.,* 2006), RP49 (Corona *et al.,* 2005), RP-S8 (Kucharski and Maleszka, 2005), and transcription factors eIF3-S8 (Grozinger *et al.,* 2003) and eF1α (Toma *et al.,* 2000; Yamazaki *et al.,* 2006). One technical difficulty with using endogenous internal RNA controls is that the amplification could come from genomic DNA that contaminates the RNA sample, instead of from the RNA. There are two solutions to this, the first being to digest the RNA sample with DNAse prior to RT-PCR. Many RNA purification kits come with this option. The other solution is to choose RT-PCR primers that are separated by an intron in the genomic copy of the gene, such that only spliced mRNA gets amplified (Bustin, 2000). For statistical reasons it is best if the amounts of target and standard are less than two orders of magnitude apart.

9.6. Designing a (real-time) RT-PCR protocol for virus detection

Designing a protocol for the detection of viruses in an RNA sample by RT-PCR involves several distinct stages. The most critical decisions concern the design of the primers since these are the primary determinants for the specificity, accuracy, reliability and sensitivity of an assay.

9.6.1. Primer design - virus considerations

Thus far, generally little attention has been paid by the honey bee virus research community to the unique challenges and restrictions regarding the design of RT-PCR primers for (honey bee) RNA viruses. The first decision concerns exactly what the protocol wishes to detect and what, as a consequence, it will not detect (Tentcheva *et al.,* 2006). Viruses, particularly RNA viruses, are supremely variable entities, so a protocol can be for the detection of a particular virus variant, for the all known variants in a species, or for a species complex of related viruses which may have to include potential species/variants as yet not identified. Although different types of primers are required for each case, the nature of the 3' most nucleotides of the primers is critical for the protocol to correctly detect what it is intended to detect. The most variable nucleotides of a virus genome are those located in the third codon position of the coding regions. These positions should be avoided for the 3' terminus of all primers, since a mismatch at the 3' terminus of just one of the primers will result in a false negative, whether screening narrowly (strain distinction) or broadly (species-complex). For forward primers, terminating on the 2nd codon position is best while for reverse primers terminating on the 1st codon position is best, since this relegates the nearest 3rd codon position to the third nucleotide from the 3' end of each primer (Table I). Another resolution of the 3' mismatch conundrum is to include deoxyinosine as the 3' nucleotide, which can pair with all nucleotides (Benjeddou *et al.,* 2001; Topley *et al.,* 2005).

For distinguishing between closely related strains the only subsequent virus-related criterion is to identify one or two nucleotides where the strains differ consistently, and locate the 3' terminus of one or both primers on these nucleotides (Nie and Singh, 2003). For distinguishing related (sub)species it is best to design the primers in a region where the viruses are most distinct (Ongus *et al.,* 2004). For identifying all potential variants within a species complex, two stretches of 20-24 nucleotides should be identified where the most disparate strains in the complex are identical, with a region of up to 400 nucleotides in between where the strains differ. The primers are derived from the conserved regions, to ensure amplification of as many strains as possible, while the intervening region is used to characterize any new strains amplified (Todd *et al.,* 2007). For merely detecting all known strains, the primers are also designed from conserved regions, but the nature of the intervening region is immaterial.

RNA virus genomes usually total about 10000 bases, with most of the genome unique and specific to the virus, providing ample genetic material from which to chose primers (Table I). Primers should be constantly

checked against a multiple sequence alignment of all known strains to assess their usefulness for the particular diagnostic aims. With careful primer design it should be possible to approach 100% correct detection (no false positives or false negatives) for most viruses with a single primer pair. However, this is very much conditional to the natural variation and variability (*i.e.* the capacity to generate new variants) for each virus. There are valid arguments that PCR is unsuitable for reliable detection of super variable viruses, such as the hepatitis viruses and HIV, even when employing several different primer sets (Gardner *et al.,* 2003). When the reliability of a primer set with respect to virus variability is in doubt, the best resolution is to employ several primer sets in parallel so that the failure of one set does not necessarily result in mis-diagnosis. Multiple primer sets also allows one to estimate the rate of misdiagnosis by different primer sets due to virus variability (Chui *et al.,* 2005). Within the honey bee viruses, multiple primer sets may be needed for reliable diagnosis within the highly variable KBV-ABPV complex and the less variable DWV-VaDV complex. Multiple primer sets have been designed and analysed for SBV (Grabensteiner *et al.,* 2001), ABPV (Bakonyi *et al.,* 2002b) and DWV (Genersch, 2005).

All these virus-related considerations concerning PCR primer design precede any subsequent primer design considerations, related to PCR performance or product detection.

9.6.2. Primer design – protocol considerations

Both amplification primers should ideally be the same length (around 20 nucleotides) with similar melting temperature (Tm) between 55-65°C. Longer primers or higher Tm leaves no room for annealing temperature optimisation below the extension temperature of 72°C, raising the possibility of non-specific amplifications. Shorter primers (lower Tm) also risks non-specific amplification, due to insufficiently unique primer sequences.

To avoid primer-dimers, there should be no complementarity between the 3' ends of the primers, with the 3'-most nucleotides most critical. Neither should there be any self-complementarity at the 3' ends of each primer. The easiest way to avoid this is to extend or shorten both primers 1 or 2 nucleotides until the 3' (self) complementarity disappears (while remembering to avoid the 3rd codon position for the 3' terminus). Long (>4) stretches of identical nucleotides should also be avoided, again especially at the 3' end.

Once the PCR primers have been defined on virus and amplification criteria, a TaqMan® probe can be designed for the intervening region. Proprietary software (Primer Express) or freeware (Primer 3) can be used

to guide the design of the probe. Most TaqMan® primer design software recommend very short amplicons (<100 nucleotides), since this shortens the cycling times, avoids incomplete amplicons and saves reagents, avoiding competition even at late cycles. However, longer amplicons (up to 500 base pairs) provide much greater flexibility in designing a suitable TaqMan® probe for the target. With the considerable virus-based restrictions to amplification primer design, finding an optimal TaqMan® probe within a larger amplicon may well justify any slight reduction in amplification efficiency. Furthermore, any new virus variants or spurious amplifications are more easily characterised with larger amplicons, through post-amplification analyses. The probe should as much as possible be devoid of secondary structures (stem-loops) and have a Tm higher than that of the amplification primers, so that it anneals to the denatured target molecules before any primer-driven polymerisation takes place. 5' G bases should be avoided since they quench fluorescence, even after cleavage (Bustin, 2000).

9.6.3. PCR optimisation

Most RT-PCR kits, whether One-Step or Two-Step (depending on whether the reverse transcriptase and PCR reactions are coupled or not), have optimised, proprietary mixes of reagents, enzymes and nucleotides, which includes optimum extension and denaturation temperatures and times. As a result, different kits perform differently with the same primers and cycling profile, and are also part of the optimisation procedure (Grabensteiner *et al.,* 2001). The only variable step, related to the unique factors in the reaction (*i.e.* primers and target), is the annealing temperature. This should be optimised with a temperature gradient, which most modern thermocyclers are capable of generating in a single run. Other components of the cycling profile can also have significant influence over the reaction dynamics (Caetano-Anolles, 1998). Primer concentration is another component that can be conveniently optimised at the same time as annealing temperature (Topley *et al.,* 2005; Todd *et al.,* 2007). The criteria for optimisation can be sensitivity, specificity or reproducibility. Higher primer concentrations and lower annealing temperatures increase sensitivity, but reduce specificity. Optimising for reproducibility usually finds a happy medium between these two. A useful rule of thumb is 60 seconds extension at 72°C for a 1000 base-pair amplicon, with usually a minimum of 15 seconds extension, although this varies for different enzymes and mixtures.

9.6.4. Reverse transcription optimisation

Often forgotten in optimisation experiments is the most delicate step in RT-PCR, the reverse transcription. The efficiency of this step can vary between 0.5% and 95%, with the type of reverse transcriptase the biggest variable, and is very sensitive to inhibitors and contaminants in the sample (Ståhlberg *et al.,* 2004b). This efficiency is furthermore also affected by both the absolute and relative amounts of target RNA in a sample, especially at very low levels of target (Ståhlberg *et al.,* 2004a, 2004b) and a variety of reaction conditions (Sing *et al.,* 2000). For this reason, neutral carrier tRNA is often added to RNA samples prior to RT-PCR to maximize target reverse transcription and detection reliability. Genetic engineering and/or prospecting has produced reverse transcriptases that can operate at around 50°C, which greatly enhances the specificity of primer-annealing during reverse transcription, and hence the reliability of detection. Apart from the choice of enzyme, the major parameter to optimise is the choice of cDNA primer, which can significantly affect quantitative estimation (Bustin, 2000). Both random primers and oligo-dT generate a larger pool of cDNA molecules to analyse, and provide normalisation between different targets (see section 8.5.1), but random primers can overestimate the original target RNA up to 19 fold (Zhang and Byrne, 1999). Target specific primers reduce potential background amplifications but can differ significantly among each other in cDNA efficiency. This can be problematic for relative quantitation of targets, but less so for absolute quantitation since the standard curves are generated with the same primer(s). Since the incubation time is rather constant at about 30 minutes, the only incubation variable to optimise is the incubation temperature. Due to the delicate and variable nature of the reverse transcriptases it is probably best to optimise this step with respect to reproducibility.

9.6.5. RT-PCR protocols for honey bee viruses

Numerous qualitative and quantitative (real-time) RT-PCR protocols have been published for the honey bee viruses DWV, KBV, SBV, ABPV, BQCV, AcIV and CBPV, using both TaqMan® and SYBR-green, summarized in Table I. With a few notable exceptions (Grabensteiner *et al.,* 2001; Topley *et al.,* 2005), only the most rudimentary optimisation has been applied, if at all, to most of these protocols. This is of less importance for qualitative detection since most primer sets are quite robust to variations in protocol (Table I). For quantitative detection however, differences in cycling profile, enzyme mixtures, primer concentrations and detecting chemistry will have significant impact on the estimation of the amount of original target, unless normalised with suitable controls. In these situations it is best to copy the entire protocol, not just the primer sequences.

10. Detection of variation

Occasionally organisms can be identified by a pattern of bands rather than a single band, or the diagnostic of interest concerns genetic polymorphisms or for other reasons variation in a pattern rather than the primary detection is of interest. The supreme importance of single nucleotide polymorphisms (SNP) in medical genetics has meant that many of the nucleic acid detection technologies discussed in sections 6 and 8 have been designed or adapted for the detection such polymorphisms, with varying degrees of success. Molecular beacons and oligonucleotide microarrays (Lieberfarb *et al.,* 2003; Lin *et al.,* 2004), target amplification methods such as NASBA or PCR, and pyrosequencing have all been adapted for the detection of polymorphisms. What follows are several additional methods specifically designed for the detection of variation, both for proteins and nucleic acids. For a history of genetic variation analyses and options see Ahmadian and Lundeberg (2002).

10.1. Protease digestion

There are several proteases (trypsin, chymotrypsin, papain, pepsin, clostripain, V8 *etc.*) available commercially with specificity for certain amino acid sequences, which can be used to detect amino acid variation in proteins directly. The fragments can be analysed by mass spectrometry, or separated by electrophoresis and visualized directly or by Western blotting with specific antisera in case of crude extract digestions (Sambrook and Russell, 2001).

10.2. Serological variability

For most serological techniques, especially the ones popular for routine diagnosis, the signal is due to the combined effects of the amount of target present and the avidity, or specificity, of the antibody for the target. This means that quantitation of the target is also a function of its variability, with no simple means to separate these two effects. The simplest means to detect serological variability between different virus strains is through spur formation in AGID tests (Fig. 13). In these tests a polyclonal antiserum to one of the strains is placed centrally while the virus strains plus controls are placed peripherally. Antibodies against common epitopes between the two strains will form the main precipitin lines while antibodies unique to the homologous virus strain will migrate through the precipitin line of the heterologous virus and precipitate behind this line with the diffusing homologous virus strain, forming a characteristic 'spur' (Madriz *et al.,* 2000). For monoclonal antibodies, ELISA and West-

ern blots the only way to assess serological variability is through dilution end-point analyses with the various strains (Harlow and Lane, 1988).

10.3. Nuclease protection assays (RPAs and SNPAs)

Nuclease protection assays are an excellent and very efficient way to analyze the genetic complexities of natural populations of organisms (Kurtah *et al.,* 1993; Kurath and Dodds, 1995; Arens, 1999; Wang and Chao, 2005). A labelled probe is hybridised to the nucleic acid sample of a population of organisms (usually viruses or other pathogens, sometimes related mRNA species) and then digested with RNAse (RNA probe) or S1-nuclease (DNA probe) which will cut the probe wherever there is a mismatch between probe and target (Fig. 17). The resulting pattern of digested probe fragments is qualitatively and quantitatively indicative of the mismatch polymorphisms present in the nucleic sample. These procedures are called RNAse protection assay (RPA) and S1-nuclease protection assay (SNPA). An example of an RPA using radiolabel is shown in Fig. 17. The power of NPAs is that entire populations can be screened for genetic complexity within the target sequence in a single reaction, which can be analysed directly by gel or capillary electrophoresis. The polymorphic sites can be mapped reasonably accurately on the genome, through the sizes of the fragments produced. The disadvantages are that the length of the probe is limited to about 300 bases for maximum efficacy of the reactions and label needs to be present throughout the probe (incorporation, global chemical labelling or dyes) in order to pick up all fragments. Quantitative signal will therefore by necessity have to be adjusted for the lengths of the digested fragments.

10.4. Restriction fragment length polymorphism (RFLP)

Something similar can also be achieved by digesting PCR products of a polymorphic target with restriction enzymes, if the polymorphism is located within a restriction site (Hauser *et al.,* 2000; Evans, 2001). PCR-r

estriction fragment length polymorphism (PCR-RFLP) can also be used in quantitative PCR if the target and competitive standard are differentiated by a restriction site (Fig. 18), or size. Again the fragments can be labelled with tagged PCR primers, incorporated direct or indirect label, or dsDNA specific dyes (ethidium bromide, SYBR dyes) and separated and quantified by capillary electrophoresis (Luyten *et al.,* 2006) or normal gel electrophoresis, each with appropriate detection-quantitation systems.

10.5. Gel retardation methods (SSCP and DGGE)

Single strand conformation polymorphism (SSCP) and denaturing gradient gel electrophoresis (DGGE) are two common techniques that use electrophoresis to differentiate directly between variants in a population of sequences (Hauser *et al.,* 2000; Stach *et al.,* 2001). In SSCP the nucleic acids are, or are made, single-stranded with a preferred secondary structure. Minor sequence variants affect this secondary conformation and hence the electrophoretic mobility of the molecule in a gel. In DGGE, the nucleic acids are separated in an electrophoretic gel containing a gradient (usually a salt) that will denature the nucleic acids (*i.e.* render them single stranded) as electrophoresis progresses, affecting their electrophoretic mobility. Nucleotide differences between the polymorphs in the sequence population will affect where in the gel this denaturation occurs, causing a mobility shift in the gel. For both methods the nucleic acids are usually transferred to a membrane post-electrophoresis and hybridised to a specific labelled probe (Fig. 24). The mobility shifts are greatest for smaller fragments, limiting its applicability and both are delicate techniques requiring careful control of conditions and equipment. Another disadvantage is that the nature and genome location of the polymorphism cannot be determined except by further experimentation. Another technique with a similar philosophy is the heteroduplex mobility shift assay, where single nucleotide mismatches between a probe and target affect the mobility of the hybridised complex during electrophoresis, allowing qualitative and quantitative distinction between different viral variants in a sample (Arens, 1999).

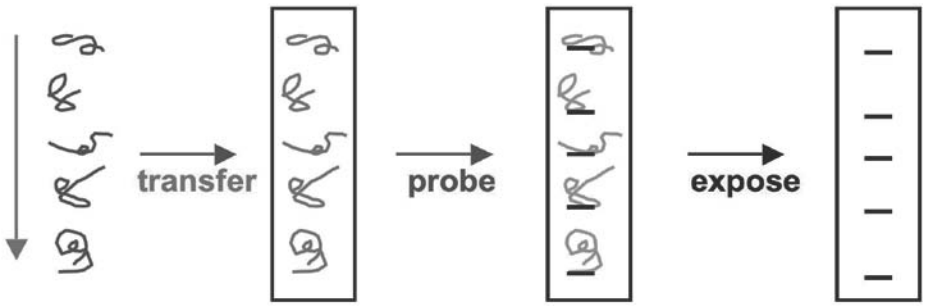


Figure 24. Illustration of the principles of gel retardation assays. Changes in single stranded conformation or denaturing characteristics of nucleic acids are resolved by electrophoresis (a). The fragments are transferred to solid support (b), detected by labeled probes (c) and visualized (d).

10.6. Sequencing

The most powerful means for detecting variation is sequencing, since every possible variant is identified and precisely mapped on the genome. There are several approaches that can be used. The purest and most expensive approach is to clone PCR products of the target(s) and sequence batches of individual clones. This also allows the relative frequencies of individual variants to be determined, even those variants occurring at very low frequencies. A second and cheaper approach is to sequence the PCR products directly and identify the polymorphisms at sites of ambiguity in the sequence. The main drawback here is that such ambiguities are also regularly produced through sequencing artefacts, making it difficult to distinguish these from true polymorphisms. Another drawback is that only major polymorphisms can be identified and that quantitation is very crude. With the development of multiplexed sequencing technologies, such as pyrosequencing (see section 8.7) and metagenomic approaches to data generation and analysis directly from complex populations (Handelsman, 2004; Xu, 2006), sequencing may become a realistic tool for analysing (virus) population variation (Service, 2006). The main drawback of sequencing in general as a tool for identifying variation is expense, although the popularity of sequencing means that this may become a minor factor in the future. See also section 8.7.

11. Errors in detection

An often neglected component in the development of new diagnostic techniques is the reliability of the diagnosis. There are two types of error, qualitative and quantitative, and both should be accounted for at all stages of process development. Below are the principal sources of both types of error for molecular methods, and how to avoid or minimize them.

11.1. Qualitative errors

These can be divided into 'false positives' (signal without target) and 'false negatives' (target without signal). Both are the primary indicators of the reliability of a diagnostic procedure, and hence are the most important variables when optimising a protocol.

11.1.1. False positives

False positives can be due to both detection errors and reporter tag errors. Reporter problems have been discussed in section 6 and are mostly due to native compounds in the sample that mimic the reporter molecule

(enzyme activity, fluorescence, luminescence *etc.*) and are largely solved by switching reporter. Illicit recognition is more common in serology than nucleic acid-based detection, probably because antibody-protein interaction is more complex and varied than nucleic acid hybridisation. It can be particularly problematic in ELISA. The most common reasons are incomplete blocking or incomplete washing, which are easily solved. However, it can also be due to the presence of non-target antibodies in the 1^{y} antiserum, consistent recognition by the 1^{y} or 2^{y} (often monoclonal) antisera of non-target compounds, or non-specific binding of antibodies by compounds in the sample. Technologies where the target is captured by antibodies before detection (DAS-ELISA, hybrid-capture etc.) are less susceptible to these problems than direct detection, since potential interfering compounds in the sample are removed before detection. False positives in nucleic acid hybridisation can be eliminated by increasing the hybridisation stringency. DNA contamination is potential source of false positives in PCR and other target amplification methods. Contamination is avoided by careful technique (particularly pipetting) and a template-free environment (room, workstations, equipment, consumables) where reactions are assembled without template, which is added elsewhere. Thermocyclers can also be a regular source of contamination. Another source of false positives in PCR is primer-dimers. These arise through momentary complementarity between the 3' ends of the primers, which generates an artificial template that is replicated with far greater efficiency than the target. They can be discounted by size separation of the PCR products or in real-time PCR through melting-curve analysis. Correct primer design, reaction optimization, reducing primer concentrations and raising the annealing temperature should solve the problem. Another problem is the amplification of non-target sequences. Often the choice of primer is decided by criteria other than optimal performance in PCR (universal primers, conserved regions for comparative studies *etc.*) and in these situations several sequences can be amplified (Fig. 16). One way to avoid this problem is to use a 'nested PCR' approach, where a secondary amplification with different primers follows the primary amplification. The involvement of a third primer increases the specificity of the detection, since only molecules containing all three primer sequences in the right orientation will be amplified. Molecular beacons and other FRET-based hybridisation probes solve many of these problems in a similar manner, by using a separate probe for identifying the correct PCR product amplified by the two PCR primers. The disadvantage of (semi)-nested RT-PCR is that there is a 50% increase of false negatives diagnosis over two primer amplification, since amplification failure due to 3' primer-target mismatches can now

occur at three sites rather than just two. Reporter problems include contaminants (native fluorescence, enzymes *etc.*) in the nucleic acid sample similar to the tag used. Better sample purification or switching reporters solves this problem.

11.1.2. False negatives

False negatives most commonly arise from the degradation of reagents, enzymes, probe or the reporter molecule. Replacement is the most practical solution. The next most common cause of false negative data is sample degradation, which unfortunately cannot be remedied. Checking the integrity of the nucleic acid prior to the assay is crucial to avoid misdiagnosis due to false negative data (see section 2.2). Other causes of false negative results are inhibitors of the reaction or the reporter molecule in the sample (Berg *et al.,* 1997; 2005), which is solved by purifying the sample and/or switching reporters. One cause of false negatives specific to PCR is 3' mismatches between primer and target, which is particularly a concern when dealing with variable sequences, such as RNA viruses. This problem can be minimized by multiple primer sets for each virus, choosing a conserved region for the primer and making the 3' end coincide with the 1st codon position, if in a coding region. With RNA based amplification systems there is also the potential for amplification product degradation (Spiess *et al.,* 2003). Care in sample preparation, plus negative and positive controls should minimize the other problems.

11.2. Quantitative errors

In essence, all diagnosis and detections are quantitative, made qualitative by thresholds that separate positive and negative diagnosis. All qualitative problems (backgrounds, enzyme inhibitors, nucleic acid competitors *etc.*) can also be manifested as quantitative problems, where there is a random or systematic departure from a predictable (usually linear) relation between the amount of target in the sample and the signal generated. Special attention should be given to uniformity in sample processing, where much of the random variability between samples originates. Signal or target amplification processes (*i.e.* PCR, enzymatic reporters) are inherently susceptible to departures from linearity. A final source of error in quantitation is the saturation levels and detection limits of the instrumentation used (Fig. 18). Standardization, replication and internal and external standards are the most obvious solutions to many of these problems.

11.3. Variability

A final element of major concern when developing a routine diagnostic procedure is reproducibility and reliability. This involves all elements of diagnosis, from sample collection to detection (Bustin, 2002). To control this variability, many commercial providers of diagnostic kits and services increasingly 'bundle' the sample processing and detection protocols, also in response to the requests from the principal end-users, which are primarily specialist diagnostic laboratories. Future developments in this area will increasingly feature individually optimized kits, tailored to pathogen and tissue/host.

12. Discussion

The future is bright for disease diagnosis and pathogen detection. The molecular biology revolution of the past quarter century has matured through the experimental, labour driven phase to become a major industrial force where quality, reliability and automation are more often the expected standard. With increased options in molecular disease diagnosis come increased expectations and this is where the next phase of development lies. Disease consists of a pathological interaction between a pathogenic agent (biological, chemical or physical) and the host, where the hosts' adverse reaction to the presence or proliferation of a pathogen causes a breakdown in the normal physiological state and, in the case of biological pathogens, often ensures or enhances the further proliferation and spread of the pathogen. The simpler component of disease diagnosis is the pathogen, and its detection. Future developments however, will increasingly focus on the host component of disease and the interplay between pathogen and host, since this is where disease prevention, treatment and cures are located. This means that future technological direction in disease diagnosis will emphasise multiplexing, miniaturisation (Fiorini and Chui, 2005) and automation (Service, 2006).

In the introduction to this chapter sensitivity, accuracy, reliability, universality, simplicity, speed and cost were identified as the principal criteria for an ideal diagnostic system. Most modern detection technologies are now sensitive enough to detect down to a single target molecule. This means that any future development will increasingly focus on quantitative detection (depending on the diagnostic requirements), with a concomitant change to a more integrated, quantitative disease management style. Accuracy of detection at the molecular level (and virus detection is largely molecular) depends essentially on the nature of the primary molecular

recognition event, *i.e.* the interaction between target and probe. In this regard, nucleic acid-based detection has a considerable advantage over protein-based detection, since the kinetics of nucleic acid hybridisation is much more predictable and reliable than that of protein interactions. This also makes nucleic acid-based detection much more adaptable to changing requirements due to the discovery or emergence of new virus variants. The principal remaining area of concern for virus detection remains therefore reliability, *i.e.* reducing incidence of misdiagnosis. The worst scenario for diagnosticians is lack of trust in diagnostic system, and this can be equated with false positive and negative results. This can be due to technological failure - which can be controlled, or pathogen evolution - which cannot be controlled, but can be monitored. A primary source of potential technological failure involves enzymatic processes, whether this is failure of reporter enzymes attached to probes or failure of target/probe amplification enzymes, due to decay, sensitivity to inhibitors (reverse transcriptases) or the 3' probe-target mismatch issue (PCR). Since all nucleic acid-based detection depends on the hybridisation of probe to target, whether or not this is followed by amplification of target, probe or signal, future developments will increasingly feature label-free or direct detection, to avoid enzymatic processes (Liepold *et al.,* 2005), as long as sensitivity, accuracy and quantitation are not compromised. Misdiagnosis due to pathogen evolution is best countered by targeting multiple regions of the viral genome, *i.e.* multiplexing (Genersch, 2005; Grabensteiner *et al.,* 2001; Bakonyi *et al.,* 2002; Genersch, 2005), which will also monitor any evolutionary drifts or the emergence of new strains and with it the need to characterise and adapt to these changes. The best technological option for multiplexing involves some form of ordered array of (oligonucleotide) probes, with various detection options, with different sensitivities, quantitation and ability to manage variability in the target. Advances in oligonucleotide design (Rouillard *et al.,* 2003) and data analysis software should improve the accuracy of quantitation in complex, interactive, multiplexed (oligonucleotide) micro- and macroarrays. The finally speed, simplicity and cost. Military and biosecurity concerns requiring rapid and local response drive technological development away from specialized laboratories towards field applications and simplified protocols (Schaad *et al.,* 2003; Higgins *et al.,* 2003), and these developments will spill over into the commercial marketplace as well. Developments in genomic, transcriptomic, proteonomic and metabolic fields will demand extensively multiplexed and quantitated analyses of biological systems, and diagnostics will follow this wave. The continuous development of new or improved technologies with wide applicability in a highly competitive market place

will ultimately drive the costs down to where disease surveillance and routine monitoring becomes cost-effective (Service, 2006), even in low priority areas like honey bee pathology.

13. Summary

This chapter analyses in detail the relative merits of the various tools and technologies currently available for the detection of viruses, as well as those that may become important in the future, and relates this to present and future requirements for disease diagnosis through a set of defined criteria. Included are both traditional disease diagnosis methods (symptomatology, microscopy and bioassays) and molecular detection methods, divided into those targeting proteins and those targeting nucleic acids. Distinction is furthermore made between the importance of different techniques in the discovery and characterisation of pathological agents, versus their potential for reliable routine detection in the context of disease diagnosis and management. Although many examples are drawn from areas outside honey bee (virus) research, the field in general is well positioned to take advantage of these developments due to the priviledged status of the honey bee as a model organism for insect and social biology, as well as its continued agricultural and ecological importance which drives the research of its diseases and pathologies.

14. Acknowledgements

Figure 1 is by Brenda V. Ball. Figure 13 was produced by Jorge Madriz in the laboratory of Dr Ana M. Espinoza at the University of Costa Rica in San Jose, Costa Rica. Figures 17 and 18 were produced by JRdM in the laboratory of Dr Marilyn J. Roossinck at the S.R. Noble Foundation in Ardmore, Oklahoma. All figures are used with permission.

References

Achenbach J.E., Topliff, C.L., Vassilev, V.B., Donis, R.O., Eskridge, K.M., Kelling, C.L. (2004) Detection and quantitation of bovine respiratory syncytical virus using real-time quantitative RT-PCR and quantitative competitive RT-PCR assays. J. Virol. Meth. 121, 1-6

Adelson, M.E., Feola, M., Trama, J., Tilton, R.C., Mordechai, E. (2005) Simultaneous detection of herpes simplex virus types 1 and 2 by real-time PCR and Pyrosequencing. J. Clin. Virol. 33, 25-34

Ahmadian, A., Lundeberg, J. (2002) A Brief History of Genetic Variation Analysis. BioTechniques 32, 1122-1124; 1126; 1128 passim

Akhavan-Tafti, H., Reddy, L.V., Siripurapu, S., Schoenfelner, B.A., Handley, R.S., Schaap, A.P.. (1998) Chemiluminescent detection of DNA in low- and medium density arrays. Clin. Chem. 44, 2065-2066

Akhavan-Tafti, H., Schaap, A.P., Arghavani, Z., deSilva, R., Eickholt, R.A., Handley, R.S., Schoenfelner, B.A., Sugioka, K., Sugioka, Y. (1994) CCD camera imaging for the chemiluminescent detection of enzymes using new ultrasensitive reagents. Biolumin. Chemilumin. 9, 155-164

Aldous, W.K., Pounder, J.I., Cloud, J.L., Woods, G.L. (2005) Comparison of six methods of extracting *Mycobacterium tuberculosis* DNA from processed sputum for testing by quantitative real-time PCR. J. Clin. Microbiol. 43, 2471-2473

Allen, M.F., Ball., B.V. (1995) Characterisation and serological relationships of strains of Kashmir bee virus. Annals Appl. Biol. 126, 471-484

Allen, M.F., Ball., B.V. (1996) The incidence and world distribution of honeybee viruses. Bee World 77, 141-162

Allen M.F., Ball B.V., White R.F., Antoniw J.F. (1986) The detection of acute paralysis virus in *Varroa jacobsoni* by the use of a simple indirect ELISA. J. Apic. Res. 25, 100-105

Al-Mahrouki, A.A., Krylov, S.N. (2005) Calibration-free quantitative analysis of mRNA. Anal. Chem. 77, 8027-8030

Alvarez, M., Carrascosa, L.G., Moreno, M., Calle, A., Zaballos, A., Lechuga, L.M., Martinez-A, C., Tamayo, J. (2004) Nanomechanics of the formation of DNA self-assembled monolayers and hybridization on microcantilevers. Langmuir 20, 9663-9668

Alvarez-Muñoz, M.T., Zaragoza-Rodriguez, S., Rojas-Montes, O., Palacios-Saucedo, G., Vazquez-Rosales, G., Gomez-Delgado, A., Torres, J., Muñoz, O. (2005) High correlation of human immunodeficiency virus type-1 viral load measured in dried-blood spot samples and in plasma under different storage conditions. Arch. Med. Res. 36, 382-386

Anderson, D.L. (1984) Comparison of serological techniques for detecting and identifying honeybee viruses. J. Invert. Path. 44, 233-243

Anderson D.L. (1985) Viruses of New Zealand honey bees. New Zealand Beekeeper. 188, 8-10

Anderson D.L. (1991) Kashmir bee virus – a relatively harmless virus of honey bee colonies. Am. Bee J. 131, 767-770

Anderson, D.L., Gibbs, A.J. (1988) Inapparent virus infections and their interactions in pupae of the honey bee (*Apis mellifera* Linnaeus) in Australia. J. Gen. Virol. 69, 1617-1625

Anderson D.L., Gibbs A.J. (1989) Transpuparial transmission of Kashmir bee virus and sacbrood virus in the honey bee (*Apis mellifera*). Ann. Appl. Biol. 114, 1-7

Antúnez, K., D'Alessandro, B., Corbella, E., Ramallo, G., Zunino, P. (2006) Honeybee viruses in Uruguay. J. Invert. Path. 91, 67-70

Antúnez, K., D'Alessandro, B., Corbella, E., Zunino, P. (2005) Detection of chronic bee paralysis virus and acute bee paralysis virus in Uruguayan honeybees. J. Invert. Path. 90, 69-72

Apfalter, P., Assadian, O., Blasi, F., Boman, J., Gaydos, C.A., Kundi, M., Makristathis, A., Nehr, M., Rotter, M.L., Hirschl, A.M. (2002) Reliability of nested PCR for detection of *Chlamydia pneumoniae* DNA in atheromas: results from a multicenter study applying standardized protocols. J. Clin. Microbiol. 40, 4428-4434

Archer, M., Christophersen, M., Fauchet, P.M. (2004) Macroporous silicon electrical sensor for DNA hybridization detection. Biomed. Microdevices. 6, 203-211

Arens, M. (1999) Methods for Subtyping and Molecular Comparison of Human Viral Genomes. Clin. Microbiol. Rev. 12, 612–626

Arens, M.Q., Mundy, L.M., Amsterdam, D., Barrett, J.T., Bigg, D., Bruckner, D., Hanna, B., Prince, H., Purington, T., Hanna, T., Hewitt, R., Kalinka, C., Koppes, T., Maxwell, S., Moe, A., Doymaz, M., Poulter, M., Saber-Tehrani, M., Simard, L., Wilkins-Carmody, D., Vidaver, J., Berger, C., Davis, A.H., Alzona, M.T. (2005) Preclinical and clinical performance of the Efoora test, a rapid test for detection of human immunodeficiency virus-specific antibodies. J. Clin. Microbiol. 43, 2399-2406

Arnold, L.J., Hammond, P.W., Wiese, W.A., Nelson, N.C. (1989) Assay formats involving acridinium-ester-labelled DNA probes. Clin. Chem. 35, 1588-1594

Ayele, W., Pollakis, G., Abebe, A., Fisseha, B., Tegbaru, B., Tesfaye, G., Mengitsu, Y., Wolday, D., van Gemen, B., Goudsmit, J., Dorigo-Zetsma, W., de Baar, M.P. (2004) Development of a nucleic acid sequence-based amplification assay that uses *gag*-based molecular beacons to distinguish between human immunodeficiency virus type 1 subtype C and C' infections in Ethiopia. J. Clin. Microbiol. 42, 1534-1541

Baeumner, A.J., Humiston, M.C., Montagna, R.A., Durst., R.A. (2001) Detection of viable oocysts of *Cryptosporidium parvum* following nucleic acid sequence based amplification. Anal. Chem. 73, 1176-1180

Baeumner, A.J., Schlesinger, N.A., Slutzki, N.S., Romano, J., Lee, E.M., Montagna, R.A. (2002) Biosensor for dengue virus detection: sensitive, rapid, and serotype specific. Anal. Chem. 74, 1442-1448

Bailey L. (1965) Paralysis of the honey bee, *Apis mellifera* Linnaeus. J. Invertebr. Pathol. 7, 132-140

Bailey, L. (1967) Acute bee paralysis virus in adult honey bees injected with sacbrood virus. Virology 33, 368

Bailey, L. (1976) Viruses attacking the honey bee. Adv. Vir. Res. 20, 271-304

Bailey, L. (1968). The multiplication of sacbrood virus in the adult honeybee. Virology 36, 312-3.

Bailey, L., Ball, B.V. (1978) *Apis* iridescent virus and clustering disease of *Apis cerana*. J. Invert. Path. 31, 368-371

Bailey, L., Ball, B.V. (1991) Honey bee pathology, 2nd ed. Academic press, London.

Bailey, L., Ball, B.V., Woods, R.D. (1976) An iridovirus from bees. J. Gen. Virol. 31, 459-461

Bailey, L., Ball, B.V., Carpenter, J.M., Woods, R.D. (1979) Small virus-like particles in honey bees associated with chronic paralysis virus and a previously undescribed disease. J. Gen. Virol. 46, 149-155

Bailey, L., Ball, B.V., Perry, J.N. (1981) The prevalence of viruses of honey bees in Britain. Annals Appl.Biol. 97, 109-118

Bailey, L., Ball, B.V., Perry, J.N. (1983a) Association of viruses with two protozoan pathogens of the honey bee. Ann. Appl.Biol. 103, 13-20

Bailey, L., Ball, B.V., Perry, J.N. (1983b) Honey bee paralysis: its natural spread and its diminished incidence in England and Wales. J. Apicult. Res. 22, 191-195

Bailey L., Carpenter J.M., Woods R.D. (1979) Egypt bee virus and Australian isolates of Kashmir bee virus. J. Gen. Virol. 43, 641-647

Bailey, L., Gibbs, A.J. (1964) Acute infection of bees with paralysis virus. J. Insect Pathol. 6, 395-407

Bailey, L., Gibbs, A.J., Woods, R.D. (1963). Two viruses from adult honey bees (*Apis mellifera* Linnaeus). Virol. 21, 390-395

Bailey, L., Milne, R.G. (1969) The multiplication regions and interactions of acute and chronic bee paralysis viruses in adult honey bees. J. Gen. Virol. 4, 9-14

Bailey, J.M., Nikfarjam, F., Shenoy, N., Shively, J.E. (1992) Automated carboxy-terminal sequence analysis of peptides and proteins using diphenyl phosphoroisothiocyanatidate. Prot. Sci.1, 1622-1633

Bailey L., Woods, R.D. (1977) Two more small RNA viruses from honey bees and further observations on sacbrood and acute bee-paralysis viruses. J. Gen. Virol. 37, 175-182

Bakonyi, T., Farkas, R., Szendroi, A., Dobos-Kovacs, M., Rusvai, M. (2002a) Detection of acute bee paralysis virus by RT-PCR in honey bee and *Varroa destructor* field samples: rapid screening of representative Hungarian apiaries. Apidologie 33, 63-74

Bakonyi, T., Grabensteiner, E., Kolodziejek, J., Rusvai, M., Topolska, G., Ritter, W., Nowotny, N. (2002b) Phylogenetic analysis of acute bee paralysis virus strains. Appl. Environ. Microbiol. 68, 6446-6450

Ball, B.V. (1985) Acute paralysis virus isolates from honey bee colonies infested with *Varroa jacobsoni*. J. Apicult. Res. 24, 115-119

Ball, B.V. (1987) The incidence of acute paralysis virus in adult honey bee and mite populations. Pcelar 3, 68-70

Ball B.V., Allen M.F. (1988) The prevalence of pathogens in honey bee (*Apis mellifera*) colonies infested with the parasitic mite *Varroa jacobsoni*. Ann. Appl. Biol. 113, 237-244

Ball, B.V., Bailey, L.(1997) Viruses, in: Morse R.A. and Flottum K. (Eds.), *Honey bee pests, predators, & diseases* 3rd Ed, A.I. Root, Medina, Ohio, pp.11-32

Becker, S., Franco, J.R., Simarro, P.P., Stich, A., Abel, P.M., Steverding, D. (2004) Real-time PCR for detection of *Trypanosoma brucei* in human blood samples. Diagn. Microbiol. Infect. Dis. 50, 193-199

Bender, F., Lange, K., Voigt, A., Rapp, M. (2004) Improvement of surface acoustic wave gas and biosensor response characteristics using a capacitive coupling technique. Anal. Chem. 76, 3837-3840

Benjeddou, M., Leat, N., Allsopp, M., Davison, S. (2001) Detection of acute bee paralysis virus and black queen cell virus from honeybees by reverse transcriptase PCR. Appl. Environ. Microbiol. 67, 2384-2387

Berényi, O., Bakonyi, T., Derakhshifar, I., Kögelberger, H., Nowotny, N. (2006) Occurence of six honeybee viruses in diseased Austrian apiaries. Appl. Environ. Microbiol. 72, 2414-2420

Berg, E.S., Anestad, G., Moi, H., Storvold, G., Skaug, K. (1997) False-negative results of a ligase chain reaction assay to detect *Chlamydia trachomatis* due to inhibitors in urine. Eur. J. Clin. Microbiol. Infect. Dis. 16, 727-731

Berg, E.S., Storvold, G., Anestad, G., Moi, H., Skaug, K. (2005) Reliability of the Amplicor internal control to disclose false-negative *Chlamydia trachomatis* PCR results. J. Microbiol. Methods. 60, 125-129

Birch, L., Dawson, C.E., Cornett, J.H. and Keer, J.T. (2001) A comparison of nucleic acid amplification techniques for the assessment of bacterial viability. Lett. Appl. Microbiol. 33, 296-301

Birch, L., English, C.A., Burns, M., Keer, J.T. (2004) Generic scheme for independent performance assessment in the molecular biology laboratory. Clin. Chem. 50, 1553-1559

Birkenmeyer, L., Armstrong, A.S. (1992) Preliminary evaluation of the ligase chain reaction for the specific detection of *Neisseria ghonorrhoeae*. J. Clin. Microbiol. 30, 3089-3094

Boltovets, P.M., Snopok, B.A., Boyko, V.R., Shevchenko, T.P., Dyachenko, N.S., Shirshov, Y.M. (2004) Detection of plant viruses using a surface plasmon resonance via complexing with specific antibodies. J. Virol. Meth. 121,101-106

Borst, A., Leverstein-van Hall, M.A., Verhoef, J., Fluit, A.C. (2001) Detection of *Candida* spp. in blood cultures using nucleic acid sequence-based amplification (NASBA). Diagn. Microbiol. Inf. Dis. 39, 155-160

Bowen-Walker, P.L., Martin, S.J., Gunn, A. (1999) The transmission of Deformed Wing Virus between honeybees (*Apis mellifera* L.) by the ectoparasitic mite *Varroa jacobsoni* Oud. J. Invertebr. Pathol. 73, 101-106

Brcak, J., Kralik, O. (1965) On the structure of the virus causing sacbrood of the honey bee. J. Invert. Pathol. 20, 110-111

Buimer, M., van Doornum, G.J., Ching, S., Peerbooms, P.G., Plier, P.K., Ram, D., Lee, H.H. (1996) Detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* by ligase chain reaction-based assays with clinical specimens from various sites: implications for diagnostic testing and screening. J. Clin. Microbiol. 34, 2395-2400

Burgener M, Candrian U, Gilgen M. (2003) Comparative evaluation of four large-volume RNA extraction kits in the isolation of viral RNA from water samples. J. Virol. Meth. 108, 165-70

Bustin, S.A. (2000) Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J. Mol. Endocrin. 25, 169-193

Bustin, S.A., Nolan, T. (2004) Pitfalls of quantitative real-time reverse-transcriptionm polymerase chain reaction. J. Biomol. Techn. 15, 155-166

Butchko, A.R., Jordan, J.A. (2004) Comparison of three commercially available serological assays used to detect human parvovirus B19-specific immunoglobulin M (IgM) and IgG antibodies in sera of pregnant women. J. Clin. Microbiol. 42, 3191-3195

Caetano-Anolles, G. (1998) DAF optimization using Taguchi methods and the effect of thermal cycling parameters on DNA amplification. Biotechniques 25, 472-6, 478-80 passim

Candotti, D., Temple, J., Owusu-Ofori, S., Allain, J.-P. (2004) Multiplex real-time quantitative RT-PCR assay for hepatitis B virus, hepatitis C virus, and human immunodeficiency virus type 1. J. Virol. Meth. 118, 39-47

Cantafora, A., Blotta, I., Pino, E., Pisciotta, L., Calandra, S., Bertolini, S. (2004) Quantitative polymerase chain reaction and microchip electrophoresis to detect major rearrangements of the low-density lipoprotein receptor gene causing familial hypercholesterolemia. Electrophoresis 25, 3882-3889

Caruso, F., Rodda, E., Furlong, D., Niikura, K., Okahata, Y. (1997) Quartz crystal microbalance study of DNA immobilization and hybridization for nucleic acid sensor development. Anal. Chem. 69, 2043-2049

Cattoli, G., Drago, A., Maniero, S., Toffan, A., Bertoli, E., Fassina, S., Terregino, C., Robbi, C., Vicenzoni, G., Capua, I. (2004) Comparison of three rapid detection systems for type A influenza virus on tracheal swabs of experimentally and naturally infected birds. Avian Pathol. 33, 432-437

Cazacu, A.C., Chung, S.E., Greer, J., Demmler, G.J. (2004a) Comparison of the Directigen Flu A+B membrane enzyme immunoassay with viral culture for the rapid detection of influenza A and B virus in respiratory tissues. J. Clin. Microbiol. 42, 3707-3710

Cazacu, A.C., Demmler, G.J., Neuman, M.A., Forbes, B.A., Chung, S., Greer, J., Alvarez, A.E., Williams, R., Bartholoma, N.Y. (2004b) Comparison of a new lateral-flow chromatographic membrane immunoassay to viral culture for rapid detection and differentiation of influenza A and B viruses in respiratory specimens. J. Clin. Microbiol. 42, 3661-3664

Chaisomchit, S., Wichajarn, R., Janejai, N., Chareonsiriwatana, W. (2005) Stability of genomic DNA in dried blood spots stored on filter paper. Southeast Asian J. Trop. Med. Public Health 36, 270-273

Chamoles, N.A., Niizawa, G., Blanco, M., Gaggioli, D., Casentini, C. (2004) Glycogen storage disease type II: enzymatic screening in dried blood spots on filter paper. Clin. Chim. Acta 347, 97-102

Chan, K.H., Maldeis, N., Pope, W., Yup, A., Ozinskas, A., Gill, J., Setto, W.H., Shortridge, K.F., Peiris, J.S.M. (2002) Evaluation of the Directigen Flu A+B test for rapid diagnosis of influenza virus type A and B infections. J. Clin. Microbiol. 40, 1675-1680

Chantawannakul, P., Ward, L., Boonham, N., Brown, M. (2006) A scientific note on the detection of honeybee viruses using real-time PCR (TaqMan) in varroa mites collected from a Thai honeybee (*Apis mellifera*) apiary. J. Invert. Pathol. 91, 69-73

Cheadle, C., Vawter, M.P., Freed, W.J., Becker, K.G. (2003) Analysis of microarray data using Z score transformation. J. Mol.Diagn. 5, 73-81

Chen, Y.P., Evans, J., Feldlaufer, M. (2006a) Horizontal and vertical transmission of viruses in the honeybee (*Apis mellifera*). J. Invert. Pathol. 92, 152-159

Chen, Y.P., Higgins, J.A., Feldlaufer, M.F. (2005a) Quantitative real-time reverse transcription-PCR analysis of Deformed Wing Virus infection in the honeybee (*Apis mellifera* L.). Appl. Environ. Microbiol. 71, 436-441

Chen, Y.P., Pettis, J.S., Collins, A. Feldlaufer, M.F. (2006b) Prevalence and transmission of honeybee viruses. Appl. Environ. Microbiol. 72, 606-611

Chen, Y.P., Pettis, J.S., Feldlaufer, M.F. (2005b) Detection of multiple viruses in queens of the honey bee (*Apis mellifera* L). J. Invert. Pathol. 90, 118-121

Chen, Y.P., Pettis, J.S., Evans, J.D., Kramer, M., Feldlaufer, M.F. (2004a)

Chen, Y.P., Smith, I.B., Collins, A.M., Pettis, J.S., Feldlaufer, M.F. (2004b) Detection of deformed wing virus infection in honey bees, *Apis mellifera* L., in the United States. Am. Bee J. 144, 557-559

Chen, Y.P., Zhao, Y., Hammond, J., Hsu, H.T., Evans, J., Feldlaufer, M.F. (2004c) Multiple virus infections in the honey bee and genome divergence of honey bee viruses. J. Invert. Pathol. 87, 84-93

Chernesky, M., Jang, D., Chong, S., Sellors, J., Mahony, J. (2003) Impact of urine collection order on the ability of assays to identify *Chlamydia trachomatis* infections in men. Sex. Transm. Dis. 30, 345-347

Chernesky, M., Smieja, M., Schachter, J., Summersgill, J., Schindler, L., Solomon, N., Campbell, K., Campbell, L., Cappuccio, A., Gaydos, C., Chong, S., Moncada, J., Phillips, J., Jang, D., Wood, B.J., Petrich, A., Hammerschlag, M., Cerney, M., Mahony, J. (2002) Comparison of an industry-derived LCx *Chlamydia pneumoniae* PCR research kit to in-house assays performed in five laboratories. J. Clin. Microbiol. 40, 2357-2362

Chiles, R.E., Green, E.N., Fang, Y., Goddard, L., Roth, A., Reisen, W.K., Scott, T.W. (2004) Blinded laboratory comparison of the *in situ* enzyme immunoassay, the VecTest wicking assay, and a reverse transcription-polymerase chain reaction assay to detect mosquitoes infected with West Nile and St. Louis encephalitis viruses. J. Med. Entomol. 41, 539-544

Chui, L., Drebot, M., Andonov, A., Petrich, A., Glushek, M., Mahony, J. (2005) Comparison of 9 different PCR primers for the rapid detection of severe acute respiratory syndrome coronavirus using 2 RNA extraction methods. J. Diagn. Microbiol. Infect. Dis. 53, 47-55

Clark, T.B. (1978). A filamentous virus of the honey bee. J. Invertebr. Pathol. 32, 332-340

Compton, J. (1991) Nucleic acid sequence-based amplification. Nature 350, 91-92

Corona, M., Hughes, K.A., Weaver, D.B., Robinson, G.E. (2005) Gene expression patterns associated with queen honey bee longevity. Mech. Ageing Dev. 126, 1230-1238

Crozier, R.H., Crozier, Y.C. (1993) The mitochondrial genome of the honeybee Apis mellifera: complete sequence and genome organization. Genetics 133, 97-117

Cui, Y, Wei, Q., Park, H., Lieber, C.M. (2001) Nanowire nanosensors for highly sensitive and selective detection of biological and chemical species. Science 293, 1289-1292

Dall, D.J. (1987) Multiplication of Kashmir bee virus in the pupae of the honeybee *Apis mellifera*. J. Invert. Path. 49, 279-290

van Dam, G.J., Wichers, J.H., Ferreira, T.M., Ghati, D., van Amerongen, A., Deelder, A.M. (2004) Diagnosis of schistosomiasis by reagent strip test for detection of circulating cathodic antigen. J. Clin. Microbiol. 42, 5458-5461

Daniel, H.D.J., Abraham, P., Raghuraman, S., Vivekanandan, P., Subramanian, T., Sridharan, G. (2005) Evaluation of a rapid assay as an alternative to conventional enzyme immuno assays for the detection of hepatitis C virus-specific antibodies. J. Clin. Microbiol. 43, 1977-1978

Dean, F.B., Hosono, S., Fang, L., Wu, X., Faruqi, A.F., Bray-Ward, P., Sun, Z., Zong, Q., Du, Y., Du, J., Driscoll, M., Song, W., Kingsmore, S.F., Egholm, M., Laksen, R.S. (2002) Comprehensive human genome amplification using multiple strand displacement amplification. Proc. Nat. Acad. Sci. USA 99, 5261-5266

Deiman, B., van Aarle, P., Sillekens, P. (2002) Characteristics and applications of nucleic acid sequence-based amplification (NASBA). Mol. Biotechnol. 20, 163-79

Delmulle, B.S., De Saeger, S.M., Sibanda, L., Barna-Vetro, I., Van Peteghem, C.H. (2005) Development of an immunoassay-based lateral flow dipstick for the rapid detection of aflatoxin B1 in pig feed. J. Agric. Food Chem. 53, 3364-3368

Denholm, C.H. (1999) Inducible honey bee viruses associated with *Varroa jacobsoni*. PhD thesis, Keele University, Staffordshire, England

Diaz-Mitoma, F., Leger, C., Miller, H., Giulivi, A., Frost, R., Shaw, L., Huebsch, L. (2003) Comparison of DNA amplification, mRNA amplification and DNA hybridisation techniques for detection of cytomegalovirus in bone marrow transplant recipients. J. Clin. Microbiol. 41, 5159-5166

Du, Z. L., Zhang, Z. B. (1985) Ultrastructural change in the hypopharyngeal glands of worker honey bees (*Apis cerana*) infected with sacbrood virus. Zool. Res. 6, 155-162

Eggeling, C., Kask, P., Winkler, D., Jäger, S. (2005) Rapid Analysis of Förster Resonance Energy Transfer by Two-Color Global Fluorescence Correlation Spectroscopy: Trypsin Proteinase Reaction. Biophys. J. 89, 605-618

Eiros, J.M., Labayru, C., Hernandez, B., Ortiz-de-Lejarazu, R., Rodriguez-Torres, A. (2002) Comparative study of two methods for RNA extraction prior to detection of resistance to human immunodeficiency virus type 1 with the line probe assay. J. Clin. Microbiol. 40, 2257-2259

Elvidge, G.P., Price, T.S., Glenny, L., Ragoussis, J. (2005) Development and evaluation of a real competitive PCR for high-throughput quantitative applications. Anal. Biochem. 339, 231-241

Erali, M., Schmidt, B., Lyon, E., Wittwer, C. (2003) Evaluation of electronic microarrays for genotyping factor-V, factor-II and MTHFR. Clin. Chem. 49, 732-739

Erdman, D.D., Weinberg, G.A., Edwards, K.M., Walker, F.J., Anderson, B.C., Winter, J., González, M., Anderson, L.J. (2003) GeneScan reverse transcription-PCR assay for detection of six common respiratory viruses in young children hospitalized with acute respiratory illness. J. Clin. Microbiol. 41, 4298-4303

Estrela, P., Migliorato, P., Takiguchi, H., Fukushima, H., Nebashi, S. (2005) Electrical detection of biomolecular interactions with metal-insulator-semiconductor diodes. Biosens. Bioelectron. 20, 1580-1586

Evans, J.D. (2001) Genetic evidence for co-infection of honeybees by acute bee paralysis and Kashmir bee viruses. J. Invertebr. Pathol. 78, 189-193

Evans, J.D. (2004) Transcriptional immune responses by honey bee larvae during invasion by the bacterial pathogen *Paenibacillus larvae*. J. Invertebr. Pathol. 85, 105-111

Evans, J.D. (2006) Beepath: an ordered quantitative-PCR array for exploring honey bee immunity and disease. J. Invertebr. Pathol. 93, 135-139

Evans, J.D., Gundersen-Rindal, D. (2003) Beenomes to *Bombyx*: future directions in applied insect genomics. Genome Biol. 4, 107

Evans, J.D., Hung, A.C.F. (2000) Molecular phylogenetics and classification of honey bee viruses. Arch. Virol. 145, 2015-2026

Evans, J.D., Lopez, D.L. (2002) Complete mitochondrial DNA sequence of the important honey bee pest, *Varroa destructor* (Acari: Varroidae). Exp. Appl. Acarol. 27, 69-78

Evans, J.D., Wheeler, D.E. (2000) Expression profiles during honeybee caste determination. Genome Biol. 2, research0001.1-0001.6

Evans, J.D., Wheeler, D.E. (2001) Gene expression and the evolution of insect polyphenisms. Bioessays 23, 62-68

Fagerstam, L.G., Frostell, A., Karlsson, R., Kullman, M., Larsson, A., Malmqvist, M., Butt, H. (1990) Detection of antigen-antibody interactions by surface plasmon resonance. Application to epitope mapping. J. Mol. Recognit. 3, 208-214

Fawcett, N., Evans, J., Lien, L., Flowers, N. (1988) Nucleic acid hybridization detected by piezoelectric resonance. Anal. Lett. 21, 1099-1114

Fernandez-Sanchez, C., McNeil, C.J., Rawson, K., Nilsson, O. (2004) Disposable noncompetitive immunosensor for free and total prostate-specific antigen based on capacitance measurement. Anal. Chem. 76, 5649-5656

Fernandez-Sanchez, C., McNeil, C.J., Rawson, K., Nilsson, O., Leung, H.Y., Gnanapragasam, V. (2005) One-step immunostrip test for the simultaneous detection of free and total prostate specific antigen in serum. J. Immunol. Meth. 307, 1-12

Ferreccio, C., Bratti, M.C., Sherman, M.E., Herrero, R., Wacholder, S., Hildesheim, A., Burk, R.D., Hutchinson, M., Alfaro, M., Greenberg, M., Morales, J., Rodriguez, A.C., Schussler, J., Eklund, C., Marshall, G., Schiffman, M. (2003) A Comparison of single and combined visual, cytologic, and virologic tests as screening strategies in a region of high risk of cervical cancer. Cancer Epidemiology, Biomarkers & Prevention 12, 815-823

Fiandaca, M.J., Hyldig-Nielsen, J.J., Gildea, B.D., Coull, J.M. (2001) Self-reporting PNA/DNA primers for PCR analysis. Genome Res. 11, 609-613

Fievet, J., Tentcheva, D., Gauthier, L., de Miranda, J.R., Cousserans, F., Colin, M.E. Bergoin, M. (2006) Localization of deformed wing virus infection in queen and drone *Apis mellifera* L. Virol. J. 3, 16

Fini, F., Gallinella, G., Girotti, S., Zerbini, M., Musiani, M. (1999) Development of a chemiluminescence competitive PCR for the detection and quantification of parvovirus B19 DNA using a microplate luminometer. Clin. Chem. 45, 1391-1396

Fiorini, G.S., Chiu, D.T. (2005) Disposable microfluidic devices: fabrication, function, and application. Biotechniques 38, 429-446

Fischer, A., Lejczak, C., Lambert, C., Servais, J., Makombe, N., Rusine, J., Staub, T., Hemmer, R., Schneider, F., Schmit, J.C., Arendt, V. (2004) Simple DNA extraction method for dried blood spots and comparison of two PCR assays for diagnosis of vertical human immunodeficiency virus type 1 transmission in Rwanda. J. Clin. Microbiol. 42, 16-20

Fixe, F., Chu, V., Prazeres, D.M., Conde, J.P. (2004) An on-chip thin film photodetector for the quantification of DNA probes and targets in microarrays. Nucl. Acids Res. 32(9), e70. doi: 10.1093/nar/gnh066

Fries, I. (1997) *Protozoa*, in: Morse R.A. and Flottum K. (Eds.), *Honey bee pests, predators, & diseases* 3rd Ed, A.I. Root, Medina, Ohio, pp.57-76

Fritz J., Cooper E.B., Gaudet S., Sorger P.K., Manalis, S.R. (2002) Electronic detection of nucleic acids by its intrinsic molecular charge. Proc. Nat. Acad. Sci. USA 99, 14142-14146

Fujiyuki, T., Takeuchi, H., Ono, M., Ohka, S., Sasaki, T., Nomoto, A., Kubo, T. (2004) Novel insect picorna-like virus identified in the brain of aggressive worker honeybees. J. Virol. 48, 1093-1100

Furgala, B., Lee, P. E. (1966) Acute bee paralysis virus, a cytoplasmic insect virus. Virol. 29, 346-348

Gabl, R., Feucht, H.D., Zeininger, H., Eckstein, G., Schreiter, M., Primig, R., Pitzer, D., Wersing, W. (2004) First results on label-free detection of DNA and protein molecules using a novel integrated sensor technology based on gravimetric detection principles. Biosens. Bioelectron. 19, 615-620

Le Gal-Reculé, G., Zwingelstein, F., Portejoie, Y., le Gall, G. (2001) Immunocapture-RT-PCR assay for detection and molecular epidemiological studies of rabbit haemorrhagic disease and European brown hare syndrome viruses. J. Virol. Meth. 97, 49-57

Garber, E.A., Eppley, R.M., Stack, M.E., McLaughlin, M.A., Park, D.L. (2005) Feasibility of immunodiagnostic devices for the detection of ricin, amanitin, and T-2 toxin in food. J. Food Prot. 68, 1294-1301

García, E.P., Dowding, L.A., Stanton, L.W., Slepnev, V.I. (2005) Scalable transcriptional analysis routine – multiplexed quantitative real-time polymerase chain reaction platform for gene expression analysis and molecular diagnostics. J. Mol. Diagn. 7, 444-454

García-Cañas, V., Cifuentes, A., González, R. (2004) Quantitation of transgenic Bt Event-176 maize using double quantitative competitive polymerase chain reaction and capillary gel electrophoresis laser-induced fluorescence. Anal. Chem. 76, 2306-2313

Gardner, S.N., Kuczmarski, T.A., Vitalis, E.A., Slezak, T.R. (2003) Limitations of TaqMan PCR for detecting divergent viral pathogens illustrated by hepatitis A, B, C and E viruses and human immunodeficiency virus. J. Clin. Microbiol. 41, 2417-2427

Gasser, B., Mattanovich, D. (2006) Antibody production with yeasts and filamentous fungi: on the road to large scale? Biotech. Lett. (in press)

van Gelder, R.N., von Zastrow, M.E., Yool, A., Dement, W.C., Barchas, J.D., Eberwine, J.H. (1990) Amplified RNA synthesized from limited quantities of heterogenous cDNA. Proc. Nat. Acad. Sci. USA 87, 1663-1667

Genersch, E. (2005) Development of a rapid and sensitive RT-PCR method for the detection of deformed wing virus, a pathogen of the honeybee (*Apis mellifera*). Vet. J. 169, 121-123

Gentry, T.J., Wickham, G.S., Schadt, C.W., He, Z., Zhou, J. (2006) Microarray applications in microbial ecology research. Microb. Ecol. 52, 159-175

Gharizadeh, B., Käller, M., Nyrén, P., Andersson, A., Uhlén, M., Lundeberg, J., Ahmadian, A. (2003) Viral and microbial genotyping by a combination of multiplex competitive hybridization and specific extension followed by hybridization to generic tag arrays. Nucl. Acids Res. 31(22), e146. doi: 10.1093/nar/gng147

Gharizadeh, B., Oggionni, M., Zheng, B., Akom, E., Pourmand, N., Ahmadian, A., Wallin, K.L., Nyrén, P. (2005) Type-specific multiple sequencing primers: a novel strategy for reliable and rapid genotyping of human papillomaviruses by pyrosequencing technology. J. Mol. Diagn. 7, 198-205

Giglio, S., Monis, P.T., Saint, C.P. (2003) Demonstration of preferential binding of SYBR Green I to specific DNA fragments in real-time multiplex PCR. Nucl.Acids Res. 31, e136

Ghosh, R.C., Ball, B.V., Willcocks, M.M., Carter, M.J. (1999) The nucleotide sequence of sacbrood virus of honey bee: an insect picorna-like virus. J. Gen. Virol. 80, 1541-1549

Gómez-Llorente, C., Antúnez, A., Blanco, S., Suarez, A., Gómez-Capilla, J.A., Farez-Vidal, M.E. (2004) Multiplex analysis of the most common mutations related to hereditary haemochromatosis: two methods combining specific amplification with capillary electrophoresis. Eur. J. Haematol. 72, 121-129

Govan, V.A., Leat, N., Allsopp, M., Davison, S. (2000) Analysis of the complete genome sequence of acute bee paralysis virus show that it belongs to novel group of insect-infecting RNA viruses. Virology 277, 457-463

Grabensteiner, E., Ritter, W., Carter, M.J., Davison, S., Pechhacker, H., Kolodziejek, J., Boecking, O., Derakhshifar, I., Moosbeckhofer, R., Licek, E., Nowotny, N. (2001) Sacbrood virus of the honeybee (*Apis mellifera*): Rapid identification and phylogenetic analysis using reverse transcription-PCR. Clin. Diagn. Lab. Immun. 8, 38-104

Gregory, P.G., Evans, J.D., Rinderer, T., deGuzman, L. (2005) Conditional immuno-gene suppression of honey bees parasitized by *Varroa* mites. J. Insect Sci. 5; 7

Gronowski, A.M., Copper, S., Baorto, D., Murray, P.R. (2000) Reproducibility problems with the Abbott laboratories LCx assay for *Chlamydia trachomatis* and *Neisseria gonorrhoeae*. J. Clin. Microbiol. 38, 2416-2418

Grove, S., Faller, R., Soleim, K.B., Dannevig, B.H. (2006) Absolute quantitation of RNA by a competitive real-time RT-PCR method using piscine nodavirus as a model. J. Virol. Meth. 132, 104-112

Grozinger, C.M., Sharabash, N.M., Whitfield, C.W., Robinson, G.E. (2003) Pheromone-mediated gene expression in the honey bee brain. Proc. Natl. Acad. Sci. USA 100, 14519-14525

Guatelli, J.C., Whitfield, K.M., Kwoh, D.Y., Barringer, K.J., Richman, D.D., Gingeras, T.R. (1990) Isothermal, *in vitro* amplification of nucleic acids by a multienzyme reaction modelled after retroviral replication. Proc. Nat. Acad. Sci. USA 87, 1874-1878

Guyot, A., Karim, S., Kyi, M.S., Fox, J. (2003) Evaluation of adjunctive HPV testing by Hybrid Capture-2® in women with minor cytological abnormalities for the diagnosis of CIN2/3 and cost comparison with coloscopy. BMC Infect. Dis. 3, 23

Habib-Bein, N.F., Beckwith, W.H. 3rd, Mayo, D., Landry, M.L. (2003) Comparison of SmartCycler real-time reverse transcription-PCR assay in a public health laboratory with direct immunofluorescence and cell culture assays in a medical center for detection of influenza A virus. J. Clin. Microbiol. 41, 3597-3601

Hafner, G.J., Yang, I.C., Wolter, L.C., Stafford, M.R., Giffard, P.M. (2001) Isothermal amplification and multimerization of DNA by Bst DNA polymerase. Biotechniques 30, 852-6, 858, 860 passim.

Handelsman, J. (2004) Metagenomics: Application of Genomics to Uncultured Microorganisms. Microbiol. Mol. Biol. Rev. 68, 669–685

Harlow, E., Lane, D.P. (1988) *Antibodies: A laboratory manual*, Cold Spring Harbor Laboratory Press, New York

Harvey, M.L. (2005) An alternative for the extraction and storage of DNA from insects in forensic entomology. J. Forensic Sci. 50, 627-629

Haugland R.P. (2002) Handbook of Fluorescent Probes and Research Products, Molecular Probes, Oregon, USA

Hauser, S., Weber, C., Vetter, G., Stevens, M., Beuve, M., Lemaire, O. (2000) Improved detection and differentiation of poleroviruses infecting beet or rape by multiplex RT-PCR. J. Virol. Meth. 89, 11-21

Heaton, R.J., Peterson, A.W., Georgiadis, R.M. (2001) Electrostatic surface plasmon resonance: direct electric field-induced hybridization and denaturation in monolayer nucleic acid films and label-free discrimination of base mismatches. Proc. Natl. Acad. Sci. USA 98, 3701-3704

Heim, A., Schumann, J. (2002) Development and evaluation of a nucleic acid sequence based amplification (NASBA) protocol for the detection of enterovirus RNA in cerebrospinal fluid samples. J. Virol. Meth. 103, 101-107.

Helguera, P.R., Taborda, R., Docampo, D.M., Ducasse, D.A. (2001) Immunocapture reverse transcription-polymerase chain reaction combined with nested PCR greatly increases the detection of Prunus necrotic ring spot virus in the peach. J. Virol. Meth. 95, 93-100

Hellyer, T.J., desJardin, L.E., Teixiera, L., Perkins, M.D., Cave, M.D., Eisenach, K.D. (1999) Detection of viable *Mycobacterium tuberculosis* by reverse transcriptase strand displacement amplification of mRNA. J. Clin. Microbiol. 37, 518-523

Henderson, K.M., Eayrs, K. (2004) Pregnancy status determination in mares using a rapid lateral flow test for measuring serum oestrone sulphate. NZ Vet. J. 52, 193-196

Herrmann, B., Larsson, V.C., Rubin, C.-J., Sund, F., Eriksson, B.-M., Arvidson, J., Yun, Z., Bondeson, K., Blomberg, J. (2004) Comparison of a duplex quantitative real-time PCR assay and the COBAS amplicor CMV monitor test for detection of cytmomegalovirus. J. Clin. Microbiol. 42, 1909-1914

Hesselink, A.T., van den Brule, A.J., Brink, A.A., Berkhof, J., van Kemenade, F.J., Verheijen, R.H., Snijders, P.J. (2004) Comparison of hybrid capture 2 with *in situ* hybridization for the detection of high-risk human papillomavirus in liquid-based cervical samples. Cancer 102, 11-18

Higgins, J.A., Nasarabadi, S., Karns, J.S., Shelton, D.R., Cooper, M., Gbakima, A., Koopman, R.P. (2003) A handheld real time thermal cycler for bacterial pathogen detection. Biosens. Bioelectron. 18, 1115-1123; Erratum in: Biosens. Bioelectron. 20, 663-664.

Hindson, B.J., Makarewicz, A.J., Setlur, U.S., Henderer, B.D., McBride, M.T., Dzenitis, J.M. (2005) APDS: the autonomous pathogen detection system. Biosens. Bioelectron. 20, 1925-1931

't Hoen, P.A.C., de Kort, F., van Ommen, G.J.B., den Dunnen, J.T. (2003) Fluorescent labeling of cRNA for microarray applications. Nucl. Acids Res. 31(5) e20. doi: 10.1093/nar/gng020

Hofkens, J., Cotlet, M., Vosch, T., Tinnefeld, P., Weston, K.D., Ego, C., Grimsdale, A., Müllen, K., Beljonne, D., Bredás, J.-L., Jordens, S., Schweitzer, G., Sauer, M., de Schryver, F. (2003) Revealing competitive Förster-type resonance energy transfer pathways in single bichromophoric molecules. Proc. Natl. Acad. Sci. USA 100, 13146-13151

Holland, P.M., Abramson, R.D., Watson, R., Gelfand, D.H. (1991) Detection of specific polymerase chain reaction product by utilizing the 5'-3' exonuclease activity of *Thermus aquaticus* DNA polymerase. Proc. Nat. Acad. Sci. USA 88, 7276-7280

Honeybee Genome Sequencing Consortium (2006) Insights into social insects from the genome of the honeybee Apis mellifera. Nature 443, 931-949

Honore-Bouakline, S., Vincensini, J.P., Giacuzzo, V., Lagrange, P.H., Herrmann, J.L. (2003) Rapid diagnosis of extrapulmonary tuberculosis by PCR: impact of sample preparation and DNA extraction. J. Clin. Microbiol. 41, 2323-2329

Hourfar, M.K., Schmidt, M., Seifried, E., Roth, W.K. (2005) Evaluation of an automated high-volume extraction method for viral nucleic acids in comparison to a manual procedure with preceding enrichment. Vox Sang. 89, 71-76

Howell, W.M., Jobs, M., Brookes, A.J. (2002) iFRET: an improved fluorescence system for DNA-melting analysis. Genome Res. 12, 1401-1407

Hu, Y., French, L.A. Jr, Radecsky, K., da Cunha, M.P., Millard, P., Vetelino, J.F. (2004) A lateral field excited liquid acoustic wave sensor. IEEE Trans. Ultrason. Ferroelectr. Freq. Control 51, 1373-1380

Hung, A.C.F. (2000) PCR detection of Kashmir bee virus in honey bee excreta. J. Apic. Res. 39, 103-106

Hung, A.C.F., Ball, B.V., Adams, J.R., Shimanuki, H., Knox, D.A. (1996) A scientific note on the detection of American strains of acute paralysis virus and Kashmir bee virus in dead bees in one US honeybee (*Apis mellifera* L.) colony. Apidologie 27, 55-56

Hung, A.C.F., Peng, C.Y.S., Shimanuki, H. (2000) Nucleotide sequence variations in Kashmir bee virus isolated from *Apis mellifera* L. and *Varroa jacobsoni* Oud.. Apidologie 31, 17-23

Hung, A.C.F., Shimanuki, H. (1999) A scientific note on the detection of Kashmir bee virus in individual honey bees and Varroa mites. Apidologie 30, 353-354

Issa, N.C., Espy, M.J., Uhl, J.R., Harmsen, W.S., Mandrekar, J.N., Gullerud, R.E., Davis, M.D., Smith, T.F. (2005) Comparison of specimen processing and nucleic acid extraction by the swab extraction tube system versus the MagNA Pure LC system for laboratory diagnosis of herpes simplex virus infections by LightCycler PCR. J. Clin. Microbiol. 43, 1059-1063

Jansson, A., Gustafsson, L.L., Mirghani, R.A. (2003) High-performance liquid chromatographic method for the determination of quinine and 3-hydroxyquinine in blood samples dried on filter paper. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 795, 151-156

Jean, J., Blais, B., Darveau, A., Fliss, I. (2001) Detection of Hepatitis A Virus by the Nucleic Acid Sequence-Based Amplification Technique and Comparison with Reverse Transcription-PCR. Appl. Environ. Microbiol. 67, 5593-5600

Jean, J., D'Souza, D.H., Jaykus, L.-A. (2004) Multiplex nucleic acid sequence-based amplification for simultaneous detection of several enteric viruses in model ready-to-eat foods. Appl. Environ. Microbiol. 70, 6603-6610

Jensen, L.E., Whitehead, A.S. (1998) Competitive reverse transcription polymerase chain reaction for quantifying pre-mRNA and mRNA of major acute phase proteins. J. Immunol. Meth. 215, 45-58

Jost, J.-P., Munch, O., Andersson, T. (1991) Study of protein-DNA interactions by surface plasmon resonance (real time kinetics). Nucl. Acids Res. 19, 2788

Karlson, H., Guthenberg, C., von Döbeln, U., Kristensson, K. (2003) Extraction of RNA from dried blood on filter papers after long-term storage. Clin. Chem. 49, 979-981

Keer, J.T., Birch, L. (2003) Molecular methods for the assessment of bacterial viability. J. Microbiol. Methods 53, 175-183

Kiatpathomchai, W., Jitrapakdee, S., Panyim, S., Boonsaeng, V. (2004) RT-PCR detection of yellow head virus (YHV) infection in *Penaeus monodon* using dried haemolymph spots. J. Virol. Meth. 119, 1-5

Kim, D.S., Jeong, Y.T., Park, H.J., Shin, J.K., Choi, P., Lee, J.H., Lim, G. (2004) An FET-type charge sensor for highly sensitive detection of DNA sequence. Biosens. Bioelectron. 20, 69-74

Kita-Matsuo, H., Yukinawa, N., Matoba, R., Saito, S., Oba, S., Ishii, S., Kato, K. (2005) Adaptor-tagged competitive polymerase chain reaction: amplification bias and quantified gene expression levels. Anal. Biochem. 339, 15-28

Knepp, J.H., Geahr, M.A., Forman, M.S., Valsamakis, A. (2003) Comparison of automated and manual nucleic acid extraction methods for detection of enterovirs RNA.

Koehne, J.E., Chen, H., Cassell, A.M., Ye, Q., Han, J., Meyyappan, M., Li, J. (2004) Miniaturized multiplex label-free electronic chip for rapid nucleic acid analysis based on carbon nanotube nanoelectrode arrays. Clin. Chem. 50, 1886-1893

Kohara, Y., Noda, H., Okano, K., Kambara, H. (2001) DNA hybridization using "bead-array": probe-attached beads arrayed in a capillary in a predetermined order. Nucl. Acids Res. Suppl. 2001, 83-84

Konnick, E.Q., Erali, M., Ashwood, E.R., Hillyard, D.R. (2005) Evaluation of COBAS amplicor HBVmonitor assay and comparison with the ultrasensitive HBV Hybrid Capture-2 assay for quantification of hepatisis B virus DNA. J. Clin. Microbiol. 43, 596-603

Konomi, N., Lebwohl, E., Zhang, D. (2002) Comparison of DNA and RNA extraction methods for mummified tissues. Mol. Cell. Probes 16, 445-451

Kucharski, R., Maleszka, R. (2005) Microarray and real-time PCR analyses of gene expression in the honeybee brain following caffeine treatment. J. Mol. Neurosc. 27, 269-276

van Kuilenburg, A.B., van Lenthe, H., Loffler, M., van Gennip, A.H. (2004) Analysis of pyrimidine synthesis "*de novo*" intermediates in urine and dried urine filter- paper strips with HPLC-electrospray tandem mass spectrometry. Clin. Chem. 50, 2117-2124

Kulincevic, J., Ball, B.V., Mladan, V. (1990) Viruses in honey bee colonies infested with *Varroa jacobsoni*: First findings in Yugoslavia. Acta Veterinaria 40, 37-42

Kurath, G., Dodds, J.A. (1995) Mutation analyses of molecularly cloned satellite tobacco mosaic virus during serial passage in plants: evidence for hotspots of genetic change. RNA 1, 491-500

Kurath, G., Heick, J.A., Dodds, J.A. (1993) RNase protection analyses show high genetic diversity among field isolates of satellite tobacco mosaic virus. Virol. 194, 414-418

Kwoh, D.Y., Davis, G.R., Whitfield, K.M., Chappelle, H.L., Di Michelle, L.J., Gingeras, T.R. (1989) Transcription-based amplification system and detection of amplified human immunodeficiency virus type 1 with a bead-based sandwich hybridization format. Proc. Natl. Acad. Sci. USA 86, 1173-1177

Labayru, C., Eiros, J.M., Hernandez, B., de Lejarazu, R.O., Torres, A.R. (2005) RNA extraction prior to HIV-1 resistance detection using Line Probe Assay (LiPA): comparison of three methods. J. Clin. Virol. 32, 265-271

Lanciotti, R.S. (2003) Molecular amplification assays for the detection of flaviviruses. Adv. Virus Res. 61, 67-99

Lanciotti, R.S., Kerst, A.J. (2001) Nucleic Acid Sequence-Based Amplification Assays for Rapid Detection of West Nile and St. Louis Encephalitis Viruses. J. Clin. Microbiol. 39, 4506-4513. doi: 10.1128/JCM.39.12.4506-4513.2001

Landry, M.L., Ferguson, D. (2000) SimulFluor respiratory screen for rapid detection of multiple respiratory viruses in clinical specimens by immunofluorescence staining. J. Clin. Microbiol. 38, 708-711

Landry, M.L., Garner, R., Ferguson, D. (2005) Real-time nucleic acid sequence based amplification using molecular beacons for detection of enterovirus RNA in clinical specimens. J. Clin. Microbiol. 43, 3136-3139

Lange, K., Bender, F., Voigt, A., Gao, H., Rapp, M. (2003) A surface acoustic wave biosensor concept with low flow cell volumes for label-free detection. Anal. Chem. 75, 5561-5566

Lanzi G., de Miranda J.R., Boniotti M.B., Cameron C.E., Lavazza A., Capucci L., Camazine S.M., Rossi C. (2006) Molecular and biological characterization of deformed wing virus of honeybees (Apis mellifera L.), J. Virol. 80, 4998-5009

Lazar, G.A., Dang, W., Karki, S., Vafa, O., Peng, J.S., Hyun, L., Chan, C., Chung, H.S., Eivazi, A., Yoder, S.C., Vielmetter, J., Carmichael, D.F., Hayes, R.J., Dahiyat, B.I. (2006a) Engineered antibody Fc variants with enhanced effector function. Proc. Natl. Acad. Sci. USA 103, 4005-4010

Lazar, G.A., Desjarlais, J.R., Jacinto, J., Karki, S., Hammond, P.W. (2006b) A molecular immunology approach to antibody humanization and functional optimization. Mol. Immunol. (in press)

Leat, N., Ball, B.V., Govan, V., Davison, S. (2000) Analysis of the complete genome sequence of black queen cell virus, a picorna-like virus of honey bees. J. Gen. Virol. 81, 2111-2119

Lee, P. E., Furgala, B. (1967) Viruslike particles in adult honey bees (*Apis mellifera* Linnaeus) following injection with sacbrood virus. Virol. 32, 11-17

LeGendre, N., Mansfield, M., Weiss, A., Matsudaira, P. (1993) Purification of proteins and peptides by SDS-PAGE, p. 71-101. In: P. Matsudaira (Ed.), A practical guide to protein and peptide purification for microsequencing, 2nd Ed., Academic Press, San Diego

Li, C.C., Seidel, K.D., Coombs, R.W., Frenkel, L.M. (2005) Detection and quantification of human immunodeficiency virus type 1 p24 antigen in dried whole blood and plasma on filter paper stored under various conditions. J. Clin. Microbiol. 43, 3901-3905

Li, N., Tan, W.G., Tsang, R.Y., Tyrrell, D.L.J., Dovichi, N.J. (2002) Quantitative polymerase chain reaction using capillary electrophoresis with lase-induced fluorescence detection: analysis of duck hepatitis B. Anal. Bioanal. Chem. 374, 269-273

Lieberfarb, M.E., Lin, M., Lechpammer, M., Li, C., Tanenbaum, D.M., Febbo, P.G., Wright, R.L., Shim, J., Kantoff, P.W., Loda, M., Meyerson, M., Sellers, W.R. (2003) Genome-wide loss of heterozygosity analysis from laser-capture microdissected prostate cancer using single nucleotide polymorphic allele (SNP) arrays and a novel bioinformatics platform dChipSNP. Cancer Res. 63, 4781-4785

Liepold, P., Wieder, H., Hillebrandt, H., Friebel, A., Hartwich, G. (2005) DNA-arrays with electrical detection: a label-free low cost technology for routine use in life sciences and diagnostics. Bioelectrochem. 67, 143-150

Lim, E.L., Tomita, A.V., Thilly, W.G., Polz, M.F. (2001) Combination of competitive quantitative PCR and constant-denaturant capillary electrophoresis for high-resolution detection and enumeration of microbial cells. Appl. Environ. Microbiol. 67, 3897-3903

Lin, M., Wei, L.J., Sellers, W.R., Lieberfarb, M., Wong, W.H., Li, C. (2004) dChipSNP: significance curve and clustering of SNP-array-based loss-of-heterozygosity data. Bioinformatics, doi: 10/1093/bioinformatics/bth069

Livak, K.J., Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods 25, 402-408

Lizardi, P.M., Guerra, C., Lomeli, H., Tussie-Luna, I., Kramer, F. (1988) Exponential amplification of recombinant-RNA hybridization probes. Biol. Technol. 6, 1197-1202

Loeffler, J., Hebart, H., Cox, P., Flues, N., Schumacher, U., Einsele, H. (2001) Nucleic acid sequence-based amplification of *Aspergillus* RNA in blood samples. J. Clin. Microbiol. 39, 1626-1629.

Lund, M., Madsen, M. (2006) Strategies for the inclusion of an internal amplification control in conventional and real time PCR detection of Campylobacter spp. In chicken fecal samples. Mol. Cell. Probes 20, 92-99

Lunello, P., Mansilla, C., Conci, V., Ponz, F. (2004) Ultra-sensitive detection of two garlic potyviruses using a real-time fluorescent (Taqman®) RT-PCR assay. J. Virol. Meth. 118, 15-21

Luthra, R., Medeiros, L.J. (2004) Isothermal multiple displacement amplification: A highly reliable approach for generating unlimited high molecular weight genomic DNA from clinical specimens. J. Mol. Diagn. 6, 236-242

Luyten, Y.A., Thompson, J.R., Morrill, W., Polz, M.F., Distel, D. (2006) Extensive variation in intracellular symbiont community composition among members of a single population of the wood-boring bivalve *Lyrodus pedicellatus* (Bivalvia: Teredinidae). Appl. Environ. Microbiol. 72, 412-417

Maaroufi, Y., Ahariz, N., Husson, M., Crokaert, F. (2004) Comparison of different methods of isolation of DNA of commonly encountered *Candida* species and its quantitation by using a real-time PCR-based assay. J. Clin. Microbiol. 42, 3159-3163

Macanovic, A., Marquette, C., Polychronakos, C., Lawrence, M.F. (2004) Impedance-based detection of DNA sequences using a silicon transducer with PNA as the probe layer. Nucl. Acids Res. 32(2), e20

Mackay, I.M., Arden, K.E., Nitsche, A. (2002) Real-time PCR in virology. Nucl. Acids Res. 30, 1292-1305

Mackay, I.M., Gardam, T., Arden, K.E., McHardy, S., Whiley, D.M., Crisante, E., Sloots, T.P. (2003) Co-detection and discrimination of six human herpesviruses by multiplex PCR-ELAHA. J. Clin. Virol. 28, 291-302

Madriz, J., Bonilla, J.A., de Miranda, J.R. Espinoza, A.M. (2000) Serological relationships between rice hoja blanca virus and Echinochloa hoja blanca virus. Uniciencia 17, 21-26

Madriz, J., de Miranda, J.R., Cabezas, E., Oliva, M., Hernandez, M., Espinoza, A.M. (1998) Echinochloa hoja blanca virus and rice hoja blanca virus occupy distinct ecological niches. J. Phytopath. 146, 305-308

Mahony, J., Chong, S., Jang, D., Luinstra, K., Faught, M., Dalby, D., Sellors, J., Chernesky, M. (1998) Urine specimens from pregnant and nonpregnant women inhibitory to amplification of *Chlamydia trachomatis* nucleic acid by PCR, ligase chain reaction, and transcription-mediated amplification: identification of urinary substances associated with inhibition and removal of inhibitory activity. J. Clin. Microbiol. 36, 3122-3126

Mahony, J.B., Song, X., Chong, S., Faught, M., Salonga, T., Kapala, J. (2001) Evaluation of the NucliSens Basic Kit for detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in genital tact specimens using nucleic acid sequence-based amplification of 16S rRNA. J. Clin. Microbiol. 39, 1429-1435

Mao, Y., Luo, C., Ouyang, Q. (2003) Studies of temperature-dependent electronic transduction on DNA hairpin loop sensor. Nucl. Acids Res. 31(18), e108

Mao, Y., Luo, C., Deng, W., Jin, G., Yu, X., Zhang, Z., Ouyang, Q., Chen, R., Yu, D. (2004) Reversibly switchable DNA nanocompartment on surfaces. Nucl. Acids Res. 32(19), e144. doi: 10.1093/nar/ghn145

Marko, N.F., Frank, B., Quackenbush, J., Lee, N.H. (2005) A robust method for amplification of RNA in the sense orientation. BMC Genomics 6(27) doi: 10.1186/1471-2164/6/27

Marozsan, A.J., Fraundorf, E., Abraha, A., Baird, H., Moore, D., Troyer, R., Nankja, I., Arts, E.J. (2004) Relationships between infectious titer, capsid protein levels, and reverse transcriptase activities of diverse human immunodeficiency virus type-1 isolates. J. Virol. 78, 11130-11141

Matsudaira, P. (1993) *A practical guide to protein and peptide purification for microsequencing*, 2nd Ed., Academic Press, San Diego

Mavropoulou, A.K., Koraki, T., Ioannou, P.C., Christopoulos, T.K. (2005) High-throughput double quantitative competitive polymerase chain reaction for determination of genetically modified organisms. Anal. Chem. 77, 4785-4791

Mekuria, G., Ramesh, S.A., Alberts, E., Bertozzi, T., Wirthensohn, M., Collins, G., Sedgley, M. (2003) Comparison of ELISA and RT-PCR for the detection of Prunus necrotic ring spot virus and prune dwarf virus in almond (*Prunus dulcis*). J. Virol. Meth. 114, 65-69

Min, J., Baeumner, A.J. (2002) Highly Sensitive and Specific Detection of Viable *Escherichia coli* in Drinking Water. Anal. Biochem. 303, 186-193

Mir, M., Katakis, I. (2005) Towards a fast-responding, label-free electrochemical DNA biosensor. Anal. Bioanal. Chem. 381, 1033-1035

de Miranda, J.R., Drebot, M., Tyler, S., Shen, M., Cameron, C.E., Stoltz, D.B., Camazine, S.M. (2004) Complete nucleotide sequence of Kashmir bee virus and comparison with acute bee paralysis virus. J. Gen. Virol. 85, 2263-2270

de Miranda, J.R., Espinoza, A.M., Hull, R. (1996a) Rapid small scale purification of rice hoja blanca and Echinochloa hoja blanca tenuivirus ribonucleoprotein. J. Vir. Meth. 56, 109-114

de Miranda, J.R., Hull, R., Bird, C. (1996b) Extraction and aphid transmission of beet western yellows luteovirus from air dried tissue. J. Vir. Meth. 58, 205-207

Mukhopadhyay, R., Lorentzen, M., Kjems, J., Besenbacher, F. (2005) Nanomechanical sensing of DNA sequences using piezoresistive cantilevers. Langmuir 21, 400-408

Mussen, E.C., and Furgala, B. (1977) Replication of sacbrood virus in larval and adult honeybees, *Apis mellifera*. J. Invert. Path. 30, 20-34

Nadeau, J.G., Pitner, J.B., Linn, C.P., Schram, J.L., Dean, C.H., Nycz, C.M. (1999) Real-time, sequence-specific detection of nucleic acids during strand displacement amplification. Anal. Biochem. 276, 177-187

Nagamine, K., Watanabe, K., Ohtsuka, K., Hase, T., Notomi, T. (2001) Loop-mediated isothermal amplification reaction using a nondenatured template. Clinic. Chem. 47, 1742-1743

Nazarenko, I., Lowe, B., Darfler, M., Ikonomi, P., Schuster, D., Rashtchian, A. (2002) Multiplex quantitative PCR using self-quenched primers labeled with a single fluorophore. Nucl. Acids Res. 30, e37

Nelson, B.P., Liles, M.R., Frederick, K.B., Corn, R.M., Goodman, R.M. (2002) Label-free detection of 16S ribosomal RNA hybridization on reusable DNA arrays using surface plasmon resonance imaging. Environ. Microbiol. 4, 735-743

Nie, X. Singh, R.P. (2000) Detection of multiple potato viruses using oligo(dT) as a common cDNA primer in multiplex RT-PCR. J. Virol. Meth. 86, 179-185

Nie, X. Singh, R.P. (2003) Specific differentiation of recombinant $PVY^{N:O}$ and PVY^{NTN} isolates by multiplex RT-PCR. J. Virol. Meth. 113, 69-77

Nielsen, P.E., Haaima, G. (1997) Peptide nucleic acid (PNA). A DNA mimic with a pseudopeptide backbone. Chem. Soc. Rev. 26, 73-78

Nieman, T. (1995) *Chemiluminescence: Theory and instrumentation*. In: Encyclopedia of analytical science, pp.608-613. Academic Press, London.

Niesters, H.G., Krajden, M., Cork, L., de Medina, M., Hill, M., Fries, E., Osterhaus, A.D. (2000) A multicenter study evaluation of the Digene Hybrid Capture II signal amplification technique for detection of hepatitis B virus DNA in serum samples and testing of EUROHEP standards. J. Clin. Microbiol. 38, 2150-2155

Nilsson, P., Persson, B., Larsson, A., Uhlen, M., Nygren, P.A. (1997) Detection of mutations in PCR products from clinical samples by surface plasmon resonance. J. Mol. Recognit. 10, 7-17

Noerholm, M., Bruus, H., Jakobsen, M.H., Telleman, P., Ramsing, N.B. (2004) Polymer microfluidic chip for online monitoring of microarray hybridizations. Lab Chip 4, 28-37

Nolan, R.L., Cai, H., Nolan, J.P., Goodwin, P.M. (2003) A simple quenching method for fluorescence background reduction and its application to the direct, quantitative detection of specific mRNA. Anal. Chem. 75, 6236-6243

Nordström, S. (2003) Distribution of deformed wing virus within honeybee (Apis mellifera) brood cells infested with the ectoparasitic mite Varroa destructor. Experim. Appl. Acarol. 29, 293-302

Nordström, S., Fries, I., Aarhus, A., Hansen, H., Korpela, S. (1999) Virus infections in Nordic honeybee colonies with no, low or severe Varroa jacobsoni infestations. Apidologie 30, 475-484

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., Hase, T. (2000) Loop-mediated isothermal amplification of DNA. Nucl. Acid. Res. 28, e63.

Nygren, M., Ronaghi, M., Nyren, P., Albert, J., Lundeberg, J. (2001) Quantification of HIV-1 using multiple quantitative polymerase chain reaction standards and bioluminometric detection. Anal. Biochem. 288, 28-38

Okahata, Y., Kawase, M., Niikura, K., Ohtake, F., Furusawa, H., Ebara, Y. (1998) Kinetic measurements of DNA hybridization on an oligonucleotide-immobilized 27-MHz quartz crystal microbalance. Anal. Chem. 70, 1288-1296

Okahata, Y., Matsunobo, Y., Ijiro, K., Mukae, M., Murakami, A., Makino, K. (1992) Hybridization of nucleic acids immobilized on a quartz crystal microbalance. J. Am. Chem. Soc. 114, 8299-8300

Ongus, J.R., Peters, D., Bonmatin, J.-M., Bengsch, E., Vlak, J.M., van Oers, M.M. (2004) Complete sequence of a picorna-like virus of the genus *Iflavirus* replicating in the mite *Varroa destructor*. J. Gen. Virol. 85, 3747-3755

Padoa, C.J., Crowther, N.J. (2006) Engineered antibodies: A new tool for use in diabetes research. Diabetes Res. Clin. Pract. 74, S51-S62

Paez, J.G., Lin, M., Beroukhim, R., Lee, J.C., Zhao, X., Richter, D.J., Gabriel, S., Herman, P., Sasaki, H., Altshuler, D., Li, C., Meyerson, M., Sellers, W.R. (2004) Genome coverage and sequence fidelity of φ29 polymerase-based multiple strand displacement whole genome amplification. Nucl. Acids Res. 32(9), e71. doi: 10.1093/nar/gnh069

Pagliarulo, V., George, B., Beil, S.J., Groshen, S., Laird, P.W., Cai, J., Willey, J., Cote, R.J., Datar, R.H. (2004) Sensitivity and reproducibility of standardized-competitive RT-PCR for transcript quantification and its comparison with real time RT-PCR. Mol. Cancer 23, 3-5

Palmieri, O., Toth, S., Ferraris, A., Andriulli, A., Latiano, A., Annese, V., Dallapiccola, B., Vecchi, M., Devoto, M., Surrey, S., Fortina, P. (2003) CARD15 Genotyping in Inflammatory Bowel Disease Patients by Multiplex Pyrosequencing. Clin. Chem. 49, 1675-1679

Patolsky, F., Zheng, G., Hayden, O., Lakadamyali, M., Zhuang, X., Lieber, C.M. (2004) Electrical detection of single viruses. Proc. Nat. Acad. Sci. USA 101, 14017-14022

Perreten, V., Vorlet-Fawer, L., Slickers, P., Ehricht, R., Kuhnert, P., Frey, J. (2005) Microarray-based detection of 90 antibiotic resistance genes of gram-positive bacteria. J. Clin. Microbiol. 43, 2291-2302

Persson, B., Stenhag, K., Nilsson, P., Larsson, A., Uhlen, M., Nygren, P. (1997) Analysis of oligonucleotide probe affinities using surface plasmon resonance: a means for mutational scanning. Anal. Biochem. 246, 34-44

Peters, I.R., Helps, C.R., Hall, E.J., Day, M.J. (2004) Real-time RT-PCR: considerations for efficient and sensitive assay design. J. Immunol. Meth. 286, 203-217

Peterson, A.W., Heaton, R.J., Georgiadis, R.M. (2001) The effect of surface probe density on DNA hybridization. Nucl. Acids Res. 29, 5163-5168

Peterson, E.M., Darrow, V., Blanding, J., Aarnaes, S., de la Maza, L.M. (1997) Reproducibility problems with the AMPLICOR PCR *Chlamydia trachomatis* test. J. Clin. Microbiol. 35, 957-959

Pfaffl, M.W., Horgan, G.W., Dempfle, L. (2002) Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucl. Acids Res. 30, e36

Pfaffl, M.W., Hageleit, M. (2001) Validities of mRNA quantification using recombinant RNA and recombinant DNA external calibration curves in real-time RT-PCR. Biotech. Lett. 23, 275-282

Pollard-Knight, D., Hawkins, E., Yeung, D., Pashby, D.P., Simpson, M., McDougall, A., Buckle, P., Charles, S.A. (1990) Immunoassays and nucleic acid detection with a biosensor based on surface plasmon resonance. Ann. Biol. Clin. 48, 642-646

Pourmand, N., Elahi, E., Davis, R.W., Ronaghi, M. (2002) Multiplex Pyrosequencing. Nucl. Acids Res. 30(7), e31

Prado, I., Rosario, D., Bernardo, L., Alvarez, M., Rodriguez, R., Vazquez, S., Guzman, M.G. (2005) PCR detection of dengue virus using dried whole blood spotted on filter paper. J. Virol. Meth. 125, 75-81

Pumannova, M., Roubalova, K., Vitek, A., Sajdova, J. (2006) Comparison of quantitative competitive polymerase chain reaction-enzyme-linked immunosorbent assay with LightCycler-based polymerase chain reaction for measuring cytomegalovirus DNA in patiens after hematopoietic stem cell transplantation. Diagn. Microbiol. Infect. Dis. 54, 115-120

Pugnale, P., Latorre, P., Rossi, C., Crovatto, K., Pazienza, V., de Gottardi, A., Negro, F. (2006) Real-time multiplex PCR assay to quantify hepatitis C virus RNA in peripheral blood mononuclear cells. J. Virol. Meth. 133, 195-204

Puolakkainen, M., Hiltunen-Back, E., Reunala, T., Suhonen, S., Lahteenmaki, P., Lehtinen, M., Paavonen, J. (1998) Comparison of performances of two commercially available tests, a PCR assay and a ligase chain reaction test, in detection of urogenital *Chlamydia trachomatis* infection. J. Clin. Microbiol. 36, 1489-1493

Qin, X., Evans, J.D., Aronstein, K.A., Murray, K.D., Weinstock, G.M. (2006) Genome sequences of the honey bee pathogens *Paenibacillus larvae* and *Ascosphaera apis*. Insect Mol. Biol. 15, 715-718

Quinlivan, M., Cullinane, A., Nelly, M., van Maanen, K., Heldens, J., Arkins, S. (2004) Comparison of sensitivities of virus isolation, antigen detection and nucleic acid amplification for detection of equine influenza virus. J. Clin. Microbiol. 42, 759-763

Radonić, A., Thulke, S., Mackay, I.M., Landt, O., Siegert, W., Nitsche, A. (2004) Guideline to reference gene selection for quantitative real-time PCR. Biochem. Biophys. Res. Commun. 313, 856-862

Reina, J., González-Gardenas, M., Ruiz de Gopegui, E., Padilla, E., Ballesteros, F., Mari, M., Munar, M. (2004) Prospective evaluation of a dot-blot enzyme immunoassay (Directigen RSV) for the antigenic detection of respiratory syncytial virus from nasopharyngeal aspirates of paediatric patients. Clin. Microbiol. Infect. 10, 967-971

Rensen, G., Smith, W., Ruzante, J., Sawyer, M., Osburn, B., Cullor, J. (2005) Development and evaluation of a real-time fluorescent polymerase chain reaction assay for the detection of bovine contaminates in cattle feed. Foodborne Pathog. Dis. 2, 152-159

Ribao, C., Torrado, I., Vilarino, M.L., Romalde, J.L. (2004) Assessment of different commercial RNA-extraction and RT-PCR kits for detection of hepatitis A virus in mussel tissues. J. Virol. Meth. 115, 177-182

Ribière, M., Faucon, J.-P., Pépin, M. (2000) Detection of chronic honey bee (Apis mellifera L.) paralysis virus infection: application to a field survey. Apidologie 31, 567-577

Ribière, M., Triboulot, C., Mathieu, L., Aurières, C., Faucon, J.-P., Pépin, M. (2002) Molecular diagnosis of chronic bee paralysis virus infection. Apidologie 33, 339-351

Richards, M.P., Poch, S.M. (2002) Quantitative analysis of gene expression by reverse transcription polymerase chain reaction and capillary electrophoresis with laser-induced fluorescence detection. Mol. Biotech. 21, 19-38

Robinson, G.E., Evans, J.D., Maleszka, R., Robertson, H.M., Weaver, D.B., Worley, K., Gibbs, R.A., Weinstock, G.M. (2006) Sweetness and light: illuminating the honey bee genome. Insect. Mol. Biol. 15, 535-539

Romano, J.W., Shurtliff, R.N., Dobratz, E., Gibson, A., Hickman, K., Markham, P.D. Pal, R. (2000) Quantitative evaluation of simian immunodeficiency virus infection using NASBA technology. J. Virol. Methods 86, 61-70

Roos-van Groningen, M.C., Eikmans, M., Baelde, H.J., de Heer, E., Bruijn, J.A. (2004) Improvement of extraction and processing of RNA from renal biopsies. Kidney Int. 65, 97-105

Rouillard, J.-M., Zuker, M., Gulari, E. (2003) OligoArray 2.0: design of oligonucleotide probes for DNA microarrays using a thermodynamic approach. Nucl. Acids Res. 31, 3057-3062

Rudi, K., Rud, I., Holck, A. (2003) A novel multiplex quantitative DNA array based PCR (MQDA-PCR) for quantification of transgenic maize in food and feed. Nucl. Acids Res. 31, e62

Rudi, K., Nogva, H.K., Moen, B., Nissen, H., Bredholt, S., Moretro, T., Naterstad, K., Holck, A. (2002) Development and application of new nucleic acid-based technologies for microbial community analyses in foods. Int. J. Food Microbiol. 78, 171-80

Ruiz, G., Peña, P., de Ory, F., Echevarría, J.E. (2005) Comparison of commercial Real-time PCR assays for quantification of Epstein-Barr virus DNA. J. Clin. Microbiol. 43, 2053-2057

Sage, L. (2004) Protein biochips go high tech. Anal. Chem. 76, 137A-142A

Sakata, T., Miyahara, Y. (2005) Potentiometric Detection of Single Nucleotide Polymorphism by Using a Genetic Field-effect transistor. Chem. Biochem. 6, 703-710

Salete-de-Paula, V., Mello-Villar, L., Coimbra-Gaspar, M.A. (2003) Comparison of four extraction methods to detect Hepatitis A virus RNA in serum and stool samples. Br. J. Infect. Dis. 7, 135-142

Sambrook, J., Russell, D.W. (2001) Molecular cloning: a laboratory manual 3rd edn., Cold Spring Harbor Laboratory Press, New York

Saunders, N.A., Clewley, J.P. (1998) DNA amplification: General concepts and methods, in: Woodford N. and Johnson A.P. (Eds.), Molecular Bacteriology: Protocols and Clinical Applications, Humana Press, Totowa, New Jersey, pp.63-82.

Scansen, B.A., Kruger, J.M., Wise, A.G., Venta, P.J., Bartlett, P.C., Maes, R.K. (2005) *In vitro* comparison of RNA preparation methods for detection of feline calicivirus in urine of cats by use of a reverse transcriptase-polymerase chain reaction assay. Am. J. Vet. Res. 66, 915-920

Schaad, N.W., Frederick, R.D., Shaw, J., Schneider, W.L., Hickson, R., Petrillo, M.D., Luster, D.G. (2003) Advances in molecular-based diagnostics in meeting crop biosecurity and phytosanitary issues. Ann. Rev. Phytopathol. 41, 305-324

Schneider, P., Wolters, L., Schoone, G., Schallig, H., Sillekens, P., Hermsen, R., Sauerwein, R. (2005) Real-time nucleic acid sequence-based amplification is more convenient than real-time PCR for quantification of *Plasmodium falciparum*. J. Clin. Microbiol. 43, 402-405

Schneider, W.L., Sherman, D.J., Stone, A.L., Damsteegt, V.D., Frederick, R.D. (2004) Specific detection and quantification of Plum pox virus by real-time fluorescent reverse transcription-PCR. J. Virol. Meth. 120, 97-105

Schoone, G.J., Oskam, L., Kroon, N.C.M., Schallig, H.D.F.H., Omar, S.A. (2000) Detection and quantification of *Plasmodium falciparum* in blood samples using quantitative nucleic acid sequence-based amplification. J. Clin. Microbiol. 38, 4072-4075

Schuurman, T., van Breda, A., de Boer, R., Kooistra-Smid, M., Beld, M., Savelkoul, P., Boom, R. (2005) Reduced PCR sensitivity due to impaired DNA recovery with the MagNA Pure LC total nucleic acid isolation kit. J. Clin. Microbiol. 43, 4616-4622

Sefc, K.M., Leonhardt, W., Steinkellner, H. (2000) partial sequence identification of grapevine-leafroll-associated virus-1 and development of a highly sensitive IC-RT-PCR detection method. J. Virol. Meth. 86, 101-106

Servais, J., Lambert, C., Fontaine, E., Plesseria, J.M., Robert, I., Arendt, V., Staub, T., Schneider, F., Hemmer, R., Burtonboy, G., Schmit, J.C. (2001) Comparison of DNA sequencing and a line probe assay for detection of human immunodeficiency virus type 1 drug resistance mutations in patients failing highly active antiretroviral therapy. J Clin Microbiol. 39, 454-459

Service, R.F. (2006) The race for the $1000 genome. Science 311, 1544-1546

Shah, J.S., Liu, J., Buxton, D., Hendricks, A., Robinson, L., Radcliffe, G., King, W., Lane, D., Olive, D.M., Klinger, J.D. (1995) Q-Beta replicase amplified assay for detection of *Mycobacterium tuberculosis* directly from clinical specimens. J. Clin. Microbiol. 33, 1435-1441

Shah, J.S., Liu, J., Smith, J., Popoff, S., Serpe, G., O'Brian, B., Radcliffe, G., Olive, D.M., Klinger, J.D. (1994) A novel ultrasensitive, Q-Beta replicase amplified hybridization assay for the detection of *Chlamydia trachomatis*. J. Clin. Microbiol. 32, 2718-2724

Sharma, S.K., Eblen, B.S., Bull, R.L., Burr, D.H., Whiting, R.C. (2005) Evaluation of lateral-flow *Clostridium botulinum* neurotoxin detection kits for food analysis. Appl. Environ. Microbiol. 71, 3935-3941

Shen, M., Cui, L., Ostiguy, N., Cox-Foster, D. (2005a) Intricate transmission routes and interactions between picorna-like viruses (Kashmir bee virus and sacbrood virus) with the honeybee host and the parasitic varroa mite. J. Gen. Virol. 86, 2281-2289

Shen, M., Cui, L., Ostiguy, N., Cox-Foster, D. (2005b) The role of varroa mites in infections of Kashmir bee virus (KBV) and deformed wing virus (DWV) in honey bees. Virol. 342, 141-149

Sherwood, C.S., Haynes, C.A., Turner, R.F. (1995) Nanogram-level micro-volume DNA assay based on the monomeric cyanine dye PO-PRO-3 iodide. Biotechniques 18, 136-141

Shimanuki, H.(1997) *Bacteria*, in: Morse R.A. and Flottum K. (Eds.), *Honey bee pests, predators, & diseases* 3rd Ed, A.I. Root, Medina, Ohio, pp.33-54

Siede, R., Büchler, R. (2003) Symptomatischer Befall von Drohnenbrut mit dem Black Queen Cell Virus auf hessische Bienenständen. Berl. Münch. Tierätrzl. Wschr. 116, 130-133

Siede, R., Büchler, R. (2004) Erstbefund des Kaschmir-Bienen-Virus in Hesse. Berl. Münch. Tierätrzl. Wschr. 117, 12-15

Siede R., Büchler R. (2006) Spatial distribution patterns of acute bee paralysis virus, black queen cell virus and sacbrood virus in Hesse, Germany. Wien. Tierätrzl. Mschr. 93, 90-93

Siede R., Derakhshifar I., Otten C., Berényi O., Bakonyi T., Köglberger H., Büchler, R. (2005) Prevalence of Kashmir bee virus in central Europe. J. Apic. Res. 44, 129

Simpkins, S.A., Chan, A.B., Hays, J., Pöpping, B., Cook, N. (2000) An RNA transcription-based amplification technique (NASBA) for the detection of viable *Salmonella enterica*. Lett. Appl. Microbiol. 30, 75-79

Singh, R.P., Nie, X., Singh, M. (2000) Duplex RT-PCR: reagent concentrations at reverse transcription stage affect the PCR performance. J. Virol. Meth. 86, 121-129

Singh-Zocchi, M., Dixit, S., Ivanov, V., Zocchi, G. (2003) Single-molecule detection of DNA hybridization. Proc. Natl. Acad. Sci. USA 100, 7605-7610

Solinas, A., Brown, L.J., McKeen, C., Mellor, J.M., Nicol, J.T.G., Thelwell, N., Brown, T. (2001) Duplex scorpion primers in SNP analysis and FRET applications. Nucl. Acids Res. 29(20), e92

Sokol, D.L., Zhang, X., Lu, P., Gewirtz, A.M. (1998) Real time detection of DNA.RNA hybridization in living cells. Nat. Biotechnol.16, 49-53

Sosnowski, R., Heller, M.J., Tu, E., Forster, A.H., Radtkey, R. (2002) Active microelectronic array system for DNA hybridization, genotyping and pharmacogenomic applications. Psychiatr. Genet. 12, 181-192

Spiess, A.-N., Mueller, N., Ivell, R. (2003) Amplified RNA degradation in T7-amplification methods results in biased microarray hybridizations. BMC Genomics 4(44), 1-13. doi: 10.1186/1471-2164/4/44

Spyres, L., Gaddis, S., Bedford, E., Arantes, S., Liburd, N., Powell, K.L., Thams, H., Mitchell, D., Walborg, E., Rouabhia, M., Aldaz, C.M., MacLeod, M.C. (2005) Quantitative high-throughput measurement of gene expression with sub-zeptomole sensitivity by capillary electrophoresis. Anal. Biochem. 345, 284-295

Stach, J.E., Bathe, S., Clapp, J.P., Burns, R.G. (2001) PCR-SSCP comparison of 16S rDNA sequence diversity in soil DNA obtained using different isolation and purification methods. FEMS Microbiol. Ecol. 36, 139-151

Ståhlberg, A., Håkansson, J., Xian, X., Semb, H., Kubista, M. (2004a) Properties of the reverse transcription reaction in mRNA quantification. Clin. Chem. 50, 509-515

Ståhlberg, A., Kubista, M., Pfaffl, M. (2004b) Comparison of reverse transcriptases in gene expression analysis. Clin. Chem. 50, 1678-

Stevens, M., Hull, R., Smith, H.G. (1997) Comparison of ELISA and RT-PCR for the detection of beet yellows closterovirus in plants and aphids. J. Virol. Meth. 68, 9-16

Stevenson, A.C., Araya-Kleinsteuber, B., Sethi, R.S., Mehta, H.M., Lowe, C.R. (2004) The application of the acoustic spectrophonometer to biomolecular spectrometry: a step towards acoustic "fingerprinting". J. Mol. Recognit. 17, 174-179

Stoltz, D., Shen, X.-R., Boggis, C., Sisson, G. (1995) Molecular diagnosis of Kashmir bee virus infection. J. Apic. Res. 34, 153-160

Stram, Y., Kuznetzova, L., Guini, M., Rogel, A., Meirom, R., Chai, D., Yadin, H., Brenner, J. (2004) Detection and quantitation of Akabane and Aino viruses by multiplex real-time reverse-transcriptase PCR. J. Virol. Meth. 116, 147-154

Stryer, L., Haugland, R.P. (1967) Energy transfer: a spectroscopic ruler. Proc. Natl. Acad. Sci. USA 58, 719-726

Su, H., Thompson, M. (1995) Kinetics of interfacial nucleic acid hybridization studied by acoustic network analysis. Biosens. Bioelectron. 10, 329-340

Sugiyama, H., Yoshikawa, T., Ihira, M., Enomoto, Y., Kawana, T., Asano, Y. (2005) Comparison of loop-mediated isothermal amplification, real-time PCR, and virus isolation for the detection of herpes simplex virus in genital lesions. J. Med. Virol. 75, 583-587

Suizdak, G. (1998) Probing viruses with mass spectrometry. J. Mass Spectrom. 33, 203-211

Suizdak, G., Bothner, B., Yeager, M., Brugidou, C., Fauquet, C.M., Hoey, K., Chang, C.-M. (1996) Mass spectrometry and viral analysis. Chem. Biol. 3, 45-48

Suzuki, H. (1998) Calcitonin levels in normal individuals with new highly sensitive chemiluminescent enzyme immunoassay. J. Clin. Lab. Anal. 12, 218-222 Erratum p. 325

Swillens, S., Goffard, J.-C., Marechal, Y., deKerchove-d'Exaerde, A., El-Housni, H. (2004) Instant evaluation of the absolute initial number of cDNA copies from a single real-time PCR curve. Nucl. Acids Res. 32, e53

Syrmis, M.W., Whiley, D.M., Thomas, M., Mackay, I.M., Williamson, J., Siebert, D.J., Nissen, M.D., Sloots, T.P. (2004) A sensitive, specific, and cost-effective multiplex reverse transcriptase-PCR assay for the detection of seven common respiratory viruses in respiratory samples. J. Mol. Diagn. 6, 125-131

Szemes, M., Klerks, M.M., van den Heuvel, J.F.J.M., Schoen, C.D. (2002) Development of a multiplex AmpliDet RNA assay for simultaneous detection and typing of potato virus Y isolates. J. Virol. Meth. 100, 83-96.

Tamarin, O., Comeau, S., Dejous, C., Moynet, D., Rebiere, D., Bezian, J., Pistre, J. (2003) Real time device for biosensing: design of a bacteriophage model using love acoustic waves. Biosens. Bioelectron. 18, 755-763

Tang, S., Mozdzanowski, J., Anumula, K.R. (1999) An Automated C-terminal Sequencing Method for Analysis of Proteins Using an ABI 473A Sequencer. J. Biomol. Tech. 10, 144-148

Tell, L.A., Foley, J., Needham, M.L., Walker, R.L. (2003) Comparison of four rapid DNA extraction techniques for conventional polymerase chain reaction testing of three *Mycobacterium* spp. that affect birds. Avian Dis. 47, 1486-1490

Tentcheva, D., Gauthier, L., Jouve, S., Canabady-Rochelle, L., Dainat, B., Cousserans, F., Colin, M.E., Ball, B.V., Bergoin, M. (2004a) Polymerase chain reaction detection of deformed wing virus (DWV) in *Apis mellifera* and *Varroa destructor*. Apidologie 35, 431-440.

Tentcheva, D., Gauthier, L., Zappulla, N., Dainat, B., Cousserans, F., Colin, M.E., Bergoin, M. (2004b) Prevalence and seasonal variations of six bee virus in *Apis mellifera* L. and *Varroa destructor* mite populations in France. Appl. Environ. Microbiol. 70, 7185-7191

Tentcheva, D., Gauthier, L., Bagny, L., Fievet, J., Dainat, B., Cousserans, F., Colin, M.E. and M. Bergoin. (2006) Comparative analysis of deformed wing virus (DWV) RNA in *Apis mellifera* L. and *Varroa destructor*. Apidologie 37, 1-7. doi: 10.1051/apido:2005054

Thelwell, N., Millington, S., Solinas, A., Booth, J. Brown, T. (2000) Mode of action and application of scorpion primers to mutation detection. Nucl. Acids Res 28, 3752-3761

Thomas, J.J., Bakhtiar, R., Suizdak, G. (2000) Mass spectrometry in viral proteonomics. Acc. Chem. Res. 33, 179-187

Thompson, M., Cheran, L.E., Zhang, M., Chacko, M., Huo, H., Sadeghi, S. (2005) Label-free detection of nucleic acid and protein microarrays by scanning Kelvin nanoprobe. Biosens. Bioelectron. 20, 1471-1481

Thomson, A. D., Smirk, B. A. (1966) Rod-shaped particles associated with a disease of alkali bees. Virol. 28, 348-350

Todd, J.H., de Miranda, J.R., Ball, B.V. (2007) Incidence and molecular characterization of viruses found in dying New Zealand honey bee (*Apis mellifera*) colonies infested with *Varroa destructor*. Apidologie (in press)

Toma, D.P., Bloch, G., Moore, D., Robinson, G.E. (2000) Changes in period mRNA levels in the brain and division of labor in honey bee colonies. Proc. Nat. Acad. Sci. USA 97, 6914-6919

Topley, E. Davison, S., Leat, N., Benjeddou, M. (2005) Detection of three honeybee viruses simultaneously by a single multiplex reverse transcriptase PCR. Afr. J. Biotech. 4, 763-767

Topolska, G., Ball, B.V., Allen, M.F. (1995) Identification of viruses in bees from two Warsaw apiaries. Medycyna Weterynaryjna 51, 145-147

Towery, R.B., Fawcett, N.C., Zhang, P., Evans, J.A. (2001) Genomic DNA hybridizes with the same rate constant on the QCM biosensor as in homogeneous solution. Biosens. Bioelectron. 16, 1-8

Tyagi, S. Kramer F.R. (1996) Molecular beacons: probes that fluoresce upon hybridization. Nat. Biotechnol. 14, 303-308

Umek R., Lin S.W., Vielmetter J., Terbrueggen R.H., Irvine B., Yu C.J., Kayyem J.F., Yowanto H., Blackburn G.F., Farkas D.H., Chen Y.P. (2001) Electronic detection of nucleic acids: a versatile platform for molecular diagnostics. J. Mol. Diag. 3, 74-84

Uslu, F., Ingebrandt, S., Mayer, D., Bocker-Meffert, S., Odenthal, M., Offenhausser, A. (2004) Labelfree fully electronic nucleic acid detection system based on a field-effect transistor device. Biosens. Bioelectron. 19, 1723-1731

Valentine-Thon, E., van Loon, A.M., Schirm, J., Reid, J., Klapper, P.E., Cleator, G.M. (2001) European proficiency testing program for molecular detection and quantitation of hepatitis B virus DNA. J. Clin. Microbiol. 39, 4407-4412

Varis, A.-L., Ball, B.V., Allen, M.F. (1992) The incidence of pathogens in honey bee (*Apis mellifera*) colonies in Finland and Great Britain. Apidologie 23, 133-137

Verheyden, B., Thielemans, A., Rombaut, B., Kronenberger, P. (2003) RNA extraction for quantitative enterovirus RT-PCR: comparison of three methods. J. Pharm. Biomed. Anal. 33, 819-823

Verkooyen, R.P., Noordhoek, G.T., Klapper, P.E., Reid, J., Schirm, J., Cleator, G.M., Ieven, M., Hoddevik, G. (2003) Reliability of nucleic acid amplification methods for detection of *Chlamydia trachomatis* in urine: results of the first international collaborative quality control study among 96 laboratories. J. Clin. Microbiol. 41, 3013-3016

Wacharapluesadee, S., Hemachudha, T. (2001) Nucleic-acid sequence based amplification in the rapid diagnosis of rabies. Lancet 358, 892-893

Walker, G.T., Fraiser, M.S., Schram, J.L., Little, M.C., Nadeau, J.G., Malinkowski, D.P. (1992a) Strand displacement amplification: an isothermal *in vitro* DNA amplification technique. Nucl.Acid Res. 20, 1691-1696

Walker, G.T., Linn, C.P., Nadeau, J.G. (1996) DNA detection by strand displacement amplification and fluorescence polarization with signal enhancement using a DNA binding protein. Nucl. Acids Res. 24, 348-353

Walker, G.T., Little, M.C., Nadeau, J.G., Shank, D.D. (1992b) Isothermal *in vitro* amplification of DNA by a restriction enzyme/DNA polymerase system. Proc. Nat. Acad. Sci. USA 89, 392-396

Walker, G.T., Nadeau, J.G., Spears, P.A., Schram, J.L., Nycz, C.M., Shank, D.D. (1994) Multiplex strand displacement amplification (SDA) and detection of DNA sequences from *Mycobacterium tuberculosis* and other mycobacteria. Nucl. Acids Res. 22, 2670-2677

Wang, S.S., Thornton, K., Kuhn, A.M., Nadeau, J.G., Hellyer, T.J. (2003) Homogenous real-time detection of single-nucleotide polymorphisms by strand displacement amplification on the BD ProbeTec ET system. Clin. Chem. 49, 1599-1607

Wang, T.-C, Chao, M. (2005) RNA Recombination of Hepatitis Delta Virus in Natural Mixed-Genotype Infection and Transfected Cultured Cells. J. Virol. 79, 2221-2229

Webby, R. Kalmakoff, J. (1998) Sequence comparison of the major capsid protein gene from 18 diverse iridoviruses. Arch. Virol. 143, 1949-1966

Weidenhammer, E.M., Kahl, B.F., Wang, L., Wang. L., Duhon, M., Jackson, J.A., Slater, M., Xu, X. (2002) Multiplexed, targeted gene expression profiling and genetic analysis on electronic microarrays. Clin. Chem. 48, 1873-1882

Weiler, J., Gausepohl, H., Hauser, N., Jensen, O.L., Hoheisel, J.D. (1997) Hybridisation based DNA screening on peptide nucleic acid (PNA) oligomer arrays. Nucl. Acids Res. 25, 2792-2799

Wetzel, T., Jardak, R., Meunier, L., Ghorbel. A., Reustle, G.M., Krczal, G. (2002) Simultaneous RT/PCR detection and differentiation of arabis mosaic and grapevine fanleaf nepoviruses in grapevines with a single pair of primers. J. Virol. Meth. 101, 63-69

Wheeler, D.E., Buck, N., Evans, J.D. (2006) Expression of insulin pathway genes during the period of caste determination in the honey bee, *Apis mellifera*. Insect Mol. Biol. 15, 597-602

Whitcombe, D., Theaker, J., Guy, S.P., Brown, T., Little, S. (1999) Detection of PCR products using self-probing amplicons and fluorescence. Nat. Biotechnol. 17, 804-807

Whitfield, C.W., Band, M.R., Bonaldo, M.F., Kumar, C.G., Liu, L., Pardinas, J.R., Robertson, H.M., Soares, M.B., Robinson, G.E. (2002) Annotated expressed sequence tags and cDNA microarrays for studies of brain and behavior in the honey bee. Genome Res. 12, 555-566

Whittier, C.A., Horne, W., Slenning, B., Loomis, M., Stoskopf, M.K. (2004) Comparison of storage methods for reverse-transcriptase PCR amplification of rotavirus RNA from gorilla (*Gorilla g. gorilla*) fecal samples. J. Virol. Meth. 116, 11-17

Wilhelm, J., Pingoud, A. (2003) Real-time polymerase chain reaction. Chem. Biol. Chem. 4, 1120-1128

Williams, K., Blake, S., Sweeney, A., Singer, J.T., Nicholson, B.L. (1999) Multiplex reverse transcriptase PCR assay for simultaneous detection of three fish viruses. J. Clin. Microbiol. 37, 4139-4141

Wilson, D., Yen-Lieberman, B., Reischl, U., Warshawsky, I., Procop, G.W. (2004) Comparison of five methods for extraction of *Legionella pneumophila* from respiratory specimens. J. Clin. Microbiol. 42, 5913-5916

Wilson, J.W., Bean, P., Robins, T., Graziano, F., Persing, D.H. (2000) Comparative evaluation of three human immunodeficiency virus genotyping systems: the HIV-GenotypR method, the HIV PRT GeneChip assay, and the HIV-1 RT line probe assay. J. Clin. Microbiol. 38, 3022-3028

Wilson, R., Akhavan-Tafti, H., de Silva, R. Schaap, A.P. (2001) Comparison between acridan ester, luminol and ruthenium chelate electrochemiluminescence. Electroanal. 13, 1083-1092

Wolcott, M.J. (1992) Advances in nucleic acid-based detection methods. Clin. Microbiol. Reviews 5, 370-386

Xu, J. (2006) Microbial ecology in the age of genomics and metagenomics: concepts, tools, and recent advances. Mol. Ecol. 15, 1713-1731

Yamazaki, Y., Shirai, K., Paul, R.K., Fujiyuki, T., Wakamoto, A., Takeuchi, H., Kubo, T. (2006) Differential expression of HR38 in the mushroom bodies of the honeybee brain depends on the caste and division of labor. FEBS letters 580, 2667-2670

Yang, X., Cox-Foster, D.L. (2005) impact of an ectoparasite on the immunity and pathology of an invertebrate: evidence for host immunosuppression and viral amplification. Proc. Natl. Acad. Sci. USA 102, 7470-7475

Yang, H., Yang, K., Khafagi, A., Tang, Y., Carey, T.E., Opipari, A.W., Lieberman, R., Oeth, P.A., Lancaster, W., Klinger, H.P., Kaseb, A.O., Metwally, A., Khaled, H., Kurnit, D.M. (2005) Sensitive detection of human papillomavirus in cervical, head/neck, and schistosomiasis-associated bladder malignancies. Proc. Nat. Acad. Sci. USA 102, 7683-7688

Yaron, R., Carmel, R., Katchalski-Katzir, E. (1979) Intramolecularly quenched fluorogenic substrates for hydrolytic enzymes. Anal. Biochem. 95, 228-235

Yue, C., Genersch, E. (2005) RT-PCR analysis of deformed wing virus (DWV) in honey bees (*Apis mellifera*) and mites (*Varroa destructor*). J. Gen. Virol.86, 3419-3424

Yuen, P.K., Li, G,. Bao, Y., Muller, U.R. (2003) Microfluidic devices for fluidic circulation and mixing improve hybridization signal intensity on DNA arrays. Lab Chip 3, 46-50

Zhang, J., Byrne, C.D. (1999) Differential priming of RNA templates during cDNA synthesis markedly affects both accuracy and reproducibility of quantitative competitive reverse transcriptase PCR. Biochem. J. 321, 769-775

Zheng, G., Patolsky, F., Cui, Y., Wang, W.U., Lieber, C.M. (2005) Multiplexed electrical detection of cancer markers with nanowire sensor arrays. Nat. Biotechnol. 23, 1294-1301

Zurfluh, M.R., Giovannini, M., Fiori, L., Fiege, B., Gokdemir, Y., Baykal, T., Kierat, L., Gartner, K.H., Thony, B., Blau, N. (2005) Screening for tetrahydrobiopterin deficiencies using dried blood spots on filter paper. Mol. Genet. Metab. 86, S96-S103

Chapter 4

IMPACT OF VIRUS INFECTIONS IN HONEY BEES

Michel F. A. AUBERT

Table of Contents

1. Introduction

Most of the honey bee viruses may exist in honey bee populations without provoking apparent symptoms. Moreover, colonies or even individuals may be simultaneously infected with different virus species. The development of sensitive molecular diagnostic tools, now reveal that non-symptomatic virus infections are much more common than previously known. The high prevalence of virus infections suggests that most infections probably are harmless. Nevertheless, virus replication may be triggered under certain circumstances to cause honey bee deaths and even colony mortality.

This chapter aims at describing and interpreting literature descriptions on the impact of virus infections in honey bee colonies. Dealing essentially with field observations, this chapter will not review the present knowledge on virology, transmission and pathogenic mechanisms of viruses or symptoms of bee virus diseases. Virus distribution and infection rates must be distinguished from diseases and disease incidence, as described in chapter 1 and will likewise not be treated here. Attention has been given in the examples given to use the terms "infection" and "disease" with the appropriate definitions (see Chapter 6).

Clearly, observed symptoms and colony mortality cannot unambiguously be attributed to a given virus in the examples given, but strong correlational data do suggest an impact from several virus infections on honey bee health.

Not all the viruses that have been isolated from honey bees will be discussed in this chapter because for most of viruses isolated, field data on their impact are simply missing.

2. Some examples of viruses pathogenic for bees

2.1. Chronic bee paralysis virus (CBPV)

Chronic bee paralysis is the only common disease of adult bees with striking well-defined symptoms. In the laboratory, it can be serially transmitted by various routes and follows a classical dose-mortality model (Burnside, 1945; Bailey *et al.*, 1963 and following years).

In Great Britain, the incidence of the disease has decreased from more than 8% of colonies in the '50s towards less than 2% in the '70s (Bailey *et al.*, 1983a). Two epidemiological scenarios of chronic paralysis are related to high densities of bees. The first is related to meteorology: bad

weather → confinement and crowding of bees in the hive → light abrasion of the cuticle → virus transmission (Ball and Bailey, 1997). The other is related to «*too many colonies per available foraging places*» (Ball and Bailey, 1997) as shown by the correlation of the incidence with the geographical density of apiaries (Fig. 1).

In France, old testimonies indicate that symptoms resembling CBPV infections were common. In 1914, Hommel wrote: «*très anciennement connue en France où elle a fréquemment causé de grands ravages... il meurt ainsi des milliers de butineuses et la ruche s'affaiblit énormément…*»[1]. Unfortunately, such testimonies are not sustained by any precise data. If chronic paralysis presently seems rare in Britain, it is now recorded more frequently in France: either it is more accurately diagnosed, or it actually became more frequent as suggested by the fact that what was called "le mal de mai" (the disease of May), is now observed as early as the end of February (Faucon *et al.*, 2002) and during other months as well (Fig. 2). The correlation between colony density and chronic paralysis virus prevalence, as demonstrated by Bailey *et al.* (1983), raises the question of whether the crowding of colonies by migratory beekeeping into large sunflower farming areas may be related to the problems with colony losses recorded in such areas. Indeed, the described symptoms resemble those related to chronic paralysis. However no sampling of sick bees are available for laboratory diagnosis on most of these occasions. An inquiry on winter mortality in France gives an indication on the potential magnitude of the problem: chronic paralysis virus was diagnosed by agarose gel immunodiffusion test (AGID) in 19% of 27 apiaries that collapsed during winter 1999 or 2000 (Faucon *et al.*, 2002).

When severe paralysis symptoms occur and CBPV infections are diagnosed in apiaries, the disease often affects a large proportion of the colonies. Five out of 11, and 6 out of 6 colonies at two separate sites are recorded by Bailey *et al.* (1981) under such circumstances. An example of severe impact of this diseases is given by Kulinčević *et al.* (1975): hairless-black syndrome repeatedly decimated colonies in their apiary in Ohio (USA). For this reason, they undertook selection of paralysis resistant and susceptible lines of honey bees. Reporting beekeeper's testimony, they add that «*this disease has caused serious losses elsewhere*», and «*the symptoms are present, at least in mild form, in almost every apiary observed in several different states*» – unfortunately for the purpose of this review, the authors provide no precise data.

---

[1] «the disease is known since old time in France where it frequently wreaked havoc ... so, thousands of foragers die and the colony is considerably weakened».

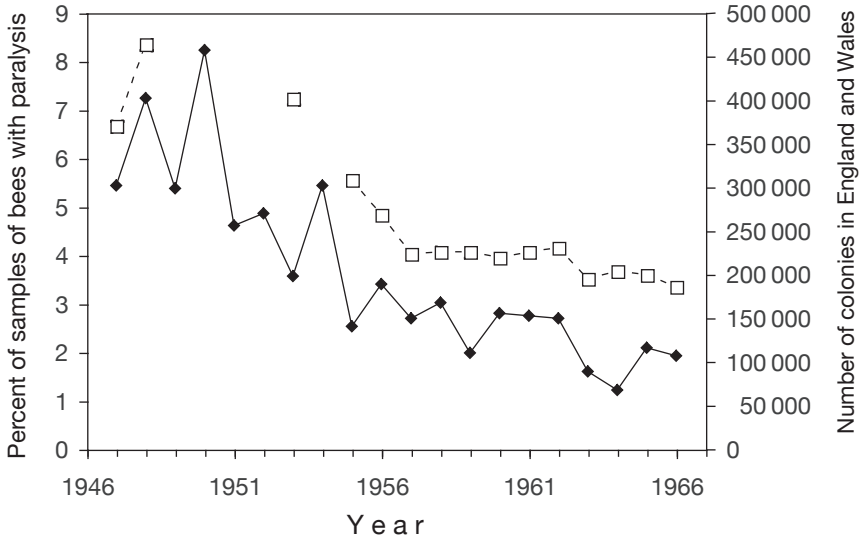


Figure 1. Percentage of samples of adult bee diagnosed as suffering from chronic paralysis (black dots) in England and Wales and total number of bee colonies in the same countries (open boxes) from 1947 to 1966 (Bailey *et al.*, 1983a).

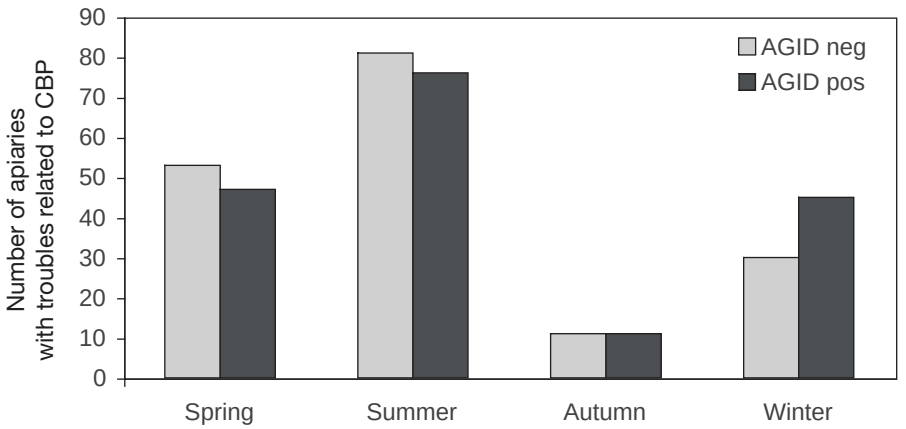


Figure 2. Number of records of apiaries with troubles associated with chronic bee paralysis virus infection in France. Cumulative data from August 2000 to November 2004. (Faucon and Ribière, 2004). AGID: when positive, the agarose gel immunodiffusion test using specific anti-serum indicates the presence of large amounts of virus antigens in the sample and confirms the aetiology of the observed troubles (see Chapter 3).

2.2. Black queen cell virus (BQCV)

BQCV also belongs to the category of bee viruses that have not been isolated incidentally but because its pathogenic properties : "*for many prepupae and a few pupae found dead in the cells in which queens were being reared*" (Bailey and Woods, 1977). Additionally, similar mortality and dead pupae of worker bees were observed in the same apiary the following year. Since then, BQCV has been shown to be a cause of death of queen larvae in Australia (Anderson, 1993) and of drone brood in Germany (Siede and Buchler, 2003).

Bailey *et al.* (1981), surveying several bee colonies demonstrated a significant correlation between the infection rate of adult bees by BQCV, BVY, *Nosema apis*, and the number of dead bees found in traps fixed to these colonies (Fig. 3). This individual bee mortality was, however, not sufficient for provoking colony losses and the effect on bee mortality from different pathogens in this case cannot be separated. Nevertheless, also limited mortality effects from pathogens on individual bees may have insidious consequences for infected colonies given another context.

Carrying on their survey, Bailey *et al.* (1983) demonstrated a strong correlation between BQPV, BVY, FV (filamentous virus), or CWV infections with colony mortality. For example, BQCV was present in 71% of dead colonies, instead of 35% of live colonies sampled during the same period. For BVY, the data are even stronger : BVY was present in one dead colony out of three, instead of one out of ten in live colonies (Fig. 4).

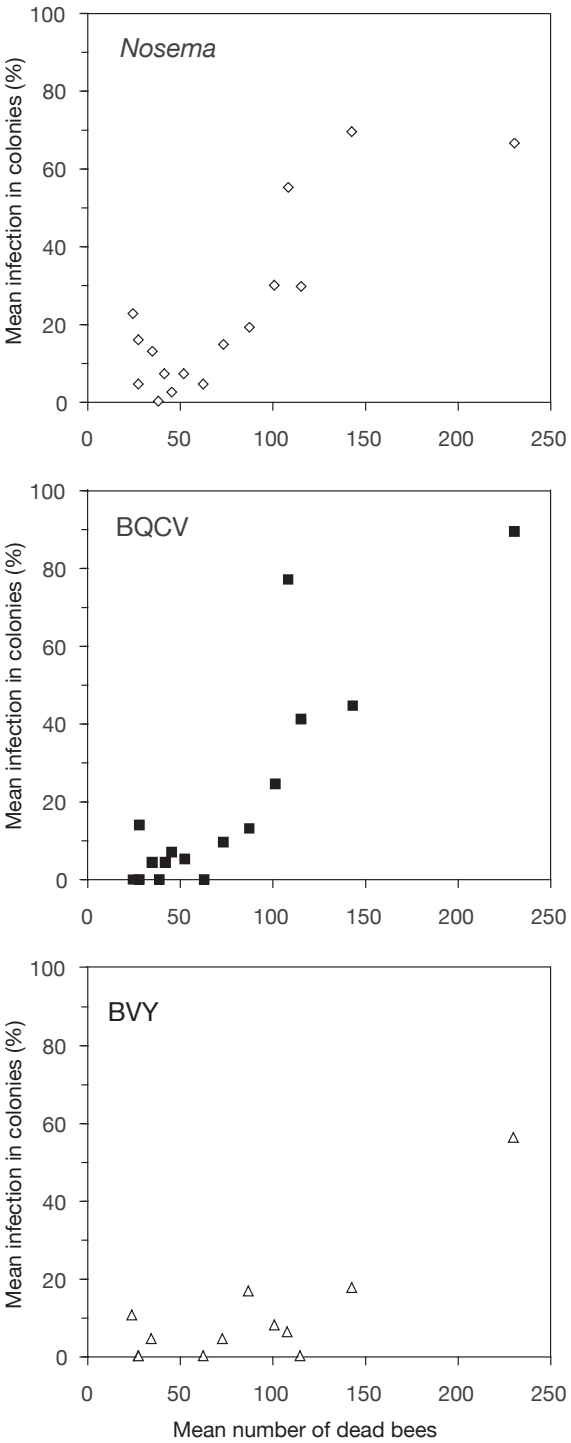
A co-infection by *Nosema apis* and BQCV and to a lesser extend with BVY appeared more harmful than infection by *Nosema apis* alone. Although only indicative, the repeated correlations demonstrate that virus infections in honey bees may be of greater importance for colony losses than presently recognized by apiculturists.

2.3. Acute bee paralysis virus (ABPV)

Despite its lethal effect when sprayed, fed, or injected into pupae and adult workers in laboratory conditions (see Chapter 1), ABPV has rarely been detected in connection with colony collapse until the *Varroa destructor* invasion into Europe. Since then, results from Ball and Allen (1988) suggest

---

Figure 3 (opposite page). Correlation between the monthly mean percentage of *Nosema apis*, BQCV or BVY infection of adult bees in 25 bee colonies and the mean monthly number of dead bees collected in traps per colony from September 1978 to January 1979 (adapted from Bailey *et al.*, 1981).


Nosema
BQCV
BVY
Mean infection in colonies (%)
Mean number of dead bees
0
20
40
60
80
100
0
50
100
150
200
250

that ABPV infections may be a primary cause of colony mortality when severely infested by *V. destructor* in Germany: the percentage of dead bees that were ABPV positive (by serology) was less than 3%, 44% or more than 80% when colony mite infestations were low, medium or high respectively.

In France, Faucon *et al.* (1992) screened 61 samples of brood with problems after observing that heavy mite infestations were followed by various problems, including acute paralysis. Using AGID for detecting ABPV and SBV, they found 6 brood samples (9.8%) ABPV positive, and the same proportion infected by SBV.

In Hungary, Bakonyi *et al.* (2002) using RT-PCR demonstrated that despite findings that ABPV was equally present at the *apiary* level in apiaries with or without problems, significantly more *colonies* were infected with ABPV in apiaries reporting problems with colony mortality (Tab. I). Thus, available field data on ABPV support the hypothesis of colony level mortality induced by virus infections.

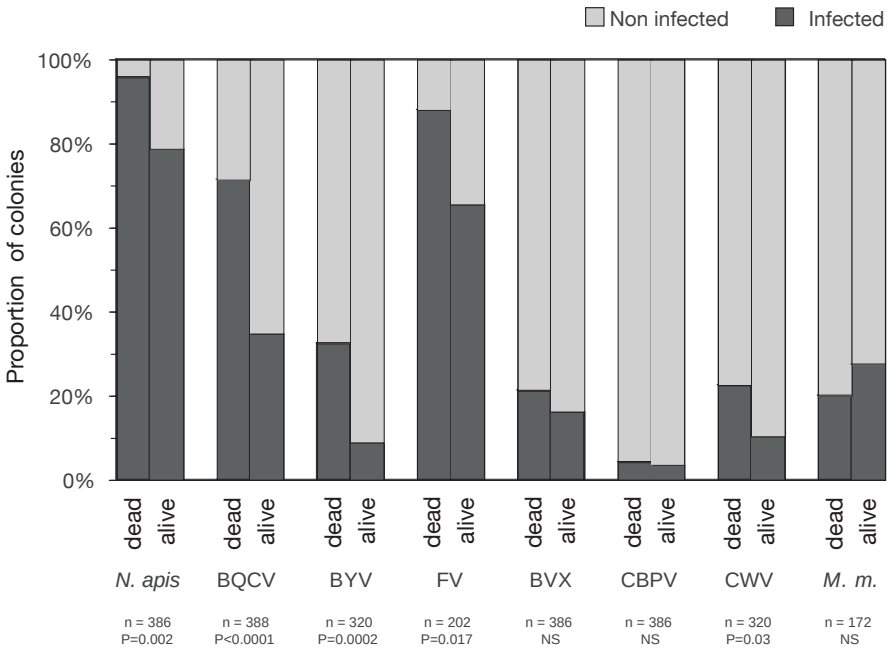


Figure 4. Percentage of colonies infected with *Nosema apis*, BQCV, BVY, FV, CBPV, CWV or *Malpighamoeba mellificae* among bee colonies found dead in late winter or in samples of dead bees from live colonies at the same period, from 1978 to 1982 in Rothamsted region (UK) (adapted from Bailey *et al.*, 1983). n: total number of colonies; P: one sided probability of the observed distribution (factorial exact Fisher test); NS: distribution is not significantly different from random.

Table I. Diagnosis of ABPV by RT-PCR in apiaries and colonies infested by *Varroa destructor* in Hungary (adapted from Bakonyi *et al.*, 2002).

	Apiaries		Colonies	
	With problems	Without problems	in one collapsed apiary	in 12 apiaries without problems
Positive	7	8	5	4; 4; 4; 2 [a]
Negative	1	4	5	23; 32; 23; 22 [a]
Fisher factorial test	P = 0.31 (NS)		P=0.04; 0.01; 0.04; 0.01 [a]	

[a] Data correspond to spring 1999, autumn 1999, spring 2000, autumn 2000 respectively. As for the following tables, statistical significance level has been evaluated using StatXact 6.0 of Cytel Software Corporation.

2.4. Deformed wing virus (DWV) and others

No doubt that DWV that was first isolated from diseased adults (in association with *Varroa destructor*) has an impact on colonies. There is a strong relationship between DWV ELISA optical density in bees and their deformity at emergence (Bowen-Walker *et al.,* 1999) (Fig. 5).

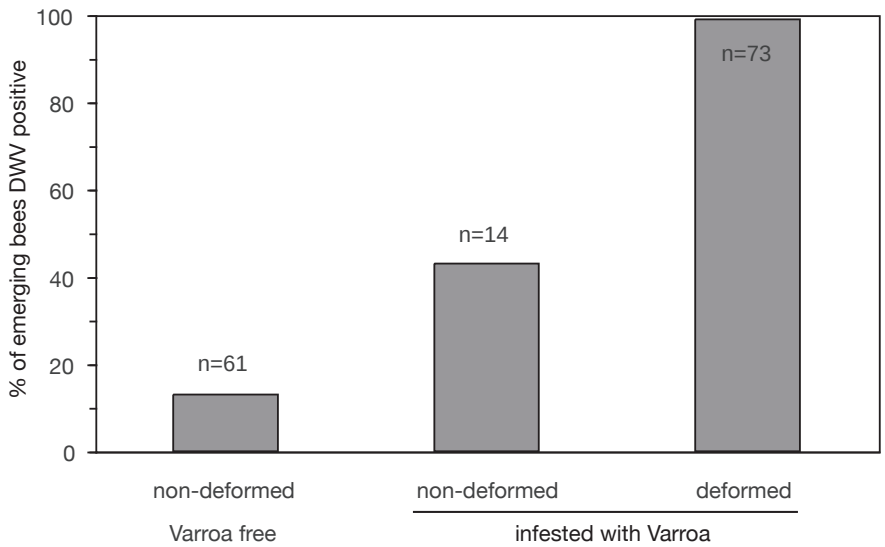


Figure 5. Relationship between the presence of DWV revealed by ELISA and deformity of bees at emergence (adapted from Bowen-Walker *et al.*, 1999).

High pupal mortality as well as adult bee mortality in an apiary near Paris led Chastel *et al.* (1990), to isolate and identify two different viruses from colonies exhibiting «*unusual symptoms*»: Egypt bee virus and DWV. Beekeepers in the area observed high colony mortality (some lost all their colonies) and wild colonies known to be established in tree holes in the vicinity of the apiaries demised. The authors attributed these deaths to the virus infections (although there is no data virus prevalence in non-symptomatic colonies in the area).

Significantly, ABPV, DWV, CWV, SPV have been more often associated with bee mortality since the spread of *Varroa destructor* (review in Ball, 1977). But, DWV infection is far from being always clearly associated with colony demise : in a context of severe infestation by *Varroa destructor*, Nordström *et al.* (1999) found DWV associated with colony collapse in one apiary in Sweden and ABPV in one apiary in Denmark but they indicate that several other apiaries were also infected without colony losses. Seven out 8 studied colonies collapsed several weeks following the first positive CWV immunodiffusion test on dead bee samples. High *V. destructor* infestation rate (measured by the number of *V. destructor* found dead on the screen bottom boards), were always associated with CWV positive test in live or dead bees.

More recent British studies have shown that severely mite-infested colonies may die from SPV or DWV, and indicate a higher prevalence of CWV in such colonies.

Unfortunately, whereas many authors agree to consider that the spreading of *Varroa destructor* is a catastrophe which enhanced the impact of several virus infection, large scale data that could provide numbered comparisons between the before- and after-*Varroa destructor* era are lacking.

3. Some recent surveys on the prevalence of several virus infections

In an area where significant losses of colonies had been reported by beekeepers, Ribière *et al.* (2002) used on the same bee samples both an agarose gel immunodiffusion (AGID) test for CBPV and a RT-PCR technique developed for the same virus. Samples were taken from apiaries with or without colony losses, all located in France. All apiaries with clinical symptoms of chronic paralysis and colony losses were positive with AGID (and for RT-PCR), while the three apiaries without losses were negative for AGID (either RT-PCR was positive or not) (Tab. II). AGID is

an appropriate method for demonstrating the presence of large amounts of virus antigen, RT-PCR is a sensitive method for localising virus RNA or at least the homologous sequence searched for. Detection of virus RNA using RT-PCR does not necessarily imply that the virus multiplies actively. In spite of the low number of apiaries tested, the study shows a correlation between overt CBPV infections and colony losses – but no correlation between the presence of CBPV and colony losses.

Tentcheva *et al.* (2004) performed specific RT-PCR for identifying ABPV, BQCV, CBPV, DWV, KBV and SBV in adults and pupae sampled during spring, summer or autumn 2002 in 36 apiaries throughout France. Few details are provided by the authors on the health status of colonies, however, they indicate that the sampled colonies «*were checked for the absence of clinical signs and were considered to be valid for honey production*». Thus, we assume that this checking was performed before the first sampling. They found that all apiaries except one were infected by at least one virus species. The only negative apiary was located in the Isle of Ouessant which so far is free from *V. destructor*. Most of the continental apiaries (all *V. destructor* infested) were found to be infected by several virus species : 34/35 by at least two viruses and 32/35 by at least 3 viruses. The most frequent virus was DWV (33/35) and the least common was KBV (6/35).

As Tentcheva *et al.* (2004) conclude that their results «*provide clear evidence that bee viruses infection occur persistently in bee colonies despite the lack of clinical signs*», we deduce that these colonies did not show any

Table II. Diagnosis of chronic bee paralysis virus infection in 12 apiaries in the areas of Sens and Limoges (France) by AGID and RT-PCR tests (adapted from Ribière *et al.*, 2002); The fact that all apiaries (and most of the colonies) with symptoms were AGID positive while apiaries (or colonies) without symptoms were AGID negative (or rarely positive) is statistically significant. On the opposite, positive RT-PCR may be obtained from apiaries (or colonies) with or without symptoms.

	Apiaries				Colonies			
	With clinical symptoms and losses		Without any symptoms		With clinical symptoms and losses		Without any symptoms	
	AGID	RT-PCR	AGID	RT-PCR	AGID	RT-PCR	AGID	RT-PCR
Positive	9	9	0	2	24	25	0	6
Negative	0	0	3	1	1	0	8	0
Fisher factorial test	AGID: P=0.004; RT-PCR: NS				AGID: P<0.0001; RT-PCR: P=0.053			

clinical sign during the whole sampling period, i.e. from March to November 2002 whereas it is not clearly stated in the article. It may be surprising that in a context where several French beekeeper associations complain for frequent and severe colony mortality, no such losses were reported from this rather large number of apiaries. This result may give a clue for an average order of magnitude for colony losses in France in the year 2002. A simple probability calculation indicates that we would have a 5% probability to observe no losses in any of 35 randomly selected apiaries: with an hypothesised loss frequency equal to 8% (8% of apiaries experience losses during the sampling period)[2]. Moreover, the authors state that colonies were «*considered to be valid for honey production*», which suggests that strong and healthy colonies were selected for this sampling.

In summary, the results of Tentcheva *et al.* (2004) demonstrate the wide distribution of honey bee viruses in French apiaries and clearly confirm that viruses are common in apparently healthy colonies. Ideally, weak colonies or colonies with symptoms should also have been tested in parallel using quantitative techniques to distinguish clearly between covert and overt infections. If quantitative PCR is not available, the use of AGID and PCR in parallel on the same colonies as performed by Ribière *et al.* (2002) is cheaper and may accomplish the same purpose.

Chen *et al.* (2004) used multiplex RT-PCR for BQCV, DWV, KBV and SBV on samples taken from 56 colonies. Compared with the previous survey, ABPV and CBPV were not searched for and the results are given for all the colonies whereas they belonged to different apiaries. All colonies except one were infected by at least one virus species. Many colonies were found to be infected by several virus species : 24/56 by at least two viruses and 5/56 by at least 3 viruses. The most frequent viruses were DWV (29/56) and BQCV (28/56). The least frequent were SBV and KBV (9/56 each). The authors provide no information on the health status of colonies, except that «*tested bees rarely had overt symptoms of infection*». For the aim of this chapter, this study has the same limitation as the previous one.

Carpana *et al.*(1990) sampled both colonies with disease symptoms and apparently healthy colonies from various parts of Italy; 21 had no clinical symptoms, the others had been observed with brood or adult mortality (33 and 5 respectively), paralysis (8) or depopulation (2). Instead of following the approach of AGID and RT-PCR, they evaluated the abundance of viruses in brood or adult bees using electron microscopy nega-

[2] Similarly, the probability to observe no symptoms in a random sample of 35 apiaries, would be as small as 1% and 0.1% if the sample was taken from a total population of apiaries with a loss frequency equal to 12 and 18% respectively.

tive staining or a specific staining using an ABPV antiserum. The highest concentrations of viruses were clearly associated with diseased colonies (Tab. III).

4. Towards bee virus control?

Control of virus diseases can only be based on preventive and indirect measures. They may consist of a) maintaining the innate defences of the host, b) controlling other pathogens that may act in synergy with viruses, c) preventing virus invasion into non-infected areas or non-infected apiaries, or their spreading between colonies within apiaries.

a) Maintaining the innate defences of bees

Viruses may be pathogenic alone or the pathogenicity may be triggered by the infection by other pathogens, toxicants, hunger or meteorological conditions. Whereas the mechanisms of virus activation remain obscure, in any case, strong colonies can better maintain homoeostasis (e.g, regulate temperature, removal of sick and dead individuals and other social activities). Therefore, common sense suggests that colonies should not be exposed to insecticides and other toxicants and must be properly cared for to avoid starvation.

Bee selection should maintain or enhance natural cleansing behaviour of adult bees aimed at eliminating sick larvae and adults (see Chapter 1 and Chapter 8).

Table III. Electron microscopy examination of viruses (ABPV or other viruses) in brood or adult bee sampled in Italian colonies during 1989-1990 (adapted from Carpana *et al.*, 1990).

Electron microscopy (specific or non specific negative staining)	Apiaries	
	With symptoms	Without any symptoms
Positive[a] (+++ or ++)	17	3
Positive (+)	1	4
Negative	29	14
Pearson's χ^2	P = 0.016	

[a] The intensity of the positive results is drawn from the authors' tables.

b) Controlling other pathogens that may act in synergy with viruses

The association of *Nosema apis* with BQCV and BVY has been evoked in this chapter. Chapter 1 describes other associations between viruses and non-viral pathogens. Good beekeeping practice recommends to control non-viral pathogens as far as efficient methods (which are beyond the scope of this chapter) are available. Appropriate control of *Varroa destructor* is obviously the appropriate method for preventing damages from virus infections associated with this parasite. To what extent *Varroa destructor* tolerance in honey bees also reflects resistance to virus infections remains to be tested.

c) preventing virus invasion or transmission

This question will be extensively discussed in Chapter 9. We will here only mention the fact that for newly discovered viruses, the original geographical distribution is unclear. That may be at the origin of controversy on whether they may have been absent until recently in some continents or present since long but never diagnosed. This is the case for the Kashmir bee virus (KBV) first described by Bailey *et al.* (1977) in workers of *Apis mellifera* injected with extracts of the Asian species *Apis cerana*. As these authors had never observed this virus in *A. mellifera* workers injected with extracts of the same species, they concluded that KBV was specific of *A. cerana*. However in strict logic, whether KBV came from *A. cerana* or the injection triggered a virus already present in *A. mellifera* is unknown. KBV was then demonstrated to occur naturally in *Apis mellifera* in Australia (Bailey *et al.*, 1979) and other countries in Australasia (see Chapter 1). Whereas KBV has been found in *A. mellifera* in other continents in the following years, the lack of specificity of strain characterization and the paucity of data on virus incidence maintain the question of whether or not this virus has been imported in Europe. If KBV is an imported virus, the fact that some authors do not consider it as a major pathogen for bees cannot justify the present disguised anarchy in inter-continental movements of bees. Perhaps we are just ignorant of the troubles KBV may provoke alone or in association with other factors. Other more pathogenic viruses may be waiting to be embarked on the next plane.

5. Discussion

5.1. Is the impact of honey bee viruses significant?

As almost all the viruses detected in honey bees have been shown to often be present in apparently healthy colonies, it is legitimate to ques-

tion if their impact is non-significant under normal conditions (i.e. if overt infections are context dependent)?

Indeed, when losses or symptoms in the field have been correlated with high virus titres in dead bees, this does not necessarily demonstrate a causal relationship between virus multiplication and the observed symptoms. In other words, viruses may actively multiply mainly in weak colonies that were already doomed, rather than colonies being weakened by virus infections. The causal relationship in the field between virus infections and individual bee and colony mortality needs to be studied in more detail. Certainly, correlational evidence suggests that virus infections may have a larger impact than is generally accepted among apiculturists. However, the causal relationships should be proven and the importance of context dependence impact of infections need to be clarified.

Which are the strongest evidence suggesting virus infections are important for honey bee health?

Firstly, most of the honey bee viruses have been discovered in dead or moribund bees (e.g. Bailey *et al.*, 1981; Sitaropoulou *et al.*, 1989 and others). Even the KBV that has been incidentally discovered by Bailey *et al.* (1975), and was considered as «*a relatively harmless virus*» (Bailey *et al.*, 1979), has been subsequently isolated from *dead* bees in Fiji (Anderson, 1990), in Canada and New-Zealand (Anderson, 1985), in Spain (Ball, unpublished), in the USA (Hung *et al.*, 1996), and in Germany (Siede and Buchler, 2004). Since detectable infections are more common in dead bees than in live bees, the infections may have caused the deaths.

Secondly, several studies demonstrate that although viruses are also common in healthy bees, many of them are found in larger amounts in dying or dead bees, which explains why less sensitive methods of virus detection are generally only positive on dead or moribund bees contrary to bio-molecular techniques as explained earlier.

Thirdly, administration of virus suspension to bees as described in laboratory experiments provokes individual changes. The addition of all the individual changes may result in severe consequences at the colony level (see references in Tab. IV).

Concluding a work on the incidence of virus diseases in the honey bee, Bailey (1967) wrote «*The sum of unseen losses in normal colonies may well exceed those in the very few colonies that become severely diseased, and virus-free colonies might be more vigorous …*». The same author (Bailey *et al.*, 1981) used traps at the hive entrance and uncovered an otherwise unseen mortality in 25 colonies co-infected by *Nosema apis*, BQCV and BVY. This mortality was at its peak in May and June, a period when natu-

ral mortality should be low considering that most of the over-wintered population of workers have already died and that most of the adult bees are young. The bee losses, probably related to the co-infection by the three pathogens, were concealed while the rapid production of young adults at this time of the year maintained a net growth of bees in most of the colonies. Considering that bees have a short life span, finding dead bees may be normal. Nevertheless, abnormal deaths may be preceded by no detectable symptoms and colonies with bees having shortened life span can stay alive during spring, summer and autumn, but fail to survive the winter. In fact, only specially designed experimental protocols may offer evidence of a reduction of the normal life span. And death is not the only unseen toll imposed by viruses. Without provoking any easy-to-observe symptom, several bee viruses provoke other detrimental effects including a lesser adaptation to cold and non-beneficial changes in brood care or foraging behaviours (Tab. IV and Chapter 1).

In other words, even when colonies seem well in May, June and July, viruses (and their associated pathogens) may have an impact, while longevity of adults is crucial for surviving long winters. Mathematical modelling applied to bee population dynamics demonstrates that colony collapse is a possible end of slightly reduced individual life expectancy of adult bees

Table IV. Some examples of "non apparent" but detrimental effects on individuals of honey bee virus infections. For more complete information see Chapter 2.

Virus	Method and main result	Reference
ABPV	Inoculation of pupae → life span of adults reduced by nearly 25% → reduction of the brood care period by nurse bees	Ponten and Ritter, 1992
BVX	Inoculation of adults → mean time for 50% mortality : 49 days (control: 69 days)	Bailey *et al.*, 1983
KBV	Virus given per os to larvae → disappearance of larvae = 16% (control: 5%)	Anderson and Gibbs, 1989
SBV	Virus given per os to adults with pollen → mean time for 50% mortality : 22 days (control: 56 days)	Bailey, 1969
	Inoculation of newly emerged adults → foraging period starts earlier → failing to forage pollen → increased susceptibility to cold	Bailey and Fernando, 1972
	Virus given per os to larvae → disappearance of larvae = 21% (control: 5%)	Anderson and Gibbs, 1989

(Martin, 2001). This suggests that even slight effects on honey bee life span of virus infections may result in colony losses. To assess the full impact at colony level of the individual effects from various virus infections, extensive long-term studies are needed that include monitoring of virus prevalence and colony survival over several years. Such studies may lead us to conclude that some viruses had been so far considered harmless from our own ignorance.

5.2. Why assessing the impact of virus infection in bees is so difficult?

Several difficulties ranging from the organisation of surveillance to the biology of bees themselves, hamper the assessment of the impact of bee virus infections.

5.2.1. Organisation of surveillance

In many situations, the surveillance system is passive : problems may be reported by beekeepers, but not studied by epidemiologists using an appropriate sampling. Several systematic surveys have consisted in series of laboratory diagnoses on samples sent by beekeepers. In such circumstances, mild problems with delayed severe impact cannot be assessed. As stated earlier, most of the pathological traits of virus infection remain unseen by the beekeeper (and even by the experimenter). When confronted to sudden depopulation or losses of colonies, it may be difficult to consider that this may be the result of problem developing over a long time period.

5.2.2. Laboratory diagnostic methods

As in any newly explored scientific field, honey bee virus detection methods are not used by many laboratories. The specificity and sensitivity of these methods vary accordingly to the technique used (serology vs molecular methods) and for the same techniques, results may vary between laboratories. Consequently, the distribution of honey bee virus is still poorly described and troubles that may cause may be wrongly attributed to other better known pathogens. Additionally, bio-molecular techniques should not only aim at identifying the presence of virus (or more precisely of homologous RNA sequences of a given virus) but should also provide information on the status of the infection (overt or covert).

5.2.3. Virus-host relationships and co-adaptation

Because honey bee viruses are often present simultaneously with other viruses or pathogens, and because several studies revealed the pres-

ence of viruses in many apparently unaffected bee colonies, it is generally difficult to identify a single specific virus as the causative agent of observed effects.

5.2.4. Bee biology considerations

Under laboratory conditions, it is impossible to accurately reproduce the appropriate living conditions for individual honey bees kept separately from their colony. The lethality induced by captivity may exacerbate the pathogenicity of any honey bee virus that would be possibly harmless under natural condition. Moreover, the stress of the artificial administration of any virus to honey bees may have the same effect. That makes the results of laboratory trials on caged bees difficult to transpose into the real world.

On the other hand, many factors affect the population dynamics of bee colonies: weather, vegetation dynamics, action of other pathogens, local flow of bee genes … None of these factors are normally under the control of the experimenters. That makes it impossible a) to process experiments on entire colonies within strictly controlled designs, b) to repeat experiments on whole colonies under the same circumstances. Hence, comparing data from various field observations is difficult or perhaps even invalid.

In any case, the use of control colonies is always a matter of discussion: if they are too close to the treated (infected) ones, they rapidly become contaminated (bees "drift" between colonies) – if they are too far apart, they are no longer real controls since they are kept under other conditions (sources of food, micro-climate, ...).

6. Conclusion

Field observations on the incidence of honey bee virus infections suggest that honey bee individuals as well as colonies may suffer severely from the infections. Furthermore, it seems likely from field reports that the development of overt infections is context dependent. In other words, honey bees may carry infections but suffer little or no consequences, but given other conditions the infection may become overt and the impact fatal at colony level, even from rather limited effects at the individual bee level. Nevertheless, it is necessary to unravel the causal relationship between colony deaths and virus infections, since available field data are not conclusive.

For many bee viruses, pathogenicity is still poorly understood. How-

ever, field observations show that several virus infections are associated with honey bees symptoms and mortality. Viruses are among the simplest parasitic forms, they are widely spread and always ready to adapt themselves to new conditions – included those created by beekeepers. Moreover, the recent introduction of *Varroa destructor* demonstrated that conditions and routes of transmission may be dramatically changed. Problems with honey bee viruses are real, they require more appropriate studies to be assessed and controlled. As stated by Ball (2004), «*we should certainly be alert and prepared for more trouble with viruses*».

References

StatXact. Version 6, Cytel Software Corporation, Cambridge (USA).

Anderson D.L. (1985) Virus of New Zealand honey bees, New Zealand Beekeep. 188, 8-10.

Anderson D.L., Gibbs A.J. (1989) Transpuparial transmission of Kashmir bee virus and sacbrood virus in the honey bee (*Apis mellifera*), Ann. Appl. Biol. 114, 1-7.

Anderson D. (1990) The disease and pest situation in the Pacific region ; the threat of exotic diseases and pests from Papua New Guinea ; diseases and disorders of queen bees, Australasian Beekeep. 92, 227-231.

Anderson D.L. (1993) Pathogens and queen bees, Australasian Beekeep. 94, 292-296.

Bailey L. (1967) The incidence of virus diseases in the honey bee, Ann. Appl. Biol. 60, 43-48.

Bailey L. (1969) The multiplication and spread of sacbrood virus of bees, Ann. Appl. Biol. 63, 483-491.

Bailey L., Fernando E.F.W. (1972) Effects of sacbrood virus on adult honeybees, Ann. Appl. Biol. 72, 27-35.

Bailey L., Gibbs A.J., Woods R.D. (1963) Two viruses from adult honey bees (*Apis mellifera* Linnaeus), Virol. 21, 390-395.

Bailey L., Ball B.V., Woods R.D. (1976) An iridovirus from bees, J. Gen. Virol. 31, 459-461.

Bailey L., Wood R.D. (1977) Two more small RNA viruses from honey bees and further observations on sacbrood and acute bee paralysis viruses, J. Gen. Virol. 37, 175-182.

Bailey L., Carpenter J.M., Woods R.D. (1979) Egypt bee virus and Australian isolates of Kashmir bee virus, J. Gen. Virol. 43, 641-647.

Bailey L., Ball B.V., Perry J.N. (1981) The prevalence of viruses of honey bees in Britain, Ann. Appl. Biol. 97, 109-118.

Bailey L., Ball B.V., Perry J.N. (1983a) Association of viruses with two protozoal pathogens of the honey bee, Ann. Appl. Biol. 103, 13-20.

Bailey L., Ball B.V., Perry J.N. (1983b) Honeybee paralysis: its natural spread and its diminished incidence in England and Wales, J. Apic. Res. 22, 191-195.

Bakonyi T., Farkas R., Szendroi A., DobosKovacs M., Rusvai M. (2002) Detection of acute bee paralysis virus by RT-PCR in honey bee and *Varroa destructor* field samples: rapid screening of representative Hungarian apiaries, Apidologie 33, 63-74.

Ball B.V. (2004) The trouble with viruses, Bee World 85, 25.

Ball B.V., Allen M.F. (1988) The prevalence of pathogens in honey bee (*Apis mellifera*) colonies infested with the parasitic mite *Varroa jacobsoni*, Ann. Appl. Biol. 113, 237-244.

Ball B. V., Bailey L.(1997) Viruses, in: Morse R.A. and Flottum K.(Ed), Honey bee pests, predators and diseases, A.I. Root Company, Medina, pp 11-32.

Bowen-Walker P.L., Martin S.J., Gunn A. (1999) The transmission of deformed wing virus between honeybees (*Apis mellifera* L.) by the ectoparasitic mite *Varroa jacobsoni*, J. Invertebr. Pathol. 73, 101-106.

Burnside C.E. (1945) The cause of paralysis of bees, Am. Bee J. 85, 354-355.

Carpana E., Vecchi M. A., Lavazza A., Bassi S., Dottori M.(1990) Prevalence of acute paralysis virus (APV) and other viral infections in honeybees in Italy. Proceedings of the international symposium on recent research on bee pathology, Ghent (Belgique),155-165.

Chastel C., Robaux P., LeGoff F.(1990) New virus from honey bee colonies. Is *Varroa* a vector or does he amplify viruses ? Proceedings of the international symposium on recent research on bee pathology, Ghent (Belgique),150-154.

Chen Y., Zhao Y., Hammond J., Hsu H., Evans J., Feldlaufer M. (2004) Multiple virus infections in the honey bee and genome divergence of honey bee viruses, J. Invertebr. Pathol. 87, 84-93.

Faucon J.P., Vitu C., Russo P., Vignoni M. (1992) Diagnostic de la paralysie aiguë: application à l'épidémiologie des maladies virales en France en 1990, Apidologie 23, 139-146.

Faucon J.P., Mathieu L., Ribière M., Martel A.C., Drajnudel P., Zeggane S., Aurières C., Aubert M. (2002) Honey bee winter mortality in France in 1999 and 2000, Bee World 83, 14-23.

Hommel R.(1914) Apiculture, Baillière, Paris.

Hung A.C.F., Shimanuki H., Knox D.A. (1996) Inapparent infection of acute paralysis virus and Kashmir bee virus in the U.S. honey bees, Am. Bee J. 136, 874-876.

Kulincevic J.M., Rothenbuhler W.C. (1975) Selection for resistance and susceptibility to hairless-black syndrome in the honeybee, J. Invertebr. Pathol. 25, 289-295.

Martin C., Provost E., Roux M., Bruchou C., Crauser D., Clement J.L., LeConte Y. (2001) Resistance of the honey bee, *Apis mellifera* to the acarian parasite *Varroa destructor* : behavioural and electroantennographic data, Physiol. Entomol 26, 362-370.

Nordström S., Fries I., Aarshus A., Hansen H., Korpela S. (1999) Virus infections in Nordic honey bee colonies with no, low or severe *Varroa jacobsoni* infestations, Apidologie 475-484.

Ponten A., Ritter W. (1992) Influence of acute paralysis virus attacks on brood care in honeybees, Apidologie 23, 363-365.

Ribière M. (2004) Les viroses de l'abeille *Apis mellifera* L., recherche des causes favorisantes. Rapport final pour le Programme Apicole Communautaire

Ribière M., Triboulot C., Mathieu L., Aurières C., Faucon J.P., Pépin M. (2002) Molecular diagnosis of chronic bee paralysis virus infection, Apidologie 33, 339-351.

Siede R., Buchler R. (2003) Symptomatic black queen cell virus infection of drone brood in Hessian apiaries, Berl. Münch. Tierärztl. Wochenschr. 116, 130-133.

Siede R., Büchler R. (2004) First detection of Kashmir bee virus in Hesse, Berl. Münch. Tierärztl. Wochenschr. 117, 12-15.

Sitaropoulou N., Neophytou E.P., Thomopoulos G.N. (1989) Structure of the nucleocapsid of a filamentous virus of the honey bee (*Apis mellifera*), J. Invertebr. Pathol. 53, 354-357.

Tentcheva D., Gauthier L., Zappulla N., Dainat B., Cousserans F., Colin M.E., Bergoin M. (2004) Prevalence and seasonal variations of six bee viruses in *Apis mellifera* L. and *Varroa destructor* mite populations in France, Appl. Environ. Microbiol. 70, 7185-7191.

Chapter 5

INFECTION STRATEGIES OF INSECT VIRUSES

Rosemary S. HAILS, Brenda V. BALL, and Elke GENERSCH

Table of contents

1. Introduction

Viruses are known to cause infections with wide-ranging consequences: from lethal infections causing death of the host to asymptomatic infections with little impact on host fitness. Depending on the progression of the infection (fast killing – slow killing – not lethal), virus infections can be transmitted vertically as well as horizontally. As a simple rule one can say that the more virulent, lethal infections are usually transmitted horizontally and the less virulent or even a-virulent, asymptomatic infections may be transmitted vertically, although the relationship between virulence and transmission strategy is not absolute. Here we review the range of infection strategies adopted by viruses, drawing some examples from vertebrates, but focusing principally on insect and bee viruses, and offer some simple rules for defining classes of infection.

2. Infection strategies

Viruses are infectious entities ranging in size between 14 nm (circoviruses) (Shivaprasad *et al.*, 1994; Tischer *et al.*, 1982) and more than 300 nm (poxviruses) (Holowczak *et al.*, 1975). Virus particles (virions) containing the viral genome are assembled from protein subunits and are sometimes enclosed by a lipid membrane (envelope). The genetic information of viruses consists of either DNA or RNA and encodes for viral structural components, some regulatory proteins (e. g. transactivators), and virus specific enzymes (e. g. proteases and polymerases). Viruses lack any own protein synthesis machinery or metabolic system but instead depend upon the host cell for reproduction and are, therefore, obligate intracellular parasites. As such they modify the host cell's processes in a way to ensure optimal viral replication, virion assembly and release of new infectious viruses.

Detailed studies on the interaction between host cells and viruses have only been feasible since the establishment of cell culture *in vitro* more than fifty years ago (Gey *et al.*, 1952; Sanford *et al.*, 1952). Since then, many primary animal and human cell cultures and cell lines have been extensively exploited in the study of viruses helping to understand the properties of animal viruses and to elucidate the mechanisms by which viruses cause disease. We now know that the interaction between viruses and host cells can result in wide-ranging consequences. The lytic cycle leading to large scale production of virus progeny and death of the host cell is not the only option. There is also the possibility of long term survival of the infected cell in the course of latent or persistent infections, and even im-

mortalization of cells can be the consequence of viral infection.

Depending on the effect on the host cell, virus infections are now categorized as follows:

1. Lytic infections characterized by large scale virus production and destruction of the host cell.
2. Persistent infections characterized by a continuous production of small amounts of viruses by the infected cells and host cell survival (Oldstone, 2006). Viral gene expression and virus production are down-regulated to a level allowing this survival of the host cell.
3. Latent infections characterized by host cell survival without production of infectious particles. Instead, the viral genome stays within the cell either integrated into the host genome or as an episome (Efstathiou and Preston, 2005; Klein, 1982). The latent stage is maintained by the expression of a few latency specific genes.
4. Infections leading to the immortalization of the host cell due to the integration of the viral genome. This integration results in the ability to infinite cell division and can result in transformation, i. e. tumorgenesis (Klein, 1972; Rapp and Westmoreland, 1976; Varmus, 1988; Wyke, 1981).

3. Infection strategies of insect viruses

To apply the above mentioned categories to a given virus-host cell system, cellular and molecular data on virus-host cell interactions are essential. E. g., target cells enabling viral replication, quantitative data on viral replication, and the state of the viral genome during infection need to be known to establish whether or not a virus truly causes a lytic, a persistent or a latent infection. Unfortunately, for bee viruses neither cell culture models nor satisfactory cellular and molecular data exist. Therefore, the above mentioned categories cannot be applied to them. Instead, the more descriptive terms *overt* and *covert* infections have to be used.

Typically, overt infections are those in which the virus-infected host develops obvious disease symptoms and covert infections are asymptomatic (Tab. I). These two broad categories cover a spectrum of infection strategies, the boundaries of which may not always be clearly defined. Even the distinction between *overt* and *covert* is not always clear cut if, like it is the case for insect viruses, the definition and observation of symptoms are difficult because clinical or laboratory diagnosis in its classical (vertebrate) sense are in most of the cases not available. In the absence of physiological and serological parameters defining disease symptoms the

only usable symptoms for insects are obvious morphological or behavioural changes or ultimately, death.

Bee viruses rarely cause easily detectable (i.e. visual) symptoms. This might suggest that they should be classified as covert infections. Occasionally, these viruses do cause symptoms (trembling, inability to flight, crippledness, death) in their hosts often accompanied by highly elevated virus titres easily detectable even by antibody-based detection methods (see Chapter 1 and 3). These overt disease outbreaks are not contradictory to a covert infection, they may rather be one possible and specific stage of a genuine covert infection as it will be explained in the following.

Table I. Outcomes of virus infections in insects (adapted from Dimmock and Primrose, 1987).

Infection type	Sub-type	Detectable virus particles	Obvious disease symptoms	Virus gene expression	Impact on host fitness	Vertical transmis-sion	Duration
Overt	Acute	Yes	Yes	Full	High	No	Short
	Chronic	Yes	Yes	Full	High	No	Long
Covert	Persist-ent	Yes	No	Full (down regulated?)	Low	Yes	Long
	Latent	No	No	Minimal and specific	Low	Yes	Long

3.1. Overt infections

Overt infections can be defined as those in which the infection is highly productive in virus antigens particles and the host exhibits disease symptoms as a result of the infection (causal relationship). The infections have a clearly defined end-point as the insect either succumbs to the infection or the infection is cleared and the host survives in the absence of further virus production. Overt infections are usually transmitted horizontally, either directly between infected and susceptible individuals, or via infectious particles that can persist external the host.

Overt infections can be sub divided further into acute and chronic infections. Acute infections are characterised by a short lived, highly productive infection that may result in symptoms of varying severity to death. Examples of acute infections caused by insect viruses include the fatal infections of lepidopteran larvae by baculoviruses (Vaughn, 1974) and irido-

virus infections in mosquito larvae (Marina *et al.*, 1999; Williams, 1996). For honey bee viruses, some examples of acute infections are also known: chronic bee paralysis virus (CBPV) in adult honey bees (Bailey, 1968; Bailey, 1976; Ball and Bailey, 1997; Ribière *et al.*, 2002; Rinderer and Green, 1976), deformed wing virus (DWV) infection vectored by *Varroa destructor* during the pupal stages of honey bees (Bailey and Ball, 1991; Ball and Allen, 1988; Martin, 2001; Martin *et al.*, 1998; Yue and Genersch, 2005), and sacbrood virus (SBV) infection of honey bee larvae (Bailey and Ball, 1991; Ball and Bailey, 1997). All these infections are short lived, highly productive in virus antigen and particles, manifest clear disease symptoms and, therefore, are classified as overt acute infections.

Chronic infections are similar to acute infections, in that the host manifests symptoms of disease and the infection produces new infectious virus particles. However, chronic infections result in long-term production of virus particles over the lifetime of the host or, in case of most insect viruses, the duration of the infected life stage. Perhaps the best known examples of chronic virus infection in insects are those established by the cypoviruses (CPV) in the midgut cells of many insect species (Belloncik and Hajime, 1998). These infections persist in this productive form throughout the larval stage, constantly re-initiating in the newly differentiated midgut cells after desquamation (Kobayashi, 1971), but do not replicate or cause disease in those larvae which survive into adulthood (Belloncik and Hajime, 1998).

In both cases, acute and chronic infections, transmission is primarily horizontal, symptoms are visible to varying degrees, and the pathogen has a significant impact on host fitness. This is the duration of the infection that distinguishes chronic from acute overt infections.

3.2. Covert infections

Covert infections are those in which the virus is present within the host in the absence of clear disease symptoms. Covert infections in insects have been described in a number of virus-host systems, but what becomes apparent on a survey of the literature is how inconsistently the common terms associated with these infections (i. e. persistent, latent, inapparent, sublethal) are applied. In many cases the modern virology definitions of persistence and latency are ignored. This no doubt reflects the complex spectrum of virus-insect interactions that are summarized under the descriptive term *covert* by default of data allowing a correct application of a more specific terminology.

Many examples of insect viruses which can be detected in their hosts in the absence of overt disease can be found in the literature. Possi-

ble covert infections of CBPV and deformed wing virus (DWV) have been detected in apparently healthy colonies of the honey bee using RT-PCR (Ribière *et al.*, 2002; Tentcheva *et al.*, 2004a). Invertebrate irisdescent virus (IIV) may also be able to induce a covert infection, as these viruses have been detected in healthy individuals of both *Simulium* spp. (Williams, 1993) and *Aedes aegypti* (Marina *et al.*, 1999) by insect bioassays. The CPV of *Heliothis virescens* can replicate in the non-target host *Diatrea grandiosella* without apparent disease (Sikorowski *et al.*, 1992). While all these examples fit with our definition of covert infection, as the virus is present in the absence of disease symptoms, they do not rule out the possibility that they are inapparent infections, rather than genuine covert infections. Inapparent infections are very similar to overt acute infections, in that they are short term infections with high levels of virus production, but, unlike acute infections, they provoke no signs or symptoms of disease (Dimmock and Primrose, 1987).

One characteristic that distinguishes a covert infection from an inapparent one is the mode of transmission. Inapparent infections are short lived, whereas a covert infection can be defined as one in which the virus persists beyond the current life stage of the host and is vertically transmitted to a subsequent generation. Evidence for the vertical transmission of baculoviruses has been in the literature for over thirty years with a number of authors reporting viral infections in the offspring of insects which had survived a virus challenge, with occasional, low levels of virus disease being observed over several subsequent generations (see Kukan, 1999 for a review). Other studies have also used the appearance of viral disease in the offspring of insects which had survived a virus challenge as evidence for vertical transmission (Burden *et al.*, 2002; Lobinger, 1991). A recent RT-PCR study detected sequences of several honey bee viruses (black queen cell virus (BQCV), Kashmir bee virus (KBV), DWV, CBPV, and SBV) in eggs (Chen *et al.*, 2006) while another study could demonstrate DWV and acute bee paralysis virus (ABPV) sequences in sperm collected from healthy looking drones (Yue *et al.*, 2006). Most recently, true vertical transmission of DWV within a colony through sperm and eggs could be demonstrated (Yue *et al.*, 2007).

The definition of a covert infection, that the virus is present within the host and is vertically transmitted in the absence of clear disease symptoms, gives us a clear distinction from an inapparent infection and places the honey bee viruses BQCV, DWV, ABPV, CBPV, KBV, and SBV among the insect viruses causing covert infections rather than inapparent ones.

There are several examples of covert virus infections re-emerging as overt infections (Burden *et al.*, 2003; Fuxa *et al.*, 1999; Ribière *et al.*, 2002).

Spontaneous outbreaks of CPV and nucleopolyhedrosis virus (NPV) disease were observed in apparently virus free cultures of *Trichoplusia ni* (Fuxa *et al.*, 1999). These outbreaks were therefore influenced by environmental stresses, being more prevalent at higher relative humidity and in more crowded conditions. However, these same conditions would promote the density dependent horizontal transmission of virus, so that a small contaminant of virus could rapidly give the appearance of a widespread spontaneous outbreak.

Spontaneous outbreaks of overt disease have also been observed for the cabbage moth (*Mamestra brassicae*) covertly infected with its specific NPV (MbNPV). These outbreaks were apparently random, often involving only one insect within a generation, but may have been influenced by environmental or genetic factors. On rare occasions up to 90% of a population died from spontaneous virus outbreaks, despite being individually reared in the larval stage, whilst other populations reared alongside were unaffected. Molecular analysis linked the virus causing the outbreak with the covert virus present in the population (Burden *et al.*, 2006).

For at least one honey bee virus, CBPV, spontaneous outbreaks of covert infections are described. Outbreaks of chronic paralytic disease have been reported in colonies of *Apis mellifera* which have previously been shown to have a covert CBPV infection (Ribière *et al.*, 2002) and it was postulated several years before that re-emergence may be linked to external factors (Allen and Ball, 1996; Giauffret, 1968) or that genetic factors may influence the emergence of clinical disease (Ball and Bailey, 1997; Rinderer and Rothenbuhler, 1975).

To summarise, covert infections are characterised by i) an absence of symptoms (although there may still be a not yet defined cost to the host) in the presence of viral particles or nucleic acid; ii) vertical transmission of the virus, possibly over many generations; and iii) occasional outbreaks of overt disease from covert infections which may be influenced by a variety of environmental and other factors (Tab. II). Following this definition, CBPV is able to cause genuine covert infections in honey bees since all the three characteristics were shown to be fulfilled (Chen *et al.*, 2006; Ribière *et al.*, 2002; Tentcheva *et al.*, 2004b). For other honey bee viruses, absence of overt disease symptoms in the presence of virus sequences as well as vertical transmission have been demonstrated (Chen *et al.*, 2006; Yue and Genersch, 2005; Yue *et al.*, 2006, Yue *et al.*, 2007). Therefore, honey bee viruses cause true covert infections with overt disease outbreaks being one state of the otherwise covert infection (Tab. II).

Table II. The levels of proof for the various stages of covert infection

	Criteria for demonstration	Some examples for bees
Detection of infection	Detection of virus particles or viral nucleic acid in apparently healthy individual insects reared in virus free conditions for longer than the incubation period of the virus.	CBPV (Ribière et al., 2002) DWV (Yue and Genersch, 2005) KBV (Anderson and Gibbs, 1988; Chen et al., 2004; Dall, 1985) SBV (Anderson and Gibbs, 1989; Bailey and Fernando, 1972; Dall, 1985) ABPV (Bailey and Gibbs, 1964)
Vertical transmission	Detection of virus particles or viral nucleic acid in eggs and sperm or in the apparently healthy offspring of insects that have survived a virus challenge.	BQCV, KBV, CBPV, SBV, DWV in eggs (Chen et al., 2006) ABPV, DWV in sperm (Yue et al., 2006)
Long term persistence	Virus particles or viral nucleic acid detectable in insects reared in virus free conditions for at least 5 generations post virus exposure.	No example so far
Re-emergence	Emergence of overt disease in insects known to harbour a covert infection and reared in virus free conditions.	CBPV (Ribière et al., 2002) KBV (Anderson and Gibbs, 1988) SBV (Bailey, 1976)

3.2.1. Latent infections

The modern definitions of virus-host interactions (Dimmock and Primrose, 1987) give two categories that may fit within the above outlined definition of a covert infection: persistent infections and latent infections. One of the remarkable advances in modern virology is the demonstration that persistent and latent viral infections exist and are common. Now we need to understand the fundamental principles by which persistence and latency are initiated and maintained, as well as the pathological consequences of continued viral presence in a host over its life. The absence of detectable disease symptoms is not equivalent with the absence of disease but may just reflect our inability to apply any correct method suitable to detect the damages causes by this presence.

In latent virus infections the viral genome is present either integrated into the host cell genome or maintained as an extrachromosomal episome. Viral gene transcription is almost completely shut down and only

a very limited number of latency associated transcripts and proteins are produced, but infectious virus particles are not formed (Dimmock and Primrose, 1987). Examples of latency include lysogenic bacteriophage infections and the long-term infection of mammalian cells by Herpes simplex virus (HSV) and Varicella zoster virus (VZV). For HSV some of the key molecular features of latency are established through the study of *in vitro* and *in vivo* experimental model systems. It is generally accepted that (i) latency is a consequence of a failure to initiate virus immediate early gene expression; (ii) during latency the virus genome exists as a non-replicating "endless" molecule consistent with the maintenance of an extrachromosomal episome; (iii) transcription during latency is restricted to a single diploid region encoding the latency associated transcripts (LATs); (iv) reactivation is a consequence of a response to cellular signals that results in the activation of virus gene expression and entry into the productive cycle (Efstathiou and Preston, 2005).

An example of latent virus infection has also been discovered in an insect system. The Hz-1 virus is a double-stranded DNA virus originally isolated from a persistently infected cell line established from ovarian tissues of *Heliothis zea* (Granados *et al.*, 1978). The virus is able to infect a wide range of cell lines, both as a latent and as a productive infection (Wood and Burand, 1986). The latent Hz-1 infection is characterised by a massive shut down of viral gene transcription with production of only a single 2.9-kb latency-associated transcript (PAT) while in a productive Hz-1 infection over 100 viral transcripts are expressed (Chao *et al.*, 1998; Chao *et al.*, 1992). In cell culture, only a small proportion of infected cells, typically less than 0.2%, become latently infected (Chao *et al.*, 1998). These latently infected cells are then immune to superinfection (Burand *et al.*, 1986) and over a period of 200 passages, cell lines covertly infected with Hz-1 cease to produce virus as the latently infected cells come to dominate the population (Lin *et al.*, 1999). The virus DNA is present in latently infected cells as both free virus episomes and fully integrated into the host chromosomal DNA, and viral DNA is equally distributed in the nuclei of both daughter cells after cell division (Lin *et al.*, 1999). The latent state does not represent a dead end for the virus as a spontaneous reactivation into a productive infection can occur in less than 0.2% of covertly infected cells over a 5 day period (Lin *et al.*, 1999). To date, latent Hz-1 infections have not been demonstrated in an insect host, only in cell culture. However, the biology of this virus-insect cell system suggests a high degree of control of the latency by the virus, and may well reflect a long and complex co-evolution between the virus and its host.

For honey bee viruses, no true latency (i.e. virus RNA integrated

into the host's genome or existing as an episome) have been demonstrated so far. For plus-stranded ssRNA viruses which replicate without a DNA intermediate it is hardly conceivable how the status of latency could be acheived. Until now, not any RNA virus derived episomal DNA has ever been reported. The only RNA viruses known to integrate a DNA version of their genome into chromosomal DNA of their hosts are the retroviruses. In this case, the RNA genomes are reverse transcribed and the resultant DNA is inserted into the host DNA by a virus-encoded integrase; these reactions are required for normal replication (Flint *et al.*, 2004; Goff, 1992; Hu and Temin, 1990). However, sequences of flavivirus-related RNA viruses were recently demonstrated to persist in a DNA form integrated in the genome of *Aedes albopictus* and *Aedes aegypti* mosquitoes (Crochu *et al.*, 2004) indicating the possibility of genetic transfer from RNA viruses to eukaryotic cells. Although the integration of small viral RNA genome fragments into host DNA is not comparable to the integration of a whole viral genome as required for latency, we cannot finally rule out that such a phenomenon might exist and waits to be demonstrated.

3.2.2. Persistent virus infections

A persistent virus infection is characterised by a constant low level production of virus particles in an infected cell. Either the infected cell survives or a minority of cells are infected, thus the spread of the virus is limited so that cell death is counterbalanced by the production of new cells, with no net loss of cells (Dimmock and Primrose, 1987). Persistent infections represent a balance between host and virus through interactions between the virus and the cells, interactions between the virus and the immune system of the host, the production of defective or interfering (DI) virus or a combination of all of these mechanisms (Dimmock and Primrose, 1987).

The three prerequisites for viral persistence are, first, that the host's immune response fails to form or fails to purge virus from the infected host. The virus disturbs the host immune system so that the forein (viral) content of an infected cell is not recognised and the spread of infection is not curtailed. Thus, viral persistence is synonymous with evasion of the host's immunological surveillance system. Second, viruses can regulate expression of both their own genes and host's genes to achieve replication and residence in a non-lytic state within the infected cells. Third, persisiting viruses are able to infect differentiated or specialized host cells over the life time of the host. The continuous replication of a viral, i.e., foreingene in a differentiated cell can selectively disorder the functions of that cell without destroying it. E.g. persisting viruses can cause disease by

aborting a specific product of a differentiated cell, thereby altering that cell's function and unballancing the host's physiology. The result is a disturbance of in the host's biologic equilibrium. Thus, one important effect of persistent virus replication is to disorder the normal homeostasis of the host and thereby cause disease without destroying (killing) the infected cells and the host. One of the best studied examples for persisting virus infection in rodents and humans is lymphocytic choriomengitis. This persisting RNA virus causes broad alterations in cell physiology, behaviour and cognitive functions, extensive injuries, and all possible courses of disease without killing infected cells (De la Torre *et al.*, 1996; De la Torre and Oldstone, 1992; de la Torre and Oldstone, 1996; Oldstone, 2002; Zinkernagel, 2002)

There are well defined examples of persistent virus infections in insects. Mosquito (*Aedes aegypti*) larvae are susceptible to infection by IIV-6, which can give rise to an overt infection. Mosquitoes which survived exposure to this virus showed a reduction in fecundity and adult size, increased juvenile development times and shorter lifespan (Marina *et al.*, 1999). This covert infection could be transmitted to uninfected females during mating with infected males (Marina *et al.*, 1999) and could also be passed to the next generation in an infectious form (Marina *et al.*, 2003) suggesting a persistent rather than latent infection.

The best characterised example of a persistent virus infection in insects is the infection of the moth *Mamestra brassicae* by MbNPV. The infection was present in all life stages of the insect and was initially thought to be present in the fat bodies (Hughes *et al.*, 1993). However, more efficient DNA extraction techniques showed that MbNPV was also present in the haemolymph and reproductive tissue of both male and female insects (Nixon, 2003). The infection was demonstrated to be persistent, as transcripts of the major occlusion body protein were present in covertly infected insects, albeit at a much lower level than in an overt MbNPV infection (Hughes *et al.*, 1997). Also, uninfected *M. brassicae* larvae which were fed the fat body cells from persistently infected larvae succumbed to an overt MbNPV infection, suggesting low levels of fully infectious virus were present (Hughes *et al.*, 1997). In addition to these laboratory results, a recent study has shown that persistent baculovirus infections were ubiquitous in then UK field populations of *M. brassicae* (Burden *et al.*, 2003). These persistent infections were also stable, persisting at high levels within the population for at least five generations after the insects were brought into the laboratory from the field.

For honey bee viruses, no true persistence has been demonstrated so far, contrary to covert infections demonstrated for BQCV, DWV, ABPV,

CBPV, KBV, and SBV. True latency as one type of covert infection is not conceivable for these RNA viruses which do not replicate via a DNA intermediate since virus integration into the host genome or maintenance as episome are at least unlikely if not impossible. Therefore, the above mentioned covert honey bee viruses fall into the category of persistent viruses.

4. Mixed infection strategies

Molecular techniques are beginning to reveal that covert infections are more common than previously supposed, and the indications are that some viruses are able to cause overt as well as covert infections, thus having mixed infection strategies. Baculoviruses previously supposed to cause overt infections are now known to cause persistent infections as well. For some honey bee viruses mixed infection strategies are also well documented.

SBV causes fatal acute and, therefore, overt infections in honey bee larvae (Bailey, 1975). SBV could also be demonstrated in pupae (Dall, 1985) and adult bees without obvious signs of disease (Anderson and Gibbs, 1989; Bailey, 1969; Bailey and Fernando, 1972) and in eggs (Chen *et al.*, 2006) indicating vertical transmission. Extracts from apparently healthy whereas SBV positive bees gave rise to overt SBV infection in the injected bees (Bailey, 1976) demonstrating that SBV causing covert infections remained fully able to cause well charaterized overt infections. Absence of clinical symptoms, vertical transmission and the covert virus' ability to still cause an overt infection fit with our definition of covert infection. Hence, SBV has a mixed infection strategy and the type of infection (overt versus covert) is depending on the life stage of the infected bee.

CBPV causes an infectious and contagious disease in adult bees displaying two complexes of symptoms (Bailey, 1968). Type I syndrome occurs at any season and is characterised by trembling and crawling bees with a few or no black, hairless individuals (Ball and Bailey, 1997). Type II syndrome is characterised by clusters of flightless, trembling and crawling bees, and some black, hairless individuals standing at the entrance of the hive (Faucon, 1992; Faucon, 1996). This type occurs in France in spring and early summer (Giauffret, 1968) (see Chapter 1). In addition , CBPV could be detected in apparently healthy individuals (Ribière *et al.*, 2002) and, like SBV, in eggs (Chen *et al.*, 2006). Healthy appearing colonies covertly infected by CBPV sometimes suffered from spontaneous CBPV outbreaks (Ribière *et al.*, 2002). Therefore, CBPV is causing overt acute as well as true covert infections.

For DWV, the situation is somewhat different. DWV sequences could be detected in all stages of development from egg to adult workers, queens and drones, all of them without any obvious signs of disease (Chen *et al.*, 2005). DWV positive sperm could be collected from healthy appearing drones and artificial insemination using this virus-positive sperm did not result in any clinical symptoms in the offspring (Yue *et al.*, 2006). These results suggest vertical transmission through sperm and eggs to larvae, pupae, and adult bees. Additionally, viral sequences could be detected in brood food indicating horizontal transmission through feeding, again resulting in an asymptomatic infection (Yue and Genersch, 2005). Therefore, DWV causes true covert infections in all life stages of the bee and these viruses are transmitted vertically as well as horizontally. Overt DWV infections are inevitably associated with *Varroa destructor*. Only when the virus is transmitted by *V. destructor* during pupal development, the characteristic clinical symptoms (malformed appendages, shortened and bloated abdomens, miscolouring) are present in the hatching bee (Ball and Allen, 1988; Bowen-Walker *et al.*, 1999; Martin, 2001; Martin *et al.*, 1998; Yue and Genersch, 2005). Hence, the infection strategy of DWV depends upon the mode of transmission. Horizontal (feeding) and vertical transmission of DWV in the absence of *V. destructor* causes asymptomatic, covert infections. Vectorial transmission of DWV by *V. destructor*, i. e., transmission by "injecting" the virus into pupae leads to overt infections. Early experiments already suggested this connection between mode of transmission and occurrence of clinical symptoms: injection bioassay with DWV using young pupal bees demonstrated the causal relationship between crippled wings of bees originating from injected pupae and DWV (Bailey and Ball, 1991).

ABPV and KBV are known to cause covert infections. For instance, individuals can carry virus loads up to 10^6 ABPV particles without affecting longevity (Bailey and Gibbs, 1964) and pupae were shown to carry ABPV and KBV without any signs of disease (Anderson and Gibbs, 1988; Dall, 1985). As typical for covert infections, persisting KBV remains fully competent and can reemerge to cause an overt infection (death): this is observed by injecting potassium buffer or insect ringer into covertly infected individuals (Anderson and Gibbs, 1988). Both viruses, KBV and ABPV, cause bee mortality and colony collapse in association with *V. destructor* (Allen and Ball, 1996; Ball and Allen, 1988). However, for both viruses it is not yet definitely demonstrated whether they need to be transmitted through the mite to cause an overt infection (like it is the case for DWV) or if mite infestation is the trigger for reactivation of the virus. Early experiments which demonstrated that both viruses can cause fatal infec-

tions when injected (Bailey and Ball, 1991; Bailey and Gibbs, 1964) do not help here since injection bioassays bypass the natural transmission routes and for many invertebrate pathogens bypassing the integument almost assures infection. Accordingly, it has been shown recently that *V. destructor* was able to transmit KBV but no clinical symptoms or bee mortality was recorded as a result of the experimental vector-borne KBV infection (Chen *et al.*, 2004). Therefore, we still need to show that transmission of KBV or ABPV through *V. destructor* (Ball, 1989) involves infectious viruses which cause overt infection before we can decide whether KBV and ABPV also use mixed infection strategies or only cause covert infections which may be reactivated through mite infestation.

5. Transmission strategies

The indications are that in insects covert infections are mainly vertically transmitted whereas overt and often fatal infections are transmitted horizontally. As transmission strategies and there epidemiological significance are well developed in Chapter 6, we will only recall that recent extensions of host-pathogen theory have considered mixed transmission strategies for directly transmitted pathogens (Lipsitch and Nowak, 1996; Lipsitch *et al.*, 1995) and that emerging from these studies, there is a fundamental opposition between horizontal and vertical modes of pathogen transmission. Horizontal transmission allows the development of more virulent forms of the pathogen with high negative impact on the fitness of the host. Vertical transmission, however, relies on host survival and reproduction, so horizontal impedes vertical transmission. Consequently, it is not immediately obvious how a pathogen with mixed infections strategies, like most if not all of the honey bee viruses, will persist in a host population. At low densities, for example, will one transmission strategy be favoured to the exclusion of the other? Furthermore, although pathogens frequently have a dramatic impact on the fitness of their hosts, this does not necessarily translate into population regulation of the host. Does the occurrence of covert infections make population regulation more or less likely?

Ecological theory is now being developed to investigate these issues in the context of host-baculovirus interactions (Bonsall *et al.*, 2005). These models show that persistent infections have a profound influence on the dynamics of host and pathogen. Two parameters important in determining the outcome are (i) the rate at which persistent infections spontaneously emerge as overt infections and (ii) the ratio of susceptibility of covertly

infected and clean hosts. When clean hosts are more susceptible to further overt infection, under a large range of reactivation rates, the stable state is between persistently infected hosts and the pathogen – susceptible hosts being excluded. Therefore, with efficient vertical transmission and minimal impact on host fitness, models predict that clean hosts could be excluded from the population altogether.

Although such models do not exist for the honey bee and its viruses they correspond well with the data collected so far in the field for this host and DWV, where all populations and nearly all individuals within each population, have been found to be carrying a persistent infection (Berenyi *et al.*, 2006; Tentcheva *et al.*, 2004a; Tentcheva *et al.*, 2004b; Yue and Genersch, 2005). In fact, clean hosts are so rare that it will be difficult to test the above made assumption that clean hosts are more susceptible to overt infections than covertly infected ones.

One key message from these models is that pathogens may well be present in host populations as persistent endemic infections, and as such may be hard to detect. Nevertheless, they may be exerting considerable regulatory influences on the host dynamics. With the development of sensitive molecular techniques, and the technology to process large numbers of samples, the prevalence and influence of covert infections of insect viruses and specifically honey bee viruses may be revealed over the coming years.

References

Allen, M.F., Ball, B.V., 1996. The incidence and world distribution of honey bee viruses. Bee World. 77, 141-162.

Anderson, D.L., Gibbs, A.J., 1988. Inapparent virus infections and their interactions in pupae of the honey bee (*Apis mellifera* Linnaeus) in Australia. J. Gen. Virol. 69, 1617-1625.

Anderson, D.L., Gibbs, A.J., 1989. Transpuparial transmission of Kashmir bee virus and sacbrood virus in the honey bee (*Apis mellifera*). Ann. Appl. Biol. 114, 1-7.

Bailey, L., 1968. The purification and properties of chronic bee-paralysis virus. J. Gen. Virol. 2, 251-260.

Bailey, L., 1969. The multiplication and spread of sacbrood virus of bees. Ann. Appl. Biol. 63, 483-491.

Bailey, L., 1975. Recent research on honey bee viruses. Bee World. 56, 55-64.

Bailey, L., 1976. Viruses attacking the honeybee. Adv. Virus Res. 20, 271-304.

Bailey, L., Ball, B.V., 1991. Honey bee pathology. Academic Press, London.

Bailey, L., Fernando, E.F.W., 1972. Effects of sacbrood virus on adult honey bees. Ann. Appl. Biol. 72, 27-35.

Bailey, L., Gibbs, A.J., 1964. Acute infection of bees with paralysis virus. J. Insect Pathol. 6, 395-407.

Ball, B.V., 1989. *Varroa jacobsoni* as a virus vector. In *Present status of varroatosis in Europe and progress in the varroa mite control*. Office for Official Publications of the European Communities, Luxembourg.

Ball, B.V., Allen, M.E., 1988. The prevalence of pathogens in honey bee (*Apis mellifera*) colonoes infested with the parasitic mite *Varroa jacobsoni*. Ann. Appl. Biol. 113, 237-244.

Ball, B.V., Bailey, L., 1997. Viruses. Honey bee pests, predators and diseases. Morse, R.A. and Flottum, K. Medina, A.I. Root Company: 11-32.

Belloncik, S., Hajime, M., 1998. Cypoviruses. The insect viruses. Miller, L.K. and Ball, L.A. New York, Plenum press: 337-369.

Berenyi, O., Bakonyi, T., Derakhshifar, I., Köglberger, H., Nowotny, N., 2006. Occurence of six honeybee viruses in diseased Austrian apiaries. Appl. Environ. Microbiol. 72, 2414-2420.

Bonsall, M.B., Sait, S.M., Hails, R.S., 2005. Invasion and dynamics of covert infection strategies in structured insect-pathogen populations. J. Anim. Ecol. 74, 464-474

Bowen-Walker, P.L., Martin, S.J., Gunn, A., 1999. The transmission of deformed wing virus between honeybees (*Apis mellifera* L.) by the ectoparasitic mite *Varroa jacobsoni* Oud. J. Invertebr. Pathol. 73, 101-106.

Burand, J.P., Kawanishi, C.Y., Huang, Y.S., 1986. Persistent baculovirus infections. The biology of baculoviruses. Granados, R.R. and Federici, B.A. Boca Raton, Fla., CRC Press Inc. 1: 159-177.

Burden, J.P., Griffiths, C.M., Cory, J.S., Smith, P., Sait, S.M., 2002. Vertical transmission of sublethal granulovirus infection in the Indian meal moth, *Plodia interpunctuella*. Mol. Ecol. 11, 547-555.

Burden, J.P., Nixon, C.P., Hodgkinson, A.E., Possee, R.D., Sait, S.M., King, L.A., Hails, R.S., 2003. Covert infections as a mechanism for long-term persistence of baculoviruses. Ecol. Lett. 6, 524-531.

Burden, J.P., Possee, R.D., Sait, S.M., King, L.A., Hails, R.S., 2006. Phenotypic and genotypic characterisation of persistent baculovirus infections in populations of the cabbage moth (Mamestra brassicae) within the British Isles. Arch. Virol. 151, 635-649.

Chao, Y.C., Lee, S.T., Chang, M.C., Chen, H.H., Chen, S.S., Wu, T.Y., Liu, F.H., Hsu, E.L., Hou, R.F., 1998. A 2.9-kilobase noncoding nuclear RNA functions in the establishment of persistent Hz-1 viral infection. J. Virol. 72, 2233-2245.

Chao, Y.C., Wood, H.A., Chang, C.Y., Lee, H.J., Shen, W.C., Lee, H.T., 1992. Differential expression of Hz-1 baculovirus genes during productive and persistent virla infections. J. Virol. 66, 1442-1448.

Chen, Y.P., Higgins, J.A., Feldlaufer, M.F., 2005. Quantitative real-time reverse transcription-PCR analysis of Deformed wing virus infection in the honeybee (*Apis mellifera* L.). Appl. Environ. Microbiol. 71, 436-441.

Chen, Y.P., Pettis, J.S., Collins, A., Feldlaufer, M.F., 2006. Prevalence and transmission of honeybee viruses. Appl. Environ. Microbiol. 72, 606-611.

Chen, Y.P., Pettis, J.S., Evans, J., Krmaer, M., Feldlaufer, M.F., 2004. Transmission of Kashmir bee virus by the ectoparasitic mite *Varroa destructor*. Apidologie. 35, 441-448.

Crochu, S., Cook, S., Attoui, H., Charrel, R.N., De Chesse, R., Belhouchet, M., Lemasson, J.-J., de Micco, P., de Lamballerie, X., 2004. Sequences of flavivirus-related RNA viruses persist in DNA form integrated in the genome of *Aedes* spp. mosquitoes. J. Gen. Virol. 85, 1971-1980.

Dall, D.J., 1985. Inapparent infection of honey bee pupae by Kashmir and sacbrood bee viruses in Australia. Ann. Appl. Biol. 106, 461-468.

De la Torre, J.C., Mallory, M., Brot, M., Gold, L., Koob, G., Oldstone, M.B.A., Masliah, E., 1996. Viral persistence in neurons alters synaptic plasticity and cognitive functions without distructuion of brain cells. Virology. 220, 508-515.

De la Torre, J.C., Oldstone, M.B.A., 1992. Selective disruption of growth hormone transcription machinery by viral infection. Proc. Natl. Acad. Sci. USA. 89, 9939-9943.

De la Torre, J.C., Oldstone, M.B.A., 1996. The anatomy of viral persistence: mechanisms of persistence and associated disease. Adv. Virus Res. 46, 311-343.

Dimmock, N.J., Primrose, S.B., 1987. Introduction to modern virology. Oxford, Blackwell Scientific Publications.

Efstathiou, S., Preston, C.M., 2005. Towards an understanding of the molecular basis of herpes simplex virus latency. Virus Res. 111, 108-119.

Faucon, J.-P., 1992. Paralysie chronique ou maladie noire. CNEVA-FNOSAD, pp. 204-212.

Faucon, J.-P., 1996. La question sanitaire. FNOSAD, Angers.

Flint, S.J., Enquist, L.W., Racaniello, V.R., Skalka, A.M., 2004. Principles of virology. Chapter 7: Reverse transcription and integration. 2nd ed., ASM Press, Washington, DC.

Fuxa, J.R., Sun, J.Z., Weidner, E.H., LaMotte, L.R., 1999. Stressors and rearing diseases of *Trichoplusia ni*: Evidence for vertical transmission of NPV and CPV. J. Invertebr. Pathol. 74, 149-155.

Gey, G., Coffman, W., Kubicek, M., 1952. Tissue culture studies of the proliferative capacity of cervical carcinoma and normal epithelium. Cancer Res. 12, 264-265.

Giauffret, A., 1968. Les viroses de l`abeille. Rev. Pathol. Comp.: 111-115.

Goff, S.P., 1992. Genetics of retroviral integration. Annu. Rev. Genet. 26, 527-544.

Granados, R.R., Nguyen, T., Cato, B., 1978. An insect cell line persistently infected with a baculovirus like particle. Intervirology. 10, 309-317.

Holowczak, J.A., Thomas, V.L., Flores, L., 1975. Isolation and characterization of vaccinia virus 'nucleoides'. Virology. 67, 506-519.

Hu, W.S., Temin, H.M., 1990. Retroviral recombination and reverse transcription. Science. 250, 1227-1233.

Hughes, D.S., Possee, R.D., King, L.A., 1993. Activation and deactivation of a latent baculovirus resembling *Mamestra brassicae* nuclear polyhedrosis virus in *M. brassicae* insects. Virology. 194, 604-615.

Hughes, D.S., Possee, R.D., King, L.A., 1997. Evidence for the presence of a low-level persistent baculovirus infection of *Mamestra brassicae* insects. J. Gen. Virol. 78, 1801-1805.

Klein, G., 1972. Herpesviruses and oncogenesis. Proc. Natl. Acad. Sci. USA. 69, 1056-1064.

Klein, R.J., 1982. The pathogenesis of acute, latent and recurrent herpes simplex virus infections. Arch. Virol. 72, 143-168.

Kobayashi, M., 1971. Replication of cytoplasmic polyhedrosis virus observed with electron microscopy. The cytoplamsic polyhedorsis of the silkworm. Aruga, H. and Tanda, Y. Tokyo, Univ. Tokyo Press: 103-127.

Kukan, B., 1999. Vertical transmission of nucleopolyhedrosisvirus in insects. J. Invertebr. Pathol. 74, 103-111.

Lin, C.L., Lee, J.C., Chen, S.S., Wood, H.A., Li, M.L., Li, C.F., Chao, Y.C., 1999. Persistent Hz-1 virus infection in insect cells: Evidence for insertion of viral DNA into host chromosomes and viral infection in a latent status. J. Virol. 73, 128-139.

Lipsitch, M., Nowak, M.A., 1996. The evolution of virulence in parasites with vertical and horizontal transmission. Evolution. 50, 1729-1741.

Lipsitch, M., Nowak, M.A., Ebert, D., May, R.M., 1995. The population dynamics of vertically and horizontally transmitted parasites. Proc. R. Soc. Lond. Ser. B - Biol. Sci. 260, 321-327.

Lobinger, G., 1991. Investigations on effect and host range of a cytoplasmic-polyhedrosis of *Dasychira pudibunda*-L. (Lep, *Lymantriidae*). J. Appl. Entomol. - Z. Angew. Entomol. 111, 442-450.

Marina, C.F., Arredondo-Jiménez, J.I., Castillo, A., Williams, T., 1999. Sublethal effects of iridovirus disease in a mosquito. Oecologia. 119, 383-388.

Marina, C.F., Ibarra, J.E., Arredondo-Jiménez, J.I., Fernandez-Salas, I., Liedo, P., Williams, T., 2003. Adverse effects of covert iridovirus infection on life history and demographic parameters of *Aedes aegypti*. Entomol. Exp. Appl. 106, 53-61.

Martin, S.J., 2001. The role of Varroa and viral pathogens in the collapse of honeybee colonies: a modelling approach. J. Appl. Ecol. 38, 1082-1093.

Martin, S.J., Hogarth, A., van Breda, J., Perrett, J., 1998. A scientific note on *Varroa jacobsoni* Oudemans and the collapse of *Apis mellifera* colonies in the United Kingdom. Apidologie. 29, 369-370.

Nixon, C.P., 2003. The biology and mechanisms of persistence in baculoviruses. Biological & Molecular Sciences. Oxford, Oxford Brookes University.

Oldstone, M.B.A., 2002. Biology and pathogenesis of lymphocytic choriomeningitis virus infection. Curr. Top. Microbiol. Immun. 263, 83-117.

Oldstone, M.B.A., 2006. Viral persistence: Parameters, mechanisms and future predictions. Virology. 344, 111-118.

Rapp, F., Westmoreland, D., 1976. Cell transformation by DNA containing viruses. Biochim. Biophys. Acta. 458, 167-211.

Ribière, M., Triboulot, C., Mathieu, L., Aurieres, C., Faucon, J.-P., Pepin, M., 2002. Molecular diagnosis of chronic bee paralysis virus infection. Apidologie. 33, 339-351.

Rinderer, T.E., Green, T.J., 1976. Serological relationship between *Chronic bee paralysis virus* anf the virus causing hairless-black syndrome in the honeybee. J. Invertebr. Pathol. 27, 403-405.

Rinderer, T.E., Rothenbuhler, W.C., 1975. Responses of three genetically different stocks of the honey bee to a virus from bees with hairless-black syndrome. J. Invertebr. Pathol. 25, 297-300.

Sanford, K.K., Likely, G.D., Bryan, W.R., Earle, W.R., 1952. The infection of cells in tissue culture with Rous sarcoma virus. J. Natl. Cancer Inst. 12, 1317-1343.

Shivaprasad, H.L., Chin, R.P., Jeffrey, J.S., Latimer, K.S., Nordhausen, R.W., Niagro, F.D., Campagnoli, R.P., 1994. Particles resembling circovirus in the bursa of Fabricius of pigeons. Avian Diseases. 38, 635-641.

Sikorowski, P.P., Luttrell, R.G., Lawrence, A.M., 1992. Inapparent infection of *Diathrea grandiosella* by *Heliothis virescens* cytoplamsic polyhedrosis-virus. Entomophaga. 37, 347-351.

Tentcheva, D., Gauthier, L., Jouve, S., Canady-Rochelle, L., Dainat, B., Cousserans, F., Colin, M.E., Ball, B.V., Bergoin, M., 2004a. Polymerase chain reaction detection of deformed wing virus (DWV) in *Apis mellifera* and *Varroa destructor*. Apidologie. 35, 431-439.

Tentcheva, D., Gauthier, L., Zappulla, N., Dainat, B., Cousserans, F., Colin, M.E., Bergoin, M., 2004b. Prevalence and seasonal variations of six bee viruses in *Apis mellifera* L. and *Varroa destructor* mite populations in France. Appl. Environ. Microbiol. 70**,** 7185-7191.

Tischer, I., Gelderblom, H., Vettermann, W., Koch, M.A., 1982. A very small porcine virus with circular single-stranded DNA. Nature. 295**,** 64-66.

Varmus, H., 1988. Retroviruses. Science. 240**,** 1427-1435.

Vaughn, J.L., 1974. Virus and rickettsial diseases. G. Cantwell (Ed.), Insect Diseases. Dekker, New York.

Williams, T., 1993. Covert iridovirus infection of blackfly larvae. Proc. R. Soc. Lond. Ser. B - Biol. Sci. 251**,** 225-230.

Williams, T., 1996. The iridoviruses. Adv. Virus Res. 46**,** 345-412.

Wood, H.A., Burand, J.P., 1986. Persistent and productive infections with the Hz-1 baculovirus. Curr. Top. Microbial Immun. 131**,** 119-134.

Wyke, J., 1981. Strategies of viral oncogenesis. Nature. 290**,** 629-630.

Yue, C., Genersch, E., 2005. RT-PCR analysis of *Deformed wing virus* (DWV) in honey bees (*Apis mellifera*) and mites (*Varroa destructor*). J. Gen. Virol. 86**,** 3419-3424.

Yue, C., Schröder, M., Bienefeld, K., Genersch, E., 2006. Detection of viral sequences in semen of honeybees (*Apis mellifera*): Evidence for vertical transmission of viruses through drones. J. Invertebr. Pathol. 92**,** 93-96.

Yue, C., Schröder, M., Gisder, S., Genersch, E., 2007. Vertical-transmission routes for deformed wing virus of honeybees (*Apis mellifera*). J. Gen. Virol. 88, 2329-2336.

Zinkernagel, R.M., 2002. Lymphocytic choriomeningitis virus and immunology. Curr. Top. Microbiol. Immun. 263**,** 1-5.

Chapter 6

EVOLUTIONARY EPIDEMIOLOGY OF VIRUS INFECTIONS IN HONEY BEES

Mark J.F. BROWN and Ingemar FRIES

Table of contents

1. Introduction

The field of evolutionary epidemiology aims to understand how parasites and hosts interact over ecological and evolutionary time. One key aspect of this aim is to determine why different parasites damage their hosts to different degrees. In other words, why are some parasites apparently benign, whilst others cause rapid mortality? The traditional view of parasite virulence suggested that over evolutionary time parasites should decrease the amount of harm that they would do to their hosts (reviewed by Bull, 1994; Lenski and May, 1994). However, over the last 25-30 years, numerous theoretical and empirical investigations have demonstrated that this simplistic view is incorrect. This body of work relies upon the insight (or assumption) that parasite virulence is related to parasite fitness through its effects on increasing or decreasing the probability of transmission. Consequently, in contrast to the traditional view, we should expect parasites to exhibit a level of virulence that maximises their own reproductive success within a complex ecological, epidemiological and evolutionary world. A large number of factors related to the biology of parasites, hosts and their interaction have been suggested to play a role in the evolution of such parasite-optimal virulence. In Section 2 we provide a general review of investigations into these factors. In Section 3 we focus on the biology of honey bees in order to highlight host-specific traits that have implications for parasite virulence in this system. In Section 4, we bring these two themes together and ask what levels of virulence we might expect to find in viral parasites/diseases of bees, and whether our knowledge is sufficient to explain the levels of virulence that we observe, especially with respect to the recent involvement of the ectoparasitic mite, *Varroa destructor*. We end with a summary of our general points and provide perspectives on where future research into the evolutionary epidemiology of viruses in bees might best be directed.

2. Evolution of virulence – important parameters/models

2.1. Defining terms

The terms 'virulence' and 'parasite' mean many things to different people. In order to provide focus and avoid confusion, we start by defining these terms.

2.1.1. What is virulence?

Parasite virulence encompasses a wide variety of effects. A

broad definition of virulence would encompass any changes caused by a parasite in its host that reduce the evolutionary fitness of said host. This could include mortality, morbidity, and partial or complete castration. These terms themselves encompass a wide variety of parasite effects. For example, host mortality could increase if (i) parasites release toxins within their hosts that induce mortality, (ii) parasites successfully compete for internal (and essential) host resources, (iii) parasites manipulate host behaviour to enhance their own transmission. At the broader level, whether a parasite causes mortality, morbidity or castration (or some combination of these factors) has implications for the evolution of virulence (Day, 2002). Nevertheless, the majority of theoretical models treat virulence as being equivalent to additional host-mortality. This should be borne in mind when extrapolating the results of such models to biological systems.

While an understanding of virulence relies on treating it as an adaptation to maximise parasite transmission, it should be noted that virulence need not be an adaptive result of host-parasite evolutionary epidemiology. Virulence may be non-adaptive if it occurs after transmission has occurred (e.g., HIV), if it results from parasites invading atypical host tissues (e.g., poliomyelitis virus), or if a host-parasite relationship is novel (e.g., *Varroa destructor* in *Apis mellifera*, see Section 4).

2.1.2. What is a parasite?

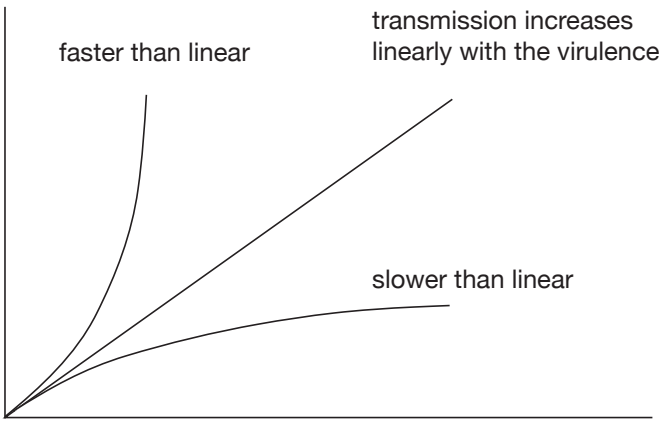
Parasitism can be defined as an ecological relationship between individuals from two species, where one lives in or on and obtains resources from and consequently damages the other. Consequently, the term 'parasite' encompasses organisms as diverse as viruses and arthropods. From the perspective of theoretical models, parasites are often divided into micro- and macroparasites. Microparasites include viruses, bacteria, fungi and protozoa, and are assumed to have significantly faster generation times and significantly higher reproductive rates than their hosts. In contrast, macroparasites include the metazoa (animals) and are assumed to have much slower generation times and lower reproductive rates than microparasites. This division is theoretically important because micro- and macroparasites are modelled in different ways, and biologically important because it has implications for the evolution of virulence. It should be noted that the majority of the models discussed below are aimed at understanding the evolutionary epidemiology of microparasites. Finally, we note that we use the terms 'parasite', 'pathogen' and 'disease' interchangeably throughout this chapter.

2.2. Transmission routes and the evolution of virulence

Parasites vary in how they get from one host to another. Horizontal transmission, which involves the infection of one individual by another through direct or indirect transfer of the parasite, is probably the most common route of transmission. However, vertical transmission can play an important role in the propagation of parasites from one generation to the next. Unsurprisingly, the type of transmission used by a particular parasite – horizontal, vertical, direct, indirect, immediate or delayed – has consequences for the amount of damage done to the host.

2.2.1. Horizontal transmission

Imagine a host-parasite system where the parasite does little harm to its host and is horizontally transmitted. What should we expect to happen over ecological and evolutionary time? Parasite fitness relies on transmission to new hosts, and thus any parasite strain or mutation that transmits more rapidly to new hosts will be ecologically and epidemiologically more successful, and will eventually replace other strains in the population. However, enhanced transmission cannot come for free. The majority of theoretical models (see references throughout) assume that enhanced transmission is the result of a higher reproductive rate of the parasite – that is, the parasite is producing more propagules or infective stages more rapidly than its competitors. To do this, the parasite needs to



this figure ha
been redrawn

Figure 1. The graph shows three possible relationships between virulence (which is assumed to be due to parasite reproduction) and transmission. Natural selection to increase parasite transmission inexorably leads to the evolution of higher virulence.

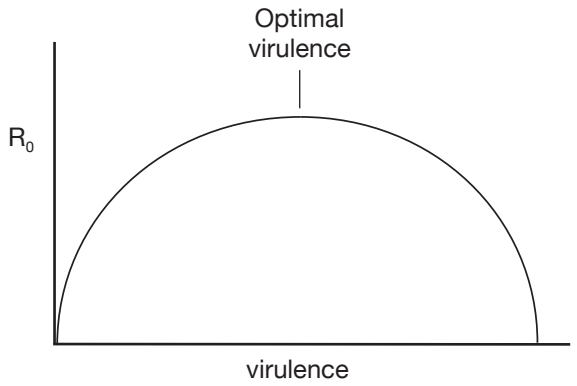


Figure 2. Host mortality constrains the evolution of virulence. The graph illustrates what happens when host mortality, either background mortality or due to parasite virulence, is incorporated into the model illustrated in Fig. 1. Because host mortality acts as a brake on parasite transmission, the relationship between virulence and transmission reaches an optimum where transmission is maximised, and thus an intermediate level of virulence is selected for.

take more resources from its host and, thus, should have a higher virulence, or impact on its host. This relationship can be modelled as a curve whereby parasite reproduction or transmission increases with increasing virulence (Fig. 1). However, an increase in parasite virulence may also result in increased host mortality, which in most cases truncates transmission. Thus, the relationship between parasite reproduction, transmission and virulence is constrained by host mortality (Fig. 2). This logic underpins the trade-off models for the evolution of virulence which assume that parasite virulence evolves as a result of selection for enhanced transmission (parasite fitness).

It should be noted that this result comes with a number of caveats. First, evidence for a positive relationship among parasite reproduction, virulence and increased transmission is rare, although it is beginning to accumulate (Ebert, 1994; Ebert and Mangin, 1997; Lipsitch and Moxon, 1997; Mackinnon and Read, 1999; Messenger *et al.*, 1999). Second, it relies on the assumption that the host and parasite populations are at equilibrium (see Section 2.3.2). Nevertheless, evidence from serial passage experiments, where the trade-off between virulence and transmission is removed, provides strong evidence to support the idea that horizontal transmission selects for higher virulence (Ebert, 1998).

2.2.2. Vertical transmission

Vertically transmitted parasites rely on passing from one generation to the next through successful reproduction of the host. In solitary organisms, this generally involves trans-ovarial transmission, while in social insects it generally implies transmission via new queens (but see Section 3 for the honey bee case). It is easily seen that, in strong contrast to horizontal transmission, vertical transmission should select for lower levels of virulence. Any vertically-transmitted parasite that decreases the fitness of its host will decrease its own reproductive success (as the number of offspring carrying it will be reduced). Consequently, natural selection will favour parasites which cause less harm to their hosts (have lower virulence), leading eventually to the evolution of completely benign parasites (or commensals). While there are exceptions to this general rule, e.g., *Wolbachia*-like organisms that manipulate host offspring sex-ratio, or even host functional gender, to increase their representation in the next generation, the prediction is a strong one.

2.2.3. Mixed horizontal and vertical transmission

If horizontal transmission selects for higher virulence and vertical transmission selects for lower virulence, what should we expect in parasites that rely on both modes of transmission? An initial verbal prediction was that there should be a continuum of virulence, ranging from low virulence in mainly vertically-transmitted parasites, to high virulence in mainly horizontally-transmitted parasites. A classic study of fig wasps and their nematode parasites provided apparently strong support for this prediction (Herre, 1993). However, more recent theoretical work has made the picture more complex. Lipsitch et al. (1996) modelled the interaction between horizontal and vertical transmission as it relates to virulence and parasite epidemiology. They examined two scenarios – one where higher rates of vertical transmission did not have to be correlated with increasing virulence, and one where higher vertical transmission came as a result of higher virulence. In the first case, they found that parasite strains showing vertical transmission coupled with low virulence dominated the parasite population (i.e., vertical transmission leads to lower virulence). In addition, they demonstrated that as rates of horizontal transmission increased (due to increased host density) horizontal transmission also selected for lower virulence. This surprising result emerges directly from the epidemiological parameters of their model. Host individuals could only contain single-strain infections and thus, as horizontal transmission increased in frequency the host population approached saturation by the parasite. At this point, vertical transmission becomes the dominant

mode of transmission and lower virulence is selected. In the second case, the parasite population became dominated by strains of higher virulence using horizontal transmission (horizontal transmission leads to higher virulence). However, as Lipsitch et al. (1996) noted, there are important caveats to their conclusions. First, they assumed an absence of multiple infections within hosts (see Section 2.4). Second, they assumed a fixed host population where prevalence levels for the parasite would increase and (potentially) saturate across time (see Section 2.3.2). Thus, it remains unclear exactly what levels of virulence we should predict for parasites which utilise both horizontal and vertical transmission.

2.2.4. Direct vs. indirect horizontal transmission

Horizontal transmission can be direct, requiring close physical proximity of the infected and uninfected individuals, or indirect, requiring a vector of some kind. Human malaria is a classic example of a parasite that uses indirect horizontal transmission, travelling between hosts via mosquitoes. Ewald (1983, 1994) was the first to propose that indirect transmission has implications for the evolution of virulence. He suggested that transmission of vectored parasites may be enhanced by the fact that virulence need not trade-off with parasite reproduction. The argument runs as follows: assume that a parasite causes morbidity (reduced locomotion) in its host. While this would reduce parasite transmission for directly horizontally transmitted parasites – by lowering the potential contact rate with susceptible individuals – for vectored parasites it might even increase transmission as transmission is determined by vectors rather than by contact rate. Consequently, the trade-off between virulence and transmission (caused by increased parasite reproduction within the host) may be lost, and thus we would expect higher virulence in vectored parasites. This verbal argument is logically appealing and some empirical evidence exists to support it. However, there are also vectored parasites that exhibit low virulence. Recent theoretical work by Day (2001, 2002) has suggested that Ewald's hypothesis only follows under very restricted conditions and, in fact, that there is no *a priori* reason to assume that vectored and directly transmitted parasites should differ in their level of virulence (but see Section 2.3.3.1).

An alternative approach to understanding virulence in vector-born parasites and diseases starts by noting that, because vectors actively search out new hosts, such parasites/diseases undergo frequency dependent transmission (O'Keefe, 2004). Despite fundamental biological and theoretical differences between mass action and frequency-dependent transmission, models of the latter still do not suggest that higher virulence should be expected in vector-born diseases.

2.2.5. Long-lived free-living propagules – the curse of the pharaoh?

The death of Lord Carnarvon from a mysterious disease after opening the tomb of Tutankhamen has inspired the suggestion that parasites with long-lived propagules will evolve high virulence. The verbal argument is based on the trade-off model, and runs that by avoiding the cost of virulence (that is, host death and the end of transmission), such parasites are free to evolve higher virulence. Bonhoeffer *et al.* (1996) explored this argument using a theoretical model and showed that, for most situations, parasite virulence should be independent of propagule longevity. However, if the host-parasite system is not at equilibrium and the parasite propagule dynamics are faster than those of the hosts, then increased longevity predicts higher virulence. More recent studies, incorporating mixed infections (see Section 2.4) and spatial structure in the host population (see Section 2.3.3) make the picture even more complicated.

2.3. Epidemiology and the evolution of virulence

The epidemiology of a parasite depends upon features of the host population and on the ability of the parasite to exist in single- and/or multiple-infections. These factors interact fundamentally with modes of transmission in determining parasite spread and prevalence within a host population, and the level of virulence to which a parasite might be expected to evolve.

2.3.1. Host demography

A key feature of host populations that interacts with both parasite epidemiology and the evolution of virulence is the background host mortality rate. The general expectation is that when hosts are long-lived, parasites should have low virulence (that is, parasite-related host mortality should be low or take a long time to occur). In contrast, when hosts are short-lived, parasites should have higher virulence (Lenski and May, 1994). This result comes directly out of the trade-off model (see Section 2.2.1). As host lifespan declines, parasites have decreased opportunities for transmission and thus the optimal virulence increases as a correlate of selection for increased transmission. This theoretical prediction has recently been supported experimentally in a study that manipulated transmission in the nuclear polyhedrosis virus of the gypsy moth (Cooper *et al.*, 2002).

Unfortunately, and as with most other aspects of the evolution of virulence, the situation is not quite that simple. Recent work by Williams and Day (2001) has emphasised the fact that increased host mortality only automatically selects for higher virulence if background mortality and parasite-induced mortality are additive. If different causes of mortality

are not additive, which is the case when virulence is context-dependent (e.g., Brown *et al.*, 2000, 2003), then reduced host longevity may or may not select for higher virulence, depending upon the exact relationship between background mortality rates and parasite-caused mortality. Nevertheless, in most cases it seems likely that this relationship is such that some increase in virulence will be selected for. Further complications result if multiple infections are taken into account (see Section 2.4).

2.3.2. Host population size

While the majority of the models discussed above assume that host populations are at a stable equilibrium, this is not necessarily true for many biological systems. Hosts may exhibit constant growth, or cyclical patterns of growth and decline. The latter pattern can be found in both annual, e.g., bumble bees, and perennial species, e.g., honey bees. This has a number of implications. First, predictions about the evolution of virulence that emerge from models relying upon equilibrium dynamics may not be applicable to more natural biological systems. Second, many parasites and diseases which appear at first sight to be endemic may more accurately be described as epidemic diseases, with patterns of rapid spread and then rapid decrease within the host population.

Of the models described above, only a few refer to systems which are not at equilibrium. Bonhoeffer et al. (1996) (see Section 2.2.5) compared the results of their analyses between host-parasite relationships in equilibrium and disequilibrium. They found that there was a significant effect of disequilibrium, with predictions for the relationship between propagule longevity and virulence depending upon whether the host or parasite was cycling faster in the system. Similarly, Lenski and May (1994) showed that in an epidemic (disequilibrium) system, where either the parasite is invading a new host population or the susceptible host population is constantly growing, higher levels of virulence evolve than in an endemic (equilibrium) system.

2.3.3. Host population structure - spatial

The epidemiological models that underlie most of our understanding of host-parasite dynamics, both in ecological and evolutionary time, depend upon the mass action principle with respect to transmission. Basically, they assume that transmission is a function of the numbers of infected and susceptible individuals in the host population. This ignores the fact that most host populations exhibit some form of spatial structure. This is especially true for social insects, where high levels of population structure (caused by division of labour) occur within colonies (Schmid-

Hempel, 1998). Only a few studies have examined the potential effect of host population structure on the epidemiology and evolution of parasites.

2.3.3.1. Structure and direct vs. indirect horizontal transmission

Boots and Sasaki (1999) used a lattice model to represent a spatially-structured host population. When parasite reproduction was local (that is, bound to the host) but transmission was global, they found that virulence evolved as expected under standard epidemiological models. However, as transmission became more locally restricted (modelling the situation where parasitised hosts are more likely to infect near- over far-neighbours) the predicted level of virulence similarly declined. This suggests that vectored diseases/parasites may indeed exhibit higher levels of virulence than directly transmitted parasites (see Section 2.2.4). However, and perhaps more importantly, it suggests that quantitative predictions about the level of parasite virulence derived from mass action or non-spatially explicit models are likely to be too high. Similar results were found by O'Keefe and Kata (2005) when spatial structure and frequency-dependent transmission were combined.

2.3.3.2. Structure and free-living propagules

Kamo and Boots (2004) examined the problem of the curse of the pharaoh (see Section 2.2.5) in spatially explicit populations (lattice models). While they found some situations in which propagule longevity correlated positively with virulence (see Section 2.5), in general they concluded that there was no evidence for the curse of the pharaoh and that in fact higher virulence may be selected for by shorter rather than longer-lived infective stages.

2.3.4. Host population structure – genetic

As well as being physically structured, host populations can exhibit genetic structure. This may be driven by low levels of offspring dispersal producing 'islands' of high genetic relatedness within the population (social insects provide an extreme example of this situation). The ability of parasites to infect and reproduce in hosts is assumed to be based on interactions between host and parasite genotypes (Schmid-Hempel, 1998). Consequently, host genetic population structure may influence both the spread and prevalence of parasites, as well as the evolution of virulence. Regoes *et al.* (2000) predicted that in a system with two host types, parasites should either evolve to be generalists, with correspondingly low levels of virulence, or to be specialists, exhibiting higher virulence on each host type. Gandon *et al.* (2002) examined the situation where host resistance varies (presumably due to genetic variation). In their model, if only sin-

gle infections occurred there was no effect on the evolution of parasite virulence (but see Section 2.4). While recent empirical work suggests a relationship (in ecological time) between the epidemiology and impact of parasites and genetic heterogeneity in social insects (bumble bees: Shykoff and Schmid-Hempel, 1991; Liersch and Schmid-Hempel, 1998; Baer and Schmid-Hempel, 1999, 2001; honey bees: Tarpy, 2003), the evolutionary implications for parasite virulence remain untested.

2.4. Single vs. multiple infections

As with all organisms, parasites compete with conspecifics for resources, i.e., hosts. If a host can only contain one infection at a time, then this competition occurs in the arena of transmission and underlies the evolutionary relationship between horizontal transmission and higher virulence (see Section 2.2.1). However, if multiple infections can infect a given host individual, competition will take place among parasite strains within a host. This has immediate implications for the epidemiology and evolution of the parasite.

Multiple infections are most likely when parasite prevalence is high, the probability of transmission is high, or when parasites mutate within their host (see Section 2.5). High host density, spatially structured populations and the coexistence of vertical and horizontal transmission may all contribute to increasing prevalence and probability of transmission. Consequently, the theoretical predictions described in Sections 2.2.1, 2.2.3, 2.2.4, 2.3.3 and 2.3.4 may all be modified by multiple infections.

Parasite competition via multiple infections can take two forms. Super-infection occurs when one parasite enters an already infected host and displaces its current parasite (superior competitiveness). Co-infection occurs when 2 or more parasites (or parasite strains) can occupy a single host at one time. In this case, competition for host resources will occur among those parasites. Both of these types of competition may lead to increases in the expected level of virulence. Parasites which utilise host resources most rapidly are likely to outcompete their conspecifics – this result is simply the extension of standard ecological competition theory. If virulence correlates with an increase in the rate of host resource use, then this competitive process should inexorably lead to an increase in parasite virulence.

Only a few theoretical studies have concentrated on the impact of single vs. multiple infections on the evolution of virulence (Claessen and de Roos, 1995; May and Nowak, 1995; van Baalen and Sabelis, 1995; Leung and Forbes, 1998; Gandon *et al.*, 2001). In general, these studies predict that multiple infections should lead both to higher virulence (Claessen

and de Roos, 1995; May and Nowak, 1995; van Baalen and Sabelis, 1995; Leung and Forbes, 1998) and competitive exclusion among the parasite strains (Van Baalen and Sabelis, 1995; Leung and Forbes, 1998). In contrast to these theoretical predictions, Taylor *et al.* (1998) showed that mixed infections of rodent malaria exhibited higher virulence, but that this was not due to higher parasite reproduction. Even more surprisingly, Ebert and Mangin (1997) found results suggestive of the idea that mixed infections could lead to lower virulence in a microsporidian parasite of invertebrates. Gandon *et al.* (2001) used a theoretical model to show that this unexpected result could occur in a host-parasite system with superinfections. Because high rates of host mortality reduce the likelihood of multiple infections (by reducing contact rate and thus the opportunity for transmission), in a system with multiple infections virulence should be higher under low rather than high host background mortality rates. This prediction reverses the relationship among horizontal transmission, virulence and host mortality described in Section 2.3.1.

In other studies, Lipsitch *et al.* (1996) acknowledged that parasites with mixed horizontal and vertical transmission would evolve to higher virulence in the presence of multiple infections. Similarly, Gandon (1998) suggested that if mixed infections could occur then long-lived parasite propagules would be associated with higher virulence (see Section 2.2.5). Finally, one explanation for the empirical results of Herre (1993), previously taken to support the idea of a virulence continuum between horizontal and vertical transmission (see Section 2.2.3) is that higher virulence was driven by competition among nematodes in mixed or multiple infections.

This view of multiple infections is driven by the idea that within-host competition occurs through resource exploitation. However, parasites may compete more directly. Massey *et al.* (2004) showed that bacteria may compete directly via bacterial-specific toxins, making mixed infections less virulent than single infections. Obviously, to understand the impact of mixed or co-infections on the evolution of virulence we need a good biological understanding of how parasites interact within hosts.

2.5 Biology of the infection

So far in this review, the biology or epidemiology of parasite infections within hosts has been treated to a large degree as a black box. Most epidemiological models of parasite virulence assume that the production of transmission stages within infected hosts and the contact rates between infected and uninfected hosts are constant over the course of an infection (reviewed in Day, 2003).

The most obvious case where such an assumption is invalid is when

parasites are semelparous, that is, host death is required for parasite transmission. Ebert and Weisser (1997) showed that in such cases parasite virulence (the timing of host death) should be higher (earlier host death) when carrying capacity within the host is low (i.e., when parasite growth and reproduction within the host is limited) or when host mortality rates are high (mirroring the general relationship between background host mortality and virulence, see Section 2.3.1). Kamo and Boots (2004) applied the idea of semelparous parasite reproduction to the relationship between longevity of free-living propagules and virulence, and found that in a spatially explicit model (see Section 2.3.3.2) this was the only scenario under which both high virulence and high propagule longevity would coevolve.

Day (2001, 2003) has examined within-host parasite biology in more detail. He has shown that when transmission occurs at an early stage within the lifespan of an infection (Day 2001) or if there is a timelag between the onset of transmission and the onset of the effects of parasite virulence (Day 2003) we should predict higher virulence.

An additional aspect which was examined by Bonhoeffer and Nowak (1994) was the effect of parasite mutation within individual hosts. They developed a model to look at the role of intra-host mutation and competition among parasite strains in the evolution of virulence. They found that intra-host competition generated a virulence polymorphism in the parasite population, shifted mean virulence beyond the optimal level for parasite transmission, and also that the parasite could evolve to intermediate levels of virulence even in the absence of a trade-off between transmission rate and virulence. Their model is particularly appropriate for viral diseases with high mutation rates and long infection periods.

From this work, it seems clear that incorporating details of the within-host biology of infections into epidemiological models will be an important step in understanding the evolutionary epidemiology of parasites.

2.5.1 Behaviour and transmission

Our focus in this chapter precludes an in-depth coverage of the impacts of parasites on host behaviour, and the obvious implications of such changes for either enhancing or controlling the spread of parasites (Schmid-Hempel, 1998). Nevertheless, such changes (due to host-manipulation by the parasite or adaptations of the host) are an integral part of the biology of host-parasite interactions. For example, sacbrood virus changes the behaviour of infected bees in a way that reduces further viral transmission (Chapter 1; Bailey and Ball, 1991). While such changes in behaviour may be implicit within transmission parameters in theoretical

models, their inclusion as explicit factors may well lead to different dynamics and predictions for the evolution of virulence.

2.5. Mixed species infections

The vast majority of empirical and theoretical work has examined the epidemiological and evolutionary properties of host-parasite systems through single-species interactions. However, hosts can support many different parasite species at the same time. How these parasites interact, directly and indirectly, and impinge on each other's epidemiology and evolution, is an area that remains to be explored. Interactions among mixed infections within hosts may increase the population of both parasites, decrease one and increase the other, or decrease the population growth of both (Cox, 2001). If virulence and transmission are the result of increased parasite reproduction then such interactions have obvious implications for parasite population dynamics and virulence in both ecological and evolutionary time. Recent work on insect pathogens has suggested that even otherwise avirulent parasites can play an important role when in mixed infections (Thomas *et al.*, 2003). Similar results have been found for viruses and mite interactions in honey bees (reviewed in Schmid-Hempel, 1998, pp. 27-31; see Section 4 below).

2.6. Discussion

It should now be clear that a large number of factors play a role in the evolutionary epidemiology of host-parasite relationships. Virulence is a key feature of such relationships, and its management is a key aim of evolutionary parasitologists. Despite this array of potential causal factors in the evolution of virulence, it may still be possible to make some preliminary generalisations. Firstly, from the parasite perspective, horizontal transmission and mixed (co-)infections are likely to result in the evolution of higher virulence. Secondly, from the host perspective, a high background host mortality rate and rapidly growing populations will also select for higher virulence in parasites. In contrast, spatially and genetically structured host populations appear, in general, to select for lower levels of virulence. Finally, vectored diseases may evolve to higher levels of virulence, but there is probably no reason to expect diseases that rely on free-living propagules for transmission to have higher levels of virulence than directly transmitted parasites and diseases. All of these factors turn out to be relevant in understanding honey bee viruses.

3. The honey bee as a host

Diseases in honey bees have been studied intensively within colonies and much is known about the intra-colonial spread and virulence of a variety of parasites. However, only a few attempts have been made to discuss honey bee epidemiology from the colony level perspective (Royce and Rossignol, 1990; Schmid-Hempel, 1998; Fries and Camazine, 2001). Here we outline why epidemiological studies in honey bees must consider both individual-level and colony-level parasite reproduction and transmission if we are to understand and manage levels of virulence in parasites of honey bees.

The field of evolutionary epidemiology is rapidly expanding (see Section 2 above) as it has become apparent that an understanding of how parasite virulence evolves may also enable the management of disease processes to decrease virulence. However, in spite of the importance of this emerging field, the perspectives provided by evolutionary epidemiology have only recently been applied to social insects. The works of Paul Schmid-Hempel (e.g. Schmid-Hempel, 1995, 1998, 2001) have developed the framework for understanding the evolution of social insect hosts and their parasites. In this section we apply these ideas to honey bees to determine how the biology of *Apis mellifera* relates to their interaction with parasites. A search in the BIOSIS data base for the years 1945-2005 revealed only one relevant hit when searching for the combination "epidemiology" and "honey bees" (using the term 'epizootiology' produced no relevant hits). The application of evolutionary epidemiological considerations to honey bees and their parasites may shed new light on our understanding of virulence in this system. While we concentrate below on *A. mellifera*, and later on their viral infections, most of our discussion is equally relevant for other species of honey bee as well as for parasites other than viruses.

In this section we discuss the biology and reproductive system of the honey bee, delineating its reproduction at both the individual and colony levels. We then discuss the consequences of this reproductive system for horizontal and vertical transmission of parasites within as well as between colonies. We also comment on the consequences of the spatial structure of the host, at both levels, for parasite transmission.

3.1. Within colony reproduction

During the foraging period in temperate climates, a colony of honey bees normally consists of one queen bee, 20-50 thousand worker bees and a few hundred drones. The queen bee is the only reproducing female

within the colony and has the capacity to produce over 2000 eggs per day under optimal conditions. The determination of offspring caste and sex is a result of two different mechanisms:

(i) worker bees are normally the product of fertilised eggs where the hatched larvae receive brood food (glandular secretions from young bees) diluted after about three days post-hatching with pollen and nectar. If these larvae were to receive brood food only, the ovaries would develop fully and the hatching bee would develop into a queen bee. Thus, caste determination is based on nutrition during larval development.

(ii) drones are normally the result of unfertilized eggs (arrhenotoky). However, it is not non-fertilization *per se* that produces drones. Honey bees (and probably most haplodiploid hymenopteran insects) have a sex-determining system with are multiple alleles at the sex locus. The gene responsible for sex determination (the complementary sex determiner, *csd*), has recently been identified in the honey bee (Beye *et al*., 2003). Heterozygosity at this locus results in females. When there is only one functional allele present at this locus as a result of homozygosity (fertilized eggs) or, as in the case of honey bees, hemizygosity (unfertilized eggs), the resulting individual is a male. Adult diploid males are never found in colonies, however, since they are detected as young larvae and eaten by the nurse bees. It has been estimated that a large bee population may hold around 15-20 sex alleles.

Thus, within a colony there is both sexual and parthenogenetic reproduction. Because the queen will be mated (mostly on a single occasion) with some 15 or so different drones, the genetic composition of the colony becomes complex. As meiosis does not occur during spermatogenesis in haploid drones, each drone produces genetically identical sperm. This produces groups of super-sisters within the colony with a relationship coefficient of 0.75 when they have the same "father". These groups of super-sisters are then related to one another by 0.25, as normal half-siblings.

While within colony reproduction is essential for colony growth and survival, the colony as a 'super-organism' (Moritz and Southwick, 1992) also has to reproduce.

3.2. Colony-level reproduction

The Darwinian fitness of a honey bee colony requires reproduction not only at the individual level, but also at the colony level. Honey bees are super-organisms that consist of individual units (bees) that have no function or survival capacity when removed from their colony context. Conceptually, this can be compared to taking simple neurons in the brain

out from their brain context. Individual (dumb) bees become a functioning (smart) colony when integrated. The simple (dumb) neurons become a thinking (at best) brain when integrated into the whole. In many social Hymenoptera, colony-level reproduction occurs with the release of new queens that found new colonies from scratch; the system in honey bees is quite different (and also seen in a few swarm-founding wasps and ants). Colony level (super-organism) reproduction in honey bees occurs when the colony swarms, that is, reproduction occurs by colony fission. When honey bees divide by swarming, the first swarm issued normally contains the old laying queen and subsequent swarms, if any, contain unmated queens. Although there is great variation in swarm sizes, each swarm issued can be expected to depart with 50-60% of the adult bee population (Winston, 1987). There is an age-related tendency to depart with the swarm, with younger workers dominating the swarm, although all age categories of bees will be represented (Muszynska, 1976; Winston, 1987). This mode of reproduction by colony fission has obvious implications for parasite transmission and is likely to be important for the evolution of parasite virulence (see Section 3.4 below).

3.3. Potential routes for parasite transmission

It is obvious from the description above that the transmission of parasites in honey bees must occur both within and between colonies. Unsurprisingly, given the complexity of honey bee biology, a variety of routes are potentially available, each of which may have implications for parasite virulence. There is the potential for vertical and horizontal transmission both among individuals and among colonies. Below we discuss these different modes of transmission within the sections for intra- colony and inter-colony transmission respectively.

3.3.1. Intra-colony transmission

Inside the colony, transmission can be either horizontal or vertical. Although recent evidence suggests a potential role for vertical transmission of deformed wing virus via eggs (transovarial transmission; Chen *et al.*, 2005), the vast majority of intra-colony transmission appears to be horizontal in nature. In general, queens are rarely carriers of parasites and when they do become infected they are replaced by bees through supersedure (where a young queen is raised and starts laying eggs before the old queen disappears from the colony). As discussed in Section 2, horizontal transmission can take a variety of routes, each of which has implications both for the epidemiology of the parasite within the honey bee colony and the evolution of virulence. There is evidence for both direct

(adult-to-adult, adult-to-larvae) and indirect transmission of parasites in honey colonies, with indirect transmission occurring both via vectors and relatively long-lived propagules (Bailey and Ball, 1991).

The spatial structure of the host may largely determine transmission. The honey bee has within colony spatial structure both at the level of brood – where the comb imposes structure – and at the level of the adult population – where division of labour imposes structure on interaction patterns and the location of individuals. Theoretical work has identified the potential importance of such structure for parasite transmission (Schmid-Hempel, 1998; Pie *et al.*, 2004). Currently, there are no specific data on whether colony comb structure and position of the brood influence transmission in honey bee colonies. However, there is some evidence to suggest that the age-dependent division of labour in honey bees may have an impact on transmission. Sacbrood virus (SBV) propagates in the hypopharyngal glands of adult bees without causing disease symptoms, and the larvae become infected as they feed on the gland secretions (Bailey, 1969). Cleaning activities (cleaning out of diseased brood) and feeding activities of young bees overlap in the age dependent sequence of tasks performed by bees (Winston, 1987). Thus, division of labour is likely to increase virus transmission rates in the case of SBV as nurse bees also become contaminated with virus particles as they clean out diseased larvae. In addition, there may even be a host response to reduce transmission, since infected adult bees are less likely to engage in feeding activities than non-infected bees (Bailey & Fernando, 1972).

3.3.2. Inter-colony parasite transmission

Mirroring the situation of intra-colony transmission, parasite transmission between colonies can be both horizontal and vertical in nature. Horizontal transmission may occur in a variety of ways, including:

1. *Drifting*. Although the colony entrance is effectively protected by guards against intruders in search of food stores, bees often enter the wrong colony within apiaries by accident. Drifting of bees may even occur over large distances between apiaries, in particular with drones that are readily accepted by any colony during some part of the season (Pfeiffer and Crailsheim, 1998). Any bee that is infected by parasites or carries infective propagules may then transfer disease to new colonies.
2. *Robbing*. When nectar sources become scarce while flying conditions prevail, honey bees will attempt to rob the stores of other colonies. If colonies cannot effectively defend the hive entrance they will soon succumb to intruders. Colonies may be weak for a variety of

reasons, and disease may be a key reason for such weakness. Robbing out of infected colonies is an effective mechanism of parasite transmission, with even mite infestations being effectively transferred in this manner (Sakofski, 1990).

3. *Contact with infectious material from the environment.* This transmission route is limited to parasites that can survive outside of the

Table I. List of common honey bee pathogens, trivial names, mode of intercolony transmission and virulence. + or +++ under transmission indicates which mode of transmission is estimated to be most important for moulding the host-parasite relationship under natural conditions; signs in parentheses indicate the impact of apiculture on transmission. Virulence is estimated from apicultural data (adapted and reworked from Fries and Camazine, 2001).

Pathogen			Transmission		Virulence	
Group	Latin name	Trivial name	Horizontal	Vertical	Individual level	Colony level
Protozoa	*Nosema apis*	Nosema disease	+ (++)	+++ (+)	Benign	Benign
	Malpighamoeba mellificae	Amoeba disease	+ (++)	+++ (+)	Benign	Benign
Fungi	*Ascosphaera apis*	Chalkbrood	+ (++)	+++ (+)	Lethal	Benign
	Aspergillus flavus	Stonebrood	+ (++)	+++ (+)	Lethal	Benign
Bacteria	*Paenibacillus larvae*	American foulbrood[1]	+ (+++)	+++ (+)	Lethal	Lethal[1]
	Melissococcus plutonius	European foulbrood	+ (++)	+++ (+)	Lethal	Benign
Virus	ABPV	Acute bee paralysis virus	+ (++)	+++ (+)	Lethal	Benign to lethal[2]
	DWV	Deformed wing virus	+ (++)	+++ (+)	Lethal	Benign to lethal[2]
Mites	*Acarapis woodi*	Tracheal mite	+ (++)	+++ (+)	Benign	Benign to lethal[3]
	Varroa destructor[4]	Varroa mite	+ (++)	+++ (+)	Benign	Benign to lethal[4]

[1] Colony level virulence may be an apicultural phenomenon – this needs to be investigated

[2] Only severe effects when vectored by varroa mites

[3] Only severe where the mite has been recently introduced or after owerwintering in cool temperate regions (McMullan and Brown, 2005)

[4] Only severe where the mite has been recently introduced and/or where mite control removes selective pressure from being virulent

host environment and may occur perhaps via flowers during foraging (as is the case for the trypanosomatid *Crithidia bombi* in bumble bees; Durrer and Schmid-Hempel, 1994) or via abandoned nest sites. This survival capacity is extreme for the brood disease American foulbrood (AFB) caused by the spore forming bacterium *Paenibacillus larvae*. Nest sites where the bees have succumbed to AFB may be infectious for decades and infected nest sites are not avoided by swarming bees (Ratnieks and Nowakowski, 1989).

Other routes of inter-colony horizontal transmission, such as contact between infected and uninfected individuals from different colonies during foraging, are probably of minor importance in general, although they may be involved in the transfer of spiroplasma (Clark, 1977).

While spatial structure of the host may be likely to influence within colony transmission (see Section 3.3.1), it must certainly have an effect on colony level transmission. In a natural system, honey bees are likely to appear as scattered units with suitable nest sites limiting colony density, as compared to the apicultural context where beekeepers crowd colonies together in apiaries. At least some evidence suggests the existence of different levels of parasite transmission depending upon the density and distribution of honey bee colonies. Adult bees from wild colonies in areas without beekeeping rarely carry detectable levels of AFB spores, whereas bees from wild colonies in areas with beekeeping are often contaminated by AFB spores (Hornitzky et al, 1996), indicating an effect of colony density on transmission. Furthermore, a clear correlation has been demonstrated between colony density and the incidence of chronic paralysis, suggesting an influence of colony density on virus transmission (Bailey *et al.*, 1983). Intuitively, inter-colony transmission of parasites in the honey bee system must be dependent on the spatial structure of the host but published data demonstrating this causal effect are, again, lacking. Unpublished data on AFB strongly support the hypothetical influence of colony density on transmission; colonies within apiaries containing clinically diseased neighbour colonies contract detectable spore levels in their honey and on adult bees faster than colonies at different distances from this apiary, with the distance from the diseased apiary determining the horizontal transmission rate (Fries & Korpela, unpublished data).

While vertical transmission may be relatively unimportant within colonies, it is another matter entirely for inter-colony transmission. As stated earlier, colony reproduction in honey bees is by fission, and thus vertical transmission may occur when a swarm leaves an infected colony. Transmission may be via either infected workers, or the transfer of infected material (e.g., honey) from the maternal hive to the new colony's

nest site. Consequently, vertical transmission has the potential to play a major role in the spread and maintenance of parasites within honey bee populations.

In Table I we list some common parasites of honey bees. The table also includes assumed main routes for disease transmission between colonies, i.e., how new colonies are most likely to contract the respective disease agents. Very little data exists on this topic for honey bee diseases in general but it is obvious from the reproductive biology of honey bees that swarming must be an important route for pathogens to become transmitted to new host colonies (Fries and Camazine 2001). All the parasites mentioned in Table I are either carried inside or outside of bees, or can be isolated from adult bees from infected colonies.

3.4. Implications of transmission for parasite evolutionary epidemiology in honey bees

From the parasite's viewpoint, it must overcome three distinct fitness hurdles in order to reproduce and disperse to new honey bee hosts:

1. The parasite must infect an individual (and usually must be able to multiply within this new host).
2. The parasite must be able to infect additional individuals within the colony to maintain itself either endemically or epidemically.
3. The parasite must successfully gain access to new colonies.

In terms of fitness, the successful transfer of a parasite's offspring to a new colony is a critical step in its life history. If a parasite fails to achieve a foothold in another host colony, it will not increase its reproductive fitness, regardless of how prolific it has been within the original host colony. Thus, the first two hurdles (intra-individual and intra-colony transmission) are important aspects of parasite fitness only to the extent that they contribute to more efficient inter-colony transmission (hurdle 3). Thus, for the parasite there are two trade-offs: (i) between growth/reproduction within and the consequent impact on individual bees vs. transmission among bees, and (ii) spread and impact within colonies vs. transmission among colonies.

4. Understanding virulence levels of honey bee viruses

In section 2.1.1 we listed various ways of defining virulence. Here we begin by explaining how we define virulence in viral diseases of honey bees. Our benchmark for virulence is host mortality, of either individual infected bees or colonies. We use mortality for the sake of simplicity, but

note that many parasites cause morbidity (that is, they lower host fitness without causing mortality), and thus the absence of mortality in any given honey bee/virus system should not be taken to mean the absence of an impact of the virus on honey bee colonies. While using mortality as a metric may seem to be straightforward, the presence of covert infections (see Chapter 5) puts a serious wrinkle into the picture. Let us take three hypothetical situations: (i) the virus is present in 80 out of 100 colonies at any point in time and always leads to colony death, (ii) the virus is present in the same number of colonies but only 1 of the 80 colonies dies due to the virus, (iii) the virus is present in the same number of colonies but in 79/80 colonies as a covert infection and the single colony with an overt infection dies. Given our current level of knowledge, each of these situations is equally likely to be true for any given virus. In the first case the virus clearly has high virulence, while in the second case, even though it causes colony mortality (and thus, in that colony, has high virulence), at the population level the virus exhibits low virulence. What about the third case? If covert and overt infections are all part of one viral population, then our conclusion from the second case applies. However, if overt and covert infections are effectively independent, perhaps due to quasispecies and mutation (see Chapter 2), then the virus must be redefined as having high virulence. We can make the same argument at the level of individual bees. Clearly, to define viral virulence accurately we need to know the level of prevalence, the level of mortality associated with it, and the relationship between covert and overt infections. At present, we do not know this for any honey bee virus. Consequently, our definitions of parasite virulence at this point in time are best guesses based on incomplete data.

4.1. In the absence of *Varroa destructor*

As detailed in section 3, honey bee colonies represent multi-level systems as far as the evolution of viral virulence is concerned. At each level, different factors may drive the evolution of virulence, and between levels these factors may act either in concert or in opposition.

1. At the lowest level, we have viral infection within individual bees. Assuming an overt infection, selection should act to favour those viral strains that replicate most rapidly. Given the presence of mutation and quasispecies, the theoretical prediction is clear (Bonhoeffer and Nowak, 1994) and we should expect the evolution of highly virulent viral strains at the level of the individual bee.
2. The next level up is the evolution of viral infections within individual colonies. At this point, trade-offs between within-host growth and between-host transmission appear. If transmission within col-

onies is vertical, this should select strongly for low levels of viral virulence and act in opposition to selective forces at the intra-individual level. However, we believe that despite recent evidence (Chen *et al.*, 2005) the vast majority of intra-colony transmission, especially during overt infections, must be horizontal in nature. Studies have demonstrated horizontal transmission of viral infections from workers to brood and workers to workers, and at least in the case of sacbrood virus this seems to be sufficient to explain the maintenance of infections within colonies (Bailey and Ball, 1991). The simple expectation from section 2 is that horizontal transmission should select for higher virulence leading to some optimum level of virulence that maximises the trade-off between within host reproduction and between host transmission. However, spatiogenetic structure within honey bee colonies (section 3.3.1) should lead to a reduction in this optimum (section 2.3.3, 2.3.4). Even if covert infections act as the equivalent of long-lived propagules, this should make no difference to patterns of virulence (section 2.7).

What about host-biology? As detailed in section 3.1, honey bee colonies go through an annual cycle of growth and decline. During the growth phase, a rapidly increasing host population should lift the upper constraint on virulence (section 2.3.2). Interestingly, an increase in background host mortality levels is also expected to lift this upper constraint (section 2.3.1), and so when colonies are growing and actively foraging, viruses should be selected for higher virulence. In contrast, as colony birth rate and foraging decrease in the autumn, selection should act to drive down the level of virulence as the opportunities for transmission decrease. So, when looking at the evolution of virulence within colonies, we should expect relatively high levels of virulence – based on the predominance of horizontal transmission among bees and viral competition within bees – to be selected.

An additional, but important factor, is the presence of multiple viral species, or virus' and other parasites, within individual bees and colonies (see Chapter 1). For example, the chronic bee paralysis-virus associate reduces the virulence of CBPV, and the impact of black queen cell virus is intimately associated with co-infection by the microsporidian, *Nosema apis* (Bailey & Ball, 1991). How such interactions may modify the evolution of viral virulence will probably turn out to be system-specific, but at present we lack sufficient empirical data about the biology of these interactions to make any specific predictions.

Table II. Transmission and virulence in viruses of honey bees (Apis mellifera). ß is as measured in apicultural systems and may not reflect the impact of these viruses in unmanaged populations. For more detailed information about mechanisms of transmission and impact, see Chapters 2 and 5.

Virus	Natural transmission[1]		Transmitted by Varroa?[2]	Virulence without Varroa		Varroa-related virulence	
	intra-colony	inter-colony		individual	colony	individual	colony
Chronic paralysis	H	H? V?	I	Severe	Severe	–	–
Cloudy wing	H	H? V?	I	Severe	Severe	–	–
Filamentous	?	H? V?	I	Benign	Benign	–	–
X	H	H? V?	No	Severe	Severe	–	–
Y	H	H? V?	No	Benign	Benign	–	–
Kashmir	H	H? V?	Yes	Severe	Benign	Severe	Severe
Deformed wing	H; V?	H? V?	Yes	Benign?	Benign?	Severe	Severe
Sacbrood	H	H? V?	Yes	Moderate	Benign	–	–
Arkansas	H?	H? V?	?	?	?	Severe?	–
Slow paralysis	H?	H? V?	Yes	Benign	Benign	Severe	–
Black queen cell	H	H? V?	?	Severe	Benign	–	–
Acute paralysis	H	H? V?	Yes	Benign	Benign	Severe	Severe
Egypt	H?	H? V?	?	?	?	Severe?	–

[1] H: horizontal transmission; V: vertical transmission; H? and V? indicate possible but untested routes

[2] Yes: Varroa destructor shown to transmit; I: injection shown to transmit, but unknown for mite; No: no transmission via mite

[3] – : no relevant data are available

3. The final level may be termed colony-level selection, and refers to our expectations for the evolution of viral virulence in a population of colonies. Theory suggests that the key point at this level is the relative importance of horizontal versus vertical transmission in maintaining the virus within the honey bee population. While opportunities for horizontal transmission among colonies are likely to be high in managed apiaries (e.g., Hornitzky, 1998), where high colony densities result in a large amount of drifting among hives (section 3.3.2), nothing is known about the potential for horizontal transmission among colonies in natural populations. However, given the likely distribution and density of wild colonies, vertical transmission during fission events to offspring swarms should be the dominant mode of transmission. If this is true then there should be strong selection at the colony-level for low virulence in viral pathogens, as highly virulent pathogens will either kill off their host colony prior to fission (and thus have no opportunity for transmission) or will reduce their growth to the point that they are unable to swarm (with similar impacts on transmission). Given that maintenance of the virus in the honey bee population ultimately depends upon inter-colony transmission, vertical transmission as the dominant route should result in viruses that have little or no effect at the colony level. In fact, unless there is some horizontal transmission among colonies, theory suggests that viruses should ultimately be expected to evolve to be commensals. Both acute paralysis virus and slow paralysis virus, which prior to the advent of *Varroa* mites were never associated with disease, provide evidence for such a scenario. However, the existence of generally low, but still noticeable in some cases (e.g. CBPV), colony level impact from virus infections (in the absence of *Varroa destructor*) suggests that some horizontal transmission among natural colonies must take place.

What about the presence of covert infections? If covert infections represent the equivalent of a reservoir for the virus, i.e., they act to maintain the virus in the same way that a seedbank maintains plant species in the absence of adult plants, then the dynamics may be substantially different. In this case, overt infections may well represent epidemic rather than endemic diseases, perhaps triggered by particular environmental conditions (e.g., bad weather or low food availability) or host contexts (e.g., overcrowding within a hive). CBPV represents a potential example of such a system (Allen and Ball, 1996). In natural populations, where opportunities for horizontal transmission are likely to be low, such an epidemic might rage through a colony, resulting in colony death, but would

be unlikely to spread through the honey bee population (that is, it would be an intra- rather than inter-colony epidemic). However, in the managed system where opportunities for horizontal transmission are high, such an epidemic might well run through the larger honey bee population, causing mass colony mortality before dying down. Thus, at the population-level, viral impact may simply be a phenomenon of honey bee management. For evidence of such dynamics related to *V. destructor* infestation, see Chapter 1.

Table II summarises what is known about transmission and virulence in viral pathogens of bees. In contrast to Table I, here we report only known modes of transmission, or the absence of such data, rather than estimations based on host biology. We do this deliberately, in the hope that it will inspire research into viral transmission routes. We have categorised virulence based on the assumption that if a virus has been known to cause mortality it has high virulence – thus, we ignore the complications of covert maintenance and parasite prevalence in the host population. It can be seen that, under natural conditions, viruses can vary in their level of virulence. However, it should be noted that even for those viruses that exhibit severe virulence (e.g., CBPV), killing individual host bees as well as colonies, for much of the time the virus is maintained within the host population in an inactive state. Thus, high prevalence may not translate into a major impact on honey bee populations even for these viruses. The main feature that can be taken home from Table II is that we know remarkably little about inter-colony transmission of viruses. Consequently, it is unclear how selective forces acting at inter- and intra-colony levels interact to determine virulence in these viral pathogens. However, the presence of variation in virulence (at the individual and colony levels) across honey bee viruses indicates that variation in modes of transmission within and among colonies is likely to be shaping the evolution of virulence in these host-parasite systems.

4.2. In the presence of *Varroa destructor*

The current increasing virus problems facing apiculture are caused by the introduction of an exotic parasitic mite, *Varroa destructor*, into European honey bee, *Apis mellifera*, populations. The mite probably feeds on adult bees by piercing through the ventral membranous inter-segmental connections on the bee abdomen and sucking up haemolymph (Bowen-Walker & Gunn, 2001). During the reproductive phase of the mite it feeds on bee pupae by piercing through the pupal skin (Bailey & Ball, 1991). The feeding activity of the mite probably triggers replication of certain virus infections in individual bees and bee larvae. The mite then acts as a vector

for virus transmission between adult bees and brood, thereby fundamentally changing the routes of within colony virus transmission of all virus types that replicate upon injection into bee haemolymph (see Table II and Chapter 1 for more details). As overt infections develop within colonies, inter-colony virus transmission rates probably also increase. Indeed, high rates of between colony horizontal transmission of deformed wing virus (DWV) have been demonstrated when mite free colonies with undetectable levels of DWV infections (using serology) develop overt virus infections within a few months after being introduced into a heavily mite infested and DWV infected apiary (Nordström *et al.*, 1999). To complicate the matter further, this virus may even replicate in the mite (Yue and Genersch, 2005). Vector borne pathogens in general are often severe to the host, but benign to the vector (e.g.malaria) (Ewald, 1993). Clearly, the introduction of *V. destructor* has fundamentally changed the transmission routes for some honey bee viruses. Because of the novelty of this change it is not yet clear what the outcome will be of these changes in modes of transmission of certain viral infections. Predictions based on evolutionary epidemiology theories suggest that more virulent variants may evolve where the virus is vectored by the mite – vectoring removes the contraint of host mortality and dramatically increases rates of horizontal transmission, both of which enhance the fitness of virulent strains that emerge within individual host bees. Given the potential for rapid mutation and mutant swarms in RNA viruses, it seems likely that these viruses have already evolved away from their pre-mite state. Because beekeepers remove the selective disadvantage of being virulent at colony level, by removing the vector through mite control measures, the current problems with virus induced colony mortality are likely to continue, or even increase, unless mite tolerant stock and/or virus resistant stock is developed. Experiments using natural selection demonstrate that honey bee populations infested by *V. destructor* and infected by DWV can not only survive , but increase in fitness, after initial high levels of mortality (Fries *et al.*, 2005). Actually, honey bee populations that do survive mite infestations – and associated virus infections - exist in several locations where man has not interfered (Rosenkranz, 1999). As a caveat, we note that mite-related virulence may be analogous to the case of Polio in humans, and thus a non-adaptive expression of virulence, but even if this was initially true, the evolutionary potential of RNA viruses suggests that virulence should rapidly evolve in this system in the ways outlined above.

The honey bee – mite – virus association probably offers an opportunity to study evolutionary epidemiology in the field. As demonstrated in natural systems, although mite infested colonies are likely to succumb to

virus infections if left untreated, the species *A. mellifera* is unlikely to perish without the involvement of apiculturists. Hypothetically, by studying transmission routes and transmission rates of virus infections within and between colonies we should be able, not only to understand the epidemiology involved, but also to follow the evolutionary process as the host-parasite system co-evolves. Without data on basic epidemiological parameters such as transmission routes and transmission rates (at the individual bee level as well as at the colony level), however, it will remain impossible to model and understand the system. With at least some data it would be possible to adapt current evolutionary epidemiology models to the complex two-level transmission system of honey bees. It is increasingly clear that theoretical models have helped in understanding epidemiology in other systems and may suggest interesting possibilities for the management of disease development and the evolution of virulence in pathogens (see Section 2). For the honey bee system, this remains to be done.

5. Summary

Evolutionary epidemiology of parasites and diseases in social insects in general, and honey bees specifically, is in an early stage of development. While theoretical models about the evolution of virulence abound we have two major hurdles to jump before we can understand the evolutionary interaction between viruses and honey bees. Firstly, we are severely lacking in good data on the true impact of viruses on their honey bee hosts and on how viruses are transmitted within colonies and between colonies, in both natural and managed systems. These data need to be collected and we indicate below in more detail what we believe the important factors are. Secondly, we need models which represent the hierarchical host system that honey bees present to viral parasites.

Important empirical questions that must be addressed include:

(i) the mode of transmission within colonies (horizontal vs. vertical)
(ii) the mode of transmission between colonies (in both managed and natural systems)
(iii) the relative rates of transmission by different modes within and between colonies
(iv) the frequency of multiple-infections (both of viral strains and viral species) within individual bees and individual colonies
(v) evidence for the presence or absence of competition/enhancement among viral strains and species, and between viruses and other co-existing pathogens (e.g., BQCV and *Nosema apis*)

(vi) the relationship between parasite reproduction within a host and its potential transmission

(vii) the relationship between covert and overt infections

(viii) the impact of the virus on individual bees and individual colonies

Once we understand the biology of the bee-virus systems at this level we should be able to parametrise a model to determine which of these factors control the levels of virulence in bee viruses. And this, in turn, will help us to know which factors to manipulate if we hope to manage their virulence.

References

Allen M., Ball B. (1996) The incidence and world distribution of honey bee viruses, Bee World, 77, 141-162.

Anderson R.M., May R.M. (1982) Co-evolution of hosts and parasites, Parasitology, 85, 411-426.

Baer B., Schmid-Hempel P. (1999) Experimental variation in polyandry affects parasite loads and fitness in a bumble-bee, Nature 397, 151-154.

Baer B., Schmid-Hempel P. (2001) Unexpected consequences of polyandry for parasitism and fitness in the bumblebee, *Bombus terrestris*, Evolution 55, 1639-1643.

Bailey L. (1969) The multiplication and spread of sacbrood virus of bees, Ann. Appl. Biol. 63, 482-491.

Bailey L., Ball B.V. (1991) Honey bee pathology 2nd Edition, Academic Press, London.

Bailey L., Ball B.V., Perry J.N. (1983) Honeybee paralysis: its natural spread and its diminished incidence in England and Wales, J. Apic. Res. 22, 191-195.

Bailey L., Fernando E.F.W. (1972) Effects of sacbrood viruse on adult honeybees, Ann. Appl. Biol., 72, 27-35.

Bailey L., Gibbs A.J. (1964) Acute infection of bees with paralysis virus, J. Insect Pathol., 6, 395-407.

Beye M., Hasselmann M., Fondrk M. K., Page R.E. Jr., Omholt S. W. (2003) The gene *csd* is the primary signal for sexual development in the honeybee and encodes an SR-type protein, Cell 14, 419-429.

Bonhoeffer S., Lenski R.E., Ebert D. (1996) The curse of the pharaoh: the evolution of virulence in pathogens with long living propagules, Proc. R. Soc. Lond. B 263, 715-721.

Bonhoeffer S., Nowak M.A. (1994) Mutation and the evolution of virulence, Proc. R. Soc. Lond. B 258, 133-140.

Boots M., Sasaki A. (1999) 'Small worlds' and the evolution of virulence: infection occurs locally and at a distance, Proc. R. Soc. Lond. B 266, 1933-1938.

Bowen-Walker P.L., Gunn, A. (2001) The effect of the ectoparasitic mite, *Varroa destructor* on adult work honeybee (*Apis mellifera*) emergence weights, water, protein, carbohydrate, and lipid levels, Entom. Exp. Appl. 101, 207-212.

Brown M.J.F., Loosli R., Schmid-Hempel P. (2000) Condition-dependent expression of virulence in a trypanosome infecting bumblebees, Oikos 91, 421-427.

Brown M.J.F., Schmid-Hempel R., Schmid-Hempel P. (2003) Strong context-dependent virulence in a host-parasite system: reconciling genetic evidence with theory, J. Anim. Ecol. 72, 994-1002.

Bull J.J. (1994) Virulence, Evolution 48, 1423-1437.

Chen Y.P., Higgins J.A., Feldlaufer M.F. (2005) Quantitative real-time reverse transcription-PCR analysis of deformed wing virus infection in the honeybee (*Apis mellifera* L.), Appl. Env. Microbiol., 71, 436-441.

Claessen D., de Roos A.M. (1995) Evolution of virulence in a host-pathogen system with local pathogen transmission, Oikos 74, 401-413.

Clark T.B. (1977) *Spiroplasma* as a new pathogen in the honeybees, J. Invertebr. Pathol. 29, 112-113.

Cooper V.S., Reiskind M.H., Miller J.A., Shelton K. A., Walther B.A., Elkinton J.S.,

Ewald P.W. (2002) Timing of transmission and the evolution of virulence of an insect virus, Proc. R. Soc. Lond. B 269, 1161-1165.

Cox F.E.G. (2001) Concomitant infections, parasites and immune responses, Parasitol. 122, S23-S38.

Day T. (2001) Parasite transmission modes and the evolution of virulence, Evolution 55, 2389-2400.

Day T. (2002) The evolution of virulence in vector-borne and directly transmitted parasites, Theor. Pop. Biol. 62, 199-213.

Day T. (2003) Virulence evolution and the timing of disease life-history events, Trends Ecol. Evol. 18, 113-118.

Durrer S., Schmid-Hempel P. (1994) Shared use of flowers leads to horizontal pathogen transmission. Proc. R. Soc. Lond. B, 258, 299-302.

Ebert D. (1994) Virulence and local adaptation of a horizontally transmitted parasite, Science 265, 1084-1086.

Ebert D. (1998) Experimental evolution of parasites, Science 282, 1432-1435.

Ebert D., Mangin K.L. (1997) The influence of host demography on the evolution of virulence of a microsporidian gut parasite, Evolution 51, 1828-1837.

Ebert D., Weisser W.W. (1997) Optimal killing for obligate killers: the evolution of life histories and virulence of semelparous parasites, Proc. R. Soc. Lond. B 264, 985-991.

Ewald P.W. (1983) Host-parasite relations, vectors and the evolution of disease severity, Ann. Rev. Ecol. Syst. 14, 465-485.

Ewald, P.W. (1993) The evolution of virulence. Scientific American, 268, 56-62.

Ewald P.W. (1994) Evolution of infectious diseases, Oxford University Press, Oxford UK.

Fries I., Camazine S. (2001). Implications of horizontal and vertical pathogen transmission for honey bee epidemiology, Apidologie, 32, 199-214.

Fries I., Rosenkranz P., Imdorf A. (2005) Survival of mite infested (*Varroa destructor*) honey bee (*Apis mellifera*) colonies in a Nordic climate. In manuscript (for Apidologie)

Gandon S. (1998) The curse of the pharaoh hypothesis, Proc. R. Soc. Lond. B. 265, 1545-1552.

Gandon S., Jansen V.A.A., van Baalen M. (2001) Host life history and the evolution of parasite virulence, Evolution 55, 1056-1062.

Gandon S., van Baalen M., Jansen V.A.A. (2002) The evolution of parasite virulence, superinfection, and host resistance, Amer. Nat. 159, 658-669.

Ganusov V.V., Antia R. (2003) Trade-offs and the evolution of virulence of microparasites: do details matter?, Theor. Pop. Biol. 64, 211-220.

Herre E.A. (1993) Population structure and the evolution of virulence in nematode parasites of fig wasps, Science 259, 1442-1445.

Hornitzky M.A.Z. (1998) The spread of *Paenibacillus larvae* subsp. *larvae* infections in an apiary, J. Apic. Res. 37, 261-265.

Hornitzky M.A.Z., Oldroyd B.P., Sommerville D. (1996) *Bacillus larvae* carrier status of swarms and feral colonies of honeybees (*Apis mellifera*) in Australia, Aus. Vet. J. 73, 116-117.

Kamo M., Boots M. (2004) The curse of the pharaoh in space: free-living infectious stages and the evolution of virulence in spatially explicit populations, J. Theor. Biol. 231, 435-441.

Koella, J.C., Restif, O. (2001) Coevolution of parasite virulence and host life history. Ecology Letters, 4, 207-214.

Lenski R.E., May R.M. (1994) The evolution of virulence in parasites and pathogens – reconciliation between 2 competing hypotheses, J. Theor. Biol. 169, 253-265.

Leung B., Forbes M.R. (1998) The evolution of virulence: a stochastic simulation model examining parasitism at individual and population levels, Evol. Ecol. 12, 165-177.

Liersch S., Schmid-Hempel P. (1998) Genetic variation within social insect colonies reduces parasite load, Proc. R. Soc. Lond. B 265, 221-225.

Lipsitch M., Moxon E.R. (1997) Virulence and transmissibility of pathogens: what is the relationship? Trends Microbiol. 5, 31-37.

Lipsitch M., Siller S., Nowak M.A. (1996) The evolution of virulence in pathogens with vertical and horizontal transmission, Evolution 50, 1729-1741.

Mackinnon M.J., Read A.F. (1999) Genetic relationships between parasite virulence and transmission in the rodent malaria *Plasmodium chabaudi*, Evolution 53, 689-703.

Massey R.C., Buckling A., Ffrench-Constant R. (2004) Interference competition and parasite virulence, Proc. R. Soc. Lond. B 271, 785-788.

May R.M., Anderson R.M. (1990) Parasite-host coevolution, Parasitology 100, 89-101

May R.M., Nowak M.A. (1995) Coinfections and the evolution of parasite virulence, Proc. R. Soc. Lond. B 261, 209-215.

McMullan J., Brown M.J.F. (2005) Brood pupation temperature affects the susceptibility of honeybees (Apis mellifera L.) to infestation by tracheal mites (*Acarapis woodi*), Apidologie 36, 97-105.

Messenger S.L., Molineux I.J., Bull J.J. (1999) Virulence evolution in a virus obeys a trade-off, Proc. R. Soc. Lond. B 266, 397-404.

Moritz R.F.A., Southwick E.E. (1992) Bees as superorganisms, Springer Verlag, Berlin.

Muszynska J.(1976) Porównanie pszczól z roju i macierzaka, Psczel. Zesz. Nauk. 20, 191-201.

Nordström S., Fries I., Aarhus A., Hansen H., Korpela S. (1999). Virus infections in Nordic honey bee colonies infested with *Varroa jacobsoni*, Apidologie 30, 475-484.

O'Keefe K.J. (2004) The evolution of virulence in pathogens with frequency-dependent transmission, J. Theor. Biol. 233, 55-64.

Pfeiffer K.J., Crailsheim K. (1998) Drifting of honeybees, Insect. Sociaux 45, 151-167.

Pfennig K.S. (2001) Evolution of pathogen virulence: The role of variation in host phenotype, Proc. Roy. Soc. Biol. Sci. Series B, 268, 755-760.

Pie M.R., Rosengaus R.B., Traniello J.F.A. (2004) Nest architecture, activity pattern, worker density and the dynamics of disease transmission in social insects, J. Theor. Biol. 226, 45-51.

Ratnieks F.L.W., Nowakowski J. (1989) Honeybee swarms accept bait hives contaminated with American foulbrood disease, Ecol. Entomol., 14, 475-478.

Regoes R.R., Nowak M.A., Bonhoeffer S. (2000) Evolution of virulence in a heterogeneous host population, Evolution 54, 64-71.

Rosenkranz P. (1999) Honey bee (*Apis mellifera* L.) tolerance to *Varroa jacobsoni* Oud. in South America, Apidologie, 30, 159-172.

Royce L.A., Rossignol P.A. (1990) Epidemiology of honey bee parasites, Parasitol. Today 6, 348-353.

Sakofski F. (1990) Transfer of *Varroa jacobsoni* between honey bee colonies, In Proc. Int. Symp. Recent Res. Bee Pathol. (ed W. Ritter), pp. 70-72. Janssen Pharmaceutica, Beerse, Belgium, 5 – 7 Sept. 1990, Gent, Belgium.

Schmid-Hempel P. (1995) Parasites and social insects, Apidologie 26, 255-271.

Schmid-Hempel P. (1998) Parasites in social insects, Princeton University Press, Princeton N.J.

Schmid-Hempel P. (2001) On the evolutionary ecology of host-parasite interactions: addressng the question with regard to bumblebees and their parasites, Naturwiss., 88, 147-158.

Shykoff J.A., Schmid-Hempel P. (1991) Parasites and the advantage of genetic variability within social insect colonies, Proc. R. Soc. Lond. B 243, 55-58.

Tarpy D.R. (2003) Genetic diversity within honey bee colonies prevents severe infections and promotes colony growth, Proc. R. Soc. Lond. B 270, 99-103.

Taylor L.H., Mackinnon M.J., Read A.F. (1998) Virulence of mixed-clone and single-clone infections of the rodent malaria *Plasmodium chabaudi*, Evolution 52, 583-591.

Thomas M.B., Watson E.L., Valverde-Garcia P. (2003) Mixed infections and insect-pathogen interactions, Ecol. Lett. 6, 183-188.

van Baalen M., Sabelis M.W. (1995) The dynamics of multiple infection and the evolution of virulence, Amer. Nat. 146, 881-910.

Williams P.D., Day T. (2001) Interactions between sources of mortality and the evolution of parasite virulence, Proc. R. Soc. Lond. B 268, 2331-2337.

Winston M.L. (1987) The biology of the honey bee, Harvard University Press, London, UK.

Yue C., Genersch E. (2005) RT-PCR analysis of *Deformed wing virus* (DWV) in honey bees (*Apis mellifera*) and mites (*Varroa destructor*), J. Gen. Virol., *in press*.

Chapter 7

INNATE IMMUNITY OF INSECTS TO INFECTION

Catherine DOSTERT, Delphine GALIANA-ARNOUX and Jean-Luc IMLER

Table of contents

1. Introduction

Insects have been extraordinarily successful over time, and have colonized all terrestrial biotopes. As a result, they are exposed to a wide panel of infectious microbes, including bacteria, fungi, eukaryotic parasites and viruses, to which they are remarkably resistant. Studies on the resistance of insects to infection were initially prompted by the realization that economically beneficial insects (eg. honey bees, silkworms) suffered from infections. Later on, insects (in particular mosquitoes) were found to transmit devastating infectious diseases to humans and cattle (eg. malaria parasite, Dengue fever virus), and the question of the insect vector's response to infection was raised. A last reason to study immunity in insects arose only recently, when it was realized that the molecular signalling cascades involved in host defence have been conserved through evolution. Thus, the analysis of innate immunity in insects may reveal novel aspects of the innate immune response in mammals, as exemplified by the discovery of Toll-like receptors. Here, we review the current state of knowledge on the innate immune response of insects to infections. The recent sequencing of the *Apis mellifera* genome reveals orthologues for most immunity-related genes identified in other species, thus paving the way for the study of immunity in honey bees.

Innate immunity is the first line of host-defence, which allows not only all animals but also plants to detect infectious microbes and to activate a set of responses to control the infection (Kurz and Ewbank, 2003; Medzhitov and Janeway, 1997; Nimchuk *et al.*, 2003; Tzou *et al.*, 2002). Adaptive immunity, which is characterized by highly specific antigen receptors expressed by lymphocytes, arose more recently and is only present in vertebrates. It is now well established that the adaptive immune response in vertebrates is triggered and shaped by a dedicated set of cytokines and co-stimulatory molecules produced by the innate immune system (Beutler, 2004). As a result, the study of innate immunity in both vertebrate and invertebrate models has been the focus of intense interest in recent years. In particular, the study of host-defence mechanisms in the fruit fly *Drosophila melanogaster* provided precious insights into the innate mechanisms of non-self recognition and signalling. A remarkable finding from this set of studies is that some of these mechanisms are evolutionarily ancient, and have been conserved through evolution (Hoffmann, 2003). In addition, recent data indicate that the vectorial capacity of insects that transmit diseases to mammalian hosts, such as *Anopheles* mosquitoes and the malaria parasite *Plasmodium*, is controlled at least in part by the insect's ability to recognize and kill the parasite (Blandin and Levashina, 2004a).

Honey bees, like all other insects, are exposed to a wide range of infectious microorganisms, including Gram-positive bacteria (in particular

the causative agent of the American foulbrood disease, *Paenibacillus larvae larvae*), fungi (in particular the Chalk brood disease agent *Ascosphera apis*), Gram-negative bacteria, spirochetes, amoebae, and last but not least, viruses (Shimanuki and Knox, 1997). In addition, bees can also be parasitized by other arthropods. This ectoparasitism can be associated with increased infectious risk, because of increased exposure to pathogens that may be vectored by the parasite on one hand, and alterations of honey bee immunity on the other hand (Shen *et al.*, 2005; Yang and Cox-Foster, 2005).

The insect host-defence system is multifaceted and involves both humoral and cellular aspects. Circulatory and membrane associated receptors recognize molecular structures from invading microorganisms, and trigger production of molecules that will directly or indirectly control the infection (antimicrobial peptides, nitric oxide synthase, phenoloxidase, thioester-containing proteins). Blood cells also participate in the detection and clearing of infection through phagocytosis or encapsulation. The most studied insect model of innate immunity is the fruit fly *Drosophila melanogaster*. This model has been used for decades to study the genetic mechanisms governing embryonic development or other aspects of its biology, and, over the years, geneticists have accumulated a wide array of genetic and molecular tools (Rubin and Lewis, 2000). More recently, *Drosophila* has emerged as a powerful model to study innate immunity (Hoffmann, 2003).

We review below the cellular, molecular and genetic mechanisms involved in the control of bacterial, fungal, parasitic or viral infections in the fruit-fly and other insects. One surprising result that emerges from these studies is that flies react differently to challenges by different types of infectious microorganisms, and mount a somewhat adapted immune response. Another striking result is the conservation of many molecules and signalling modules operating in innate immunity between *Drosophila* and mammals, which points to the ancient origin of host-defence mechanisms in animals, and suggests that studies in the fruit-fly can have relevance for the study of honey bees.

2. The humoral response to bacterial and fungal infections

The best characterised aspect of the insect response to infection, is arguably the inducible synthesis and secretion by the fat body into the haemolymph (blood) of a cocktail of potent cationic antimicrobial peptides (AMP) active against bacteria and/or fungi. Inducible antimicrobial peptides have been isolated from all holometabolous insects studied to date (Bulet *et al.*, 2004). We present below the structure and function of

the main families of antimicrobial peptides, as well as their regulation. Extensive genetic analysis of the response to infection in the *Drosophila* model has led to the identification of several infection susceptibility loci, and the definition of the signalling pathways. The *Drosophila* genes involved in the control of infection are conserved in other insect species, including *A. mellifera*. The goal of the following parts of this chapter is to illustrate how functional genomics can help to unravel host-parasite interactions in honey bees.

2.1. Inducible antimicrobial peptides

Septic injury of higher insects triggers the induction of strong antimicrobial activity in the blood of infected animals. Biochemical analysis revealed that this activity can be attributed to several families of antimicrobial peptides (Tab. I). As mentioned above, expression of AMPs is strongly induced in the fat body in response to infection. In addition, these peptides are also expressed, either constitutively or upon infection, in hemocytes and surface epithelia (Tzou *et al.*, 2000). Secretion of AMPs is a conserved feature of innate immune responses in vertebrates and invertebrates (Boman, 1995; Bulet *et al.*, 2004; Ganz, 2003). However, whereas the human arsenal of AMPs is limited to three main families (Defensins, Cathelicidins and Histatins), *Drosophila* AMPs belong to seven distinct structural classes, which can be classified in three different groups depending on their main microbial targets (Tab. I): (i) Defensin (Lambert *et al.*, 1989) is mostly active on a large panel of Gram-positive bacteria. It is composed of an α-helix linked to antiparallel β-strands by two disulfide bridges (CSαβ motif), and is present at a concentration of 1 µM in the blood of infected flies; (ii) Drosocin (Bulet *et al.*, 1993), Cecropins (Steiner *et al.*, 1981), Attacins (Hultmark *et al.*, 1983) and Diptericins (Dimarcq *et al.*, 1988) are mostly active against Gram-negative bacteria. Both Drosocin and Diptericin are O-glycosylated and contain a high proportion of proline (Drosocin) and glycine (Diptericin) residues. Drosocin reaches concentrations of 40 µM in the haemolymph of infected flies. Cecropins were the first inducible AMPs discovered in animals. These linear α-helical peptides are largely distributed in higher insects, and the circulating concentration of Cecropin A is estimated at 20 µM during the course of infection. Attacins are the largest antimicrobial peptides in *Drosophila*, with nearly 200 residues. The proline-rich pro-domain of one of the four Attacins, Attacin C, is stable in the haemolymph and synergizes with Cecropin in *in vitro* antibacterial assays (Rabel *et al.*, 2004); (iii) Drosomycin (Fehlbaum

et al., 1994) and Metchnikowin (Levashina *et al.*, 1995) are efficient against fungi. Like Defensin, Drosomycin is composed of an α-helix linked to antiparallel β-strands, but contains an additional disulfide bridge: as a result, the structure of Drosomycin is strongly reminiscent of that of plant Defensins. The concentration of Drosomycin reaches an astonishing 100 µM in the haemolymph during infection. The other antifungal peptide, Metchnikowin, is a linear proline-rich peptide.

Honey bees also rely on a systemic humoral immune response to counter infections, and thanks in particular to the efforts of P. Casteels and colleagues in the early 1990s, several antimicrobial peptides have been isolated from the haemolymph of infected bees. These include two defensins, one of which is also called Royalisin (Fujiwara *et al.*, 1990; Klaudiny *et al.*, 2005); the short (18 amino-acids) peptides Apidaecins (Casteels *et al.*, 1989), Abaecin (Casteels *et al.*, 1990), and Hymenoptaecin (Casteels *et al.*, 1993). Other antimicrobial peptides isolated from honey bees are the Jelleines (Fontana *et al.*, 2004), which are produced constitutively and secreted into the royal jelly, the lysozymes, and mellitin (Sun *et al.*, 2005), the major protein component of honey bee venom. The antimicrobial peptides isolated from the haemolymph of immune challenged bees share several properties with their *Drosophila* counterparts, including their small size, cationic nature, and broad activity spectrum. Abaecin and Apidaecins share with Drosocin and Metchnikowin a high content of proline residues, whereas Hymenoptaecin is rich in glycine residues, like Diptericins and Attacins (Tab. I). Sequence homology between the signal peptides of Abaecin and Drosocin is also apparent (Casteels-Josson *et al.*, 1994). Finally, Apidaecins share with Drosocin a mode of action that is neither lytic, nor membrane active, and involves stereoselective recognition of a chiral target (Casteels and Tempst, 1994). The strong induction of antimicrobial activity in the haemolymph is a critical aspect of innate immunity in all holometabolous insects. As we will see below, induction is mediated at the transcriptional level (Casteels-Josson *et al.*, 1994; Evans, 2004). An original strategy is used in the case of Apidaecins to amplify the antibacterial response: indeed, inducible transcripts encode multipeptide precursors, which are processed to generate active Apidaecins (Casteels-Josson *et al.*, 1993).

2.2. Genetic control of the humoral immune response

AMPs are encoded by inducible genes. Interestingly, genetic analysis in *Drosophila* revealed the existence of two signalling pathways that differentially regulate expression of the genes encoding AMPs: induction of the *diptericin* gene, but not that of *drosomycin*, is abolished in *immune deficiency* (*imd*) mutant flies (Lemaitre *et al.*, 1995). Conversely, induction of

Table I. Inducible antimictobial peptides in *Drosophila.*

AMP family	Structural class	Number of genes	Activity*	Concentration in the blood (humoral response)	Epithelial expression	Related peptide in honey bee
Diptericin	Gly-rich	2	Gram-negative bacteria	0.5 µm	Midgut	Hymenoptaecin
Drosocin	Pro-rich	1	Gram-negative bacteria	40 µM	Female reproductive tract, tracheae	Abaecin, apidaecin
Attacin	Gly-rich	4	Gram-negative bacteria	1-5 µM	Midgut	
Cecropin	α-helical	4	Gram-negative bacteria	50 µM	Male and female reproductive tract, Malpighian tubules	
Defensin	CSαβ	1	Gram-positive bacteria	1 µM	Female reproductive tract, labellar glands	Defensin-1, -2
Metchnikowin	Pro-rich	1	Fungi	40 µM	Labellar glands, Malpighian tubules	
Drosomycin	CSαβ	7	Fungi	100 µM	Female reproductive tract, labellar glands, tracheae, salivary glands	

* main biological activity, at phyisiological concentration

drosomycin, but not *diptericin*, is abolished in *Toll* mutant flies (Lemaitre *et al.*, 1996) (Fig. 1).

2.2.1. The Drosophila Toll pathway controls the response to fungi and Gram-positive bacteria.

The Toll pathway was named after the transmembrane receptor Toll (Belvin and Anderson, 1996). Toll is activated by the neurotrophin-related cytokine Spaetzle (DeLotto and DeLotto, 1998; Morisato and Anderson, 1994). Spaetzle is synthesized as an inactive precursor that is unable to bind to Toll. Upon infection by fungi or Gram-positive bacteria, circulating serine proteases are activated and proteolytically process Spaetzle to release a 12kDa C-terminal fragment, which binds to and activates Toll (Weber *et al.*, 2003). At the plasma membrane, Toll interacts with a complex of three death domain (DD) containing proteins, DmMyD88, Tube and the kinase Pelle (Charatsi *et al.*, 2003; Horng and Medzhitov, 2001; Sun *et al.*, 2002; Tauszig-Delamasure *et al.*, 2002). Activation of this complex triggers phosphorylation of the cytoplasmic inhibitor protein Cactus, which is subsequently polyubiquitinated and degraded. This releases the transcription factor DIF, which can translocate into the nucleus and mediate transcriptional induction of dozens of genes including those encoding Drosomycin and Metchnikowin (Rutschmann *et al.*, 2000a) (Fig. 1). DIF is a member of the Rel family of transcription factors, to which NF-κB (a critical transcription factor for immune responses in mammals) belongs. Similarly, Cactus is the homologue of the mammalian inhibitor of NF-κB, IκB. Overall, the Toll pathway is reminiscent of the signalling pathway activated by the inflammatory cytokine interleukin-1 (IL-1) in mammals (Hoffmann, 2003). Furthermore, the spectacular phenotype of Toll mutant flies, which rapidly succumb to infections by fungi or Gram-positive bacteria (although they resist infections by Gram-negative bacteria), prompted the search for homologues of these receptors in mammals, and led to the discovery of the family of Toll like receptors (TLRs)(Medzhitov *et al.*, 1997; Poltorak *et al.*, 1998; Rock *et al.*, 1998). TLRs are now recognized as pivotal receptors of the mammalian immune system, which are activated by a wide array of microbial ligands (Akira and Takeda, 2004). Upon activation, TLRs trigger both the expression of effector molecules of the innate immune system (TNF, iNOS, AMPs) and also the cytokines and co-stimulatory molecules that induce and shape the adaptive immune response. Orthologues of all genes encoding components of the Toll pathway are found in the *A. mellifera* genome (Tab. II), strongly suggesting that a similar pathway operates to control infections in honey bees.

Interestingly, the *Drosophila* genome encodes a family of 9 Toll re-

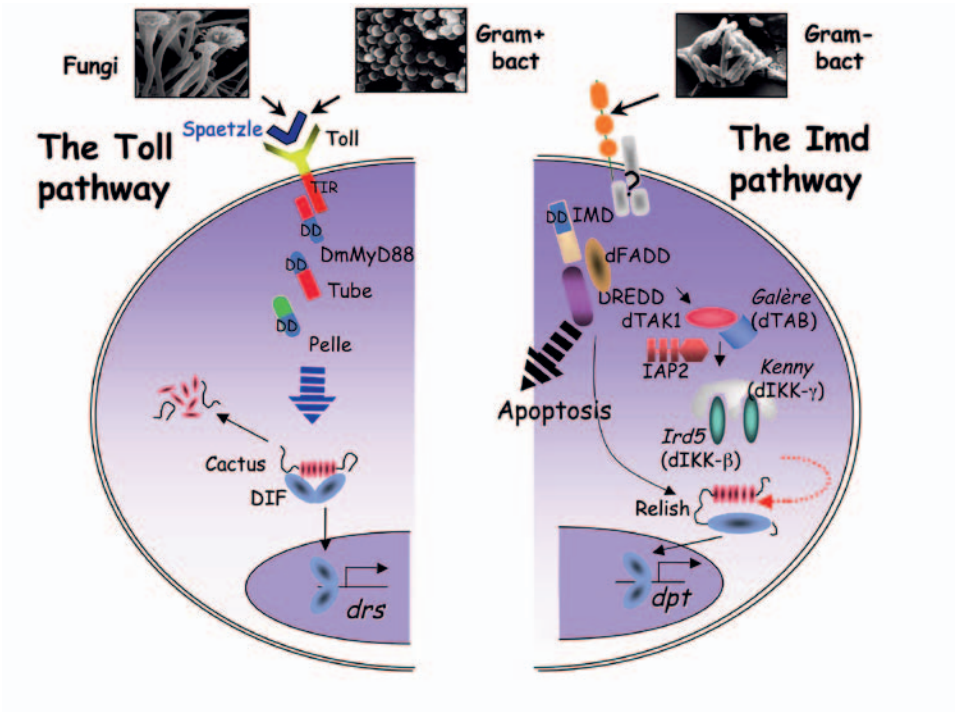


Figure 1. The *Drosophila* humoral response is controlled by the Toll and Imd pathways. Expression of the gene encoding the antifungal peptide Drosomycin (*drs*) is induced upon infection by fungi or Gram-positive bacteria. Recognition of infectious agents by soluble innnate immunity receptors in the haemolymph activates a proteolytic cascade that leads to processing of the cystein-knot growth factor Spaetzle, and generation of an active ligand for the receptor Toll. Toll regulates activation of the NF-κB related protein DIF. Expression of the gene encoding the antibacterial peptide Diptericin (*dpt*) is controlled by a distinct member of the NF-κB family of transcription factors, Relish. The transmembrane receptor PGRP-LC detects infection by Gram-negative bacteria, and activates the Imd pathway. See the text for details. DD: death domain.

ceptors (Tauszig *et al.*, 2000). The other members of the family have not been assigned immune functions, and in fact have been poorly characterized at this stage. Genetic analysis indicates that two members of the family, *18-wheeler* (*18w* or *Toll-2*) and *Toll-8*, have developmental functions (Ligoxygakis *et al.*, 2002a; Seppo *et al.*, 2003). As a matter of fact, most *Drosophila Tolls* were shown to be highly expressed, in a stage and tissue-specific manner during embryogenesis and metamorphosis (Chiang

and Beachy, 1994; Eldon *et al.*, 1994; Kambris *et al.*, 2002; Tauszig *et al.*, 2000). Hence, these genes may primarily carry developmental rather than immune functions. Consistent with these observations, induction of AMP in response to infection is wild-type in *18w* mutant flies (Ligoxygakis *et al.*, 2002a). Interestingly, an orthologue of *18w* was recently described in honey bee (*Am18w*), and silencing of this gene by RNA interference did not appear to affect induction of AMP following septic injury (Aronstein and Saldivar, 2005).

Importantly, induction of the antibacterial peptide genes *diptericin* and *drosocin* is not affected in *Toll* mutant flies (Lemaitre *et al.*, 1996), indicating that a second pathway is involved in the regulation of antimicrobial peptide gene expression.

Table II. Innate immunity pathways components in *Drosophila*, and their honey bee orthologues.

Pathway	*D. melanogaster* gene	*A. mellifera* gene
Toll	PGRP-SA	GB15371
	GNBP-1	GB19492
	Spz	GB15688
	Toll	GB18520
	DmMyD88	GB12344
	tube	GB13742
	pelle	GB16397
	cactus	GB10655; GB13520; GB11883
	dorsal/Dif	GB19066; GB18032
Imd	PGRP-LC	GB17188
	Imd	GB18606
	dFADD	DanH3
	DREDD	DanH2
	dTAK1	GB14664
	dTAB	GB18650
	IAP2	GB11057
	ird5 (dIKKβ)	GB15273
	Kenny (dIKKγ)	GB17106
	relish	GB13742

2.2.3. The Drosophila Imd pathway controls resistance to infection by Gram-negative bacteria.

The *Drosophila* gene *imd* encodes a cytosolic protein containing a DD most closely related to that of the TNF receptor interacting protein RIP in mammals (Georgel *et al.*, 2001). It interacts with the DD adapter dFADD, which in turn binds to and activates the caspase DREDD (Leulier *et al.*, 2000; Leulier *et al.*, 2002; Naitza *et al.*, 2002). Imd also activates the serine-threonine protein kinase dTAK1 (Vidal *et al.*, 2001), which is thought to phosphorylate and activate the IκB kinase (IKK) complex (Silverman *et al.*, 2000), composed of the catalytic subunit IKKβ encoded by the gene *ird5*, and the regulatory subunit IKKγ encoded by *kenny* (Lu *et al.*, 2001; Rutschmann *et al.*, 2000b). The TAK1 cofactor TAB2, encoded by the gene *galère*, is also required for the full activation of Imd target genes (Geuking *et al.*, 2005; Kleino *et al.*, 2005) (D. Ferrandon, personal communication). In mammals, molecules of the TNF Receptor Associated Factor (TRAF) family play a critical role in TNF signalling downstream of RIP and upstream of TAK1. The role of the *Drosophila* TRAF molecules is still debated, but the molecule IAP2, which contains a RING finger, like TRAFs, was recently shown to be an important component of the Imd pathway (Gesellchen *et al.*, 2005; Kleino *et al.*, 2005).

The Imd pathway targets another *Drosophila* NF-κB related protein named Relish (Hedengren *et al.*, 1999). Like the mammalian NF-κB protein p105, Relish has a composite structure containing an N-terminal Rel domain, linked to an inhibitory C-terminal domain. Activation of Relish involves phosphorylation by *Drosophila* IKKβ (Silverman *et al.*, 2000), and proteolytic cleavage, possibly by DREDD or another caspase (Stoven *et al.*, 2003). Upon infection with Gram-negative bacteria, the Imd pathway is activated and mediates rapid endoproteolysis of Relish. The released 68kD amino-terminal domain of the transcription factor then translocates into the nucleus and mediates induction of *diptericin* together with many other genes (Fig. 1). Flies deficient for *imd*, or any other gene of the pathway, resist infection by fungi and Gram-positive bacteria much like wild-type flies, but are extremely susceptible to infection with Gram-negative bacteria (Lemaitre *et al.*, 1995). Interestingly again, orthologues of most genes of the Imd pathway are present in the genome of honey bees (Tab. II), suggesting that these insects use similar mechanisms to counter bacterial infections. As a matter of fact, functional studies in other Dipteran insects, the mosquitoes *Anopheles gambiae* (Anophelinae) and *Aedes aegypti* (Culicinae), point to the existence of two pathways controlling distinct NF-κB related molecules to control infections by bacteria and fungi. In the malaria vector *A. gambiae*, the orthologue of Relish, REL2, produces two

protein isoforms through alternative splicing. The small isoform, REL2-S, regulates the response to the Gram-negative bacteria *E. coli*, whereas the long isoform, REL2-F (as well as the Imd orthologue), is involved in the response to the Gram-positive bacteria *Staphylococus aureus*, and to the malaria parasite *Plasmodium berghei* (Meister *et al.*, 2005). No immune function has so far been assigned to the DIF orthologue REL1 in *A. gambiae* (also known as Gambif1). In the vector of yellow and dengue fever viruses, *A. aegypti*, the orthologue of *Relish*, the *REL2* gene produces three protein isoforms, two of which are similar to the *A. gambiae* REL2-F and –S. The *Aedes* REL2-S factor was shown to be required for the response to infection by Gram-negative bacteria, as in *Anopheles* mosquitoes (Shin *et al.*, 2003). The *A. aegypti* orthologue of DIF, REL1, appears to be on the other hand specifically required to counter fungal infections (Bian *et al.*, 2005; Shin *et al.*, 2005).

2.3. Sensing infection by bacteria and fungi in insects

The *Drosophila* Toll pathway is activated both by fungi and Gram-positive bacteria, whereas the Imd pathway is activated by Gram-negative bacteria, thus raising the question of the receptors that sense infectious non-self in insects. The breakthrough in this field came as the result of a random mutagenesis screen that led to the discovery of the *Drosophila semmelweiss* (*seml*) mutation (Michel *et al.*, 2001). Indeed, *seml* mutant flies are defective for induction of *drosomycin* in response to Gram-positive bacteria, but exhibit wild-type responses to infections by fungi or Gram-negative bacteria. The gene *seml* was cloned and found to encode a secreted member of the Peptidoglycan (PGN) Recognition Protein (PGRP) family, PGRP-SA (Fig. 2). PGRPs were originally isolated from the haemolymph of larger insects, such as the silkworm *Bombyx mori*, using affinity purification on PGN decorated beads (reviewed in Steiner, 2004).

PGN is an important component of the cell wall of all bacteria. These microbial molecules can be grouped in two major types, the so-called Lysine-type PGN, and diaminopimelic acid (DAP)-type PGN. The long carbohydrate chains of alternating N-Acetyl Glucosamine and N-Acetyl muramic acid of PGN are connected via cross-linked stem peptides. The Lys-PGN found in the cell wall of most Gram-positive bacteria contains a Lysine at the third position of the stem peptide. By contrast, the DAP-PGN, found in Gram-negative bacteria and in some Gram-positives (genus *Bacillus*), contains a DAP residue instead of the Lysine (Steiner, 2004). *In vitro* studies established that PGRP-SA binds Lys-PGN characteristic of most Gram-positive bacteria (Werner *et al.*, 2000), in good agreement with the immunodeficient phenotype of *seml* mutant flies.

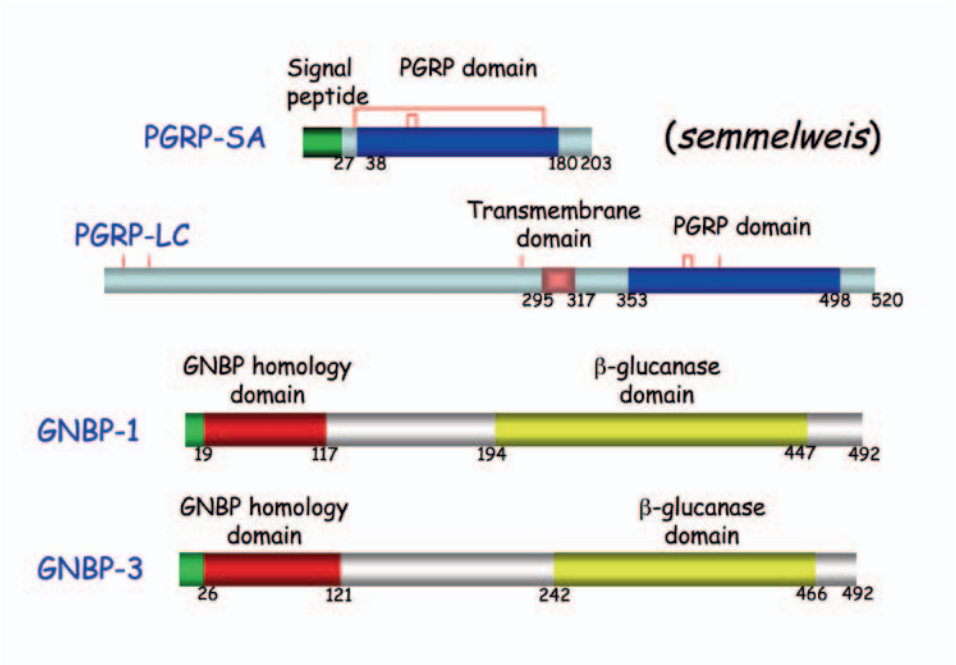


Figure 3: Receptors sensing infection in *Drosophila*.

Peptidoglycan recognition proteins (PGRPs) detect Lys-PGN from Gram-positive bacteria (PGRP-SA, encoded by the gene *semmelweis*) or DAP-PGN from Gram-negative bacteria, through their shared PGRP domain. Some PGRPs are secreted, like PGRP-SA, whereas other are inserted in the plasma membrane, like PGRP-LC. β-glucan recognition proteins (βGRPs) share a C-terminal non functional β-glucanase domain, and an amino-terminal homology domain. GNBP-1 is required for the recognition of Lys-PGN containing bacteria, together with PGRP-SA, whereas GNBP-3 is involved in the recognition of fungi. See the text for details.

The *Drosophila* PGRP family encompasses 13 members, divided into two subfamilies based on their size (20 kDa for the S or short forms; 30-90kDa for the L or long forms; Werner *et al.*, 2000) (fig. 2). Several structures of PGRP domains have been solved recently. The PGRP domain bears significant sequence homology with bacteriophage T3 and T7 lysozymes, which are N-Acetyl muramoyl-L-alanine amidases, and the X-ray structure of PGRP domains confirmed this similarity. The structure exhibits a cleft in which PGN is accommodated (Chang *et al.*, 2005; Chang *et al.*, 2004; Guan *et al.*, 2004; Reiser *et al.*, 2004).

The striking phenotype of *seml* mutant flies prompted studies on the other members of the PGRP family. Surprisingly, it was found that PGRP-

LC, which is located at the plasma membrane, is a component of the receptor for the Imd pathway (Choe *et al.*, 2002; Gottar *et al.*, 2002; Ramet *et al.*, 2002), and is activated by DAP-PGN predominantly found in the inner cell wall of Gram-negative bacteria (Kaneko *et al.*, 2004; Leulier *et al.*, 2003). These results indicate that the Toll and Imd pathways are activated by receptors of the PGRP family, which can discriminate between the PGN types of invading bacteria. More recently, other members of the PGRP family were shown to participate in the immune response: the secreted PGRP-SD participates in the activation of the Toll pathway (Bischoff *et al.*, 2004), as does PGRP-SA, whereas PGRP-LE acts in the Imd pathway, together with PGRP-LC (Takehana *et al.*, 2004). The association of various members of the family may generate the repertoire of receptors required to accommodate the wide variety of microbial inducers exposed during the course of an infection. Other members of the PGRP family have a functional amidase domain, and function as scavengers to degrade PGN (Mellroth *et al.*, 2003).

PGRPs are not the sole receptors involved in recognition of infectious non-self in *Drosophila*, and members of the β-glucan recognition proteins (βGRP) also play an important role. βGRPs are 50kDa secreted proteins with a C-terminal β-glucanase-like domain, preceded by a conserved N-terminal domain. Like PGRPs, these proteins were originally isolated by affinity purification from the blood of large insects, on the basis of their interaction with components of the cell wall of fungi or, in one case, Gram-negative bacteria (reviewed in Ferrandon *et al.*, 2004). The *Drosophila* genome encodes three members of this family, GNBP1 to 3, plus three additional smaller versions harbouring only the conserved amino-terminal domain (GNBP-like 1 to 3) (Ferrandon *et al.*, 2004). The mutation *osiris*, which affects the gene encoding GNBP1 was found to have the same phenotype as the *seml* mutation, indicating that GNBP1 acts in concert with PGRP-SA (and in some instances with PGRP-SD) to recognize infection by Lys-PGN containing bacteria (Gobert *et al.*, 2003). Interestingly, another member of the GNBP family, GNBP3 encoded by the gene *hades*, is required for activation of the Toll pathway by fungi (D. Ferrandon *et al.*, in preparation).

Hence, it appears that the fruit-fly relies on two families of innate immunity receptors, the PGRPs and the GNBPs/βGRPs to detect bacteria and fungi, and to trigger activation of the humoral response. While PGRPs are also present in mammals, where they function either as scavengers, or as antimicrobial molecules, (reviewed in Steiner, 2004), βGRPs are only present in invertebrates. Importantly, members from both families of recognition proteins are encoded by the *A. mellifera* genome (Tab. II).

3. Blood cells and the control of parasite infections

In addition to the humoral response, host-defence in insects relies on cellular reactions, which involve specialized blood cell types (Meister, 2004; Theopold *et al.*, 2004). Mucosal epithelia, which are in contact with large numbers of microorganisms, also actively participate to insect host-defence.

3.1. Haemocyte lineages

The cells found in insect haemolymph (collectively called haemocytes) can be grouped in different classes, according to their morphology, function, and, in some rare occasions, expression of molecular markers. In *Drosophila*, the importance of haemocytes in the control of infection is best illustrated by studies in larvae mutant for the *domino* gene, which have reduced hemocyte counts, and increased sensitivity to infection (Braun *et al.*, 1998). Similarly, in fly or mosquito adults, saturation of hemocytes by injection of polystyrene beads sensitizes flies to bacterial infections (Elrod-Erickson *et al.*, 2000; Moita *et al.*, 2005). *Drosophila* haemocytes belong to three lineages: the plasmatocytes, the crystal cells, and the lamellocytes (Evans *et al.*, 2003; Meister, 2004) (Fig. 3).

Plasmatocytes are the major cell type present in the circulation and within tissues (Lanot *et al.*, 2001). They are dedicated phagocytes that resemble the cells of the mammalian monocyte/macrophage lineage. These circulating cells patrol tissues and help clear apoptotic corpses and cellular debris, in addition to microorganisms. They may also signal infection to the fat body (Agaisse *et al.*, 2003; Foley and O'Farrell, 2003). These cells probably combine the functions of the plasmatocytes and granular cells found in lepidopterans. Crystal cells represent 5% or less of the blood cells in larvae and adults (Lanot *et al.*, 2001). As their name indicates, they contain crystalline inclusions that correspond to enzymes involved in humoral melanization, in particular the zymogen prophenoloxidase. Once secreted by crystal cells and activated upon cleavage by the serine protease PAE (phenoloxidase activating enzyme), phenoloxidase catalyzes the oxidation of tyrosine-derived phenols to quinines, which polymerize to form melanin. These reactions are accompanied by the local production of cytotoxic free radicals, quinones or semi-quinones, which contribute to the killing of invading microorganisms or parasites (De Gregorio *et al.*, 2002; Kanost *et al.*, 2004; Ligoxygakis *et al.*, 2002b). These cells are functionally equivalent to the oenocytoid cell type described in lepidopterans.

The last cell type, the lamellocyte, is rarely seen in healthy animals. Lamellocyte precursors are restricted to the lymph glands (the haemat-

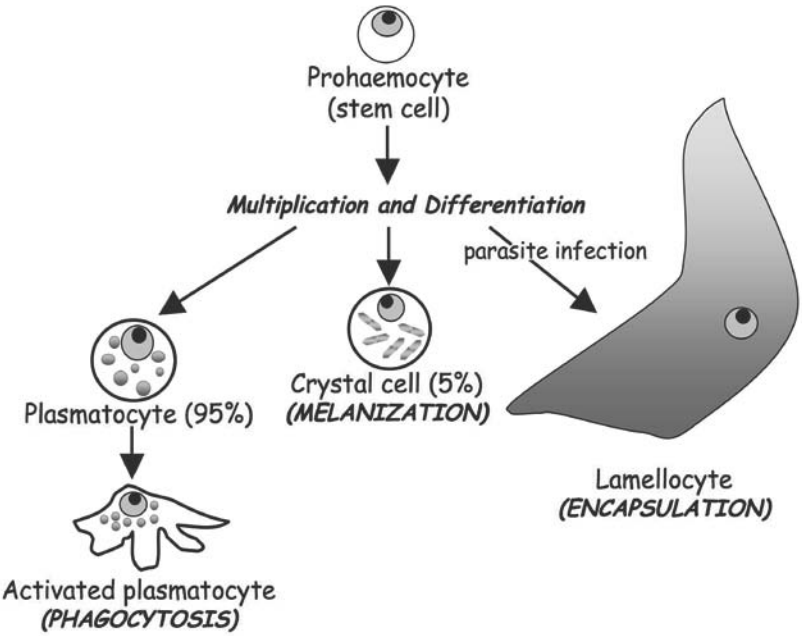


Figure 3. *Drosophila* blood cells.

There are three types of blood cells in *Drosophila*: the most abundant are the plasmatocytes, which can be activated upon infection into phagocytes. Crystal cells are rare, representing only 5% of the blood cells in larvae or adults. These small cells (10-12 µm diameter, similar to plasmatocytes) contain high concentrations of enzymes necessary for activation of the melanisation cascade. Finally, the lamellocytes are large, flat cells (30-40 µm diameter), that only differenciate in response to infection by metazoan parasites, which are too large to be phagocytosed. All three cell-types derive from a common precursor, the prohaemocyte.

opoietic organ in larvae), and only differentiate into lamellocytes under specific conditions, such as parasitization by invaders too large to be phagocytosed by plasmatocytes (Meister, 2004).

Recent work on the regulation of haematopoiesis in the fruit fly revealed the importance of evolutionary conserved pathways or transcription factors such as the Notch and JAK/STAT pathways, and the orthologues of the transcription factors GATA and Early B cell factor (EBF) (Evans *et al.*, 2003; see also below).

3.2. The *Drosophila* response to parasitic wasp infections.

Lamellocytes are large, adhesive flat cells that associate with one another to form capsules around large invaders (Lanot *et al.*, 2001). A common immune threat encountered by flies is parasitism by hymenopters, which lay their eggs in fly larvae. The Hymenopteran parasite is first recognized by plasmatocytes, which bind to the egg chorion. The current model is that plasmatocytes then send a differentiation signal to the lymph glands, thus explaining the spectacular changes induced in the haematopoietic organ: enhanced proliferation, increased number of crystal cells, and massive differentiation of lamellocytes. Once released from the lymph gland, lamellocytes in turn bind to the Hymenopteran egg, and form a multilayered capsule around the invader, which is ultimately melanized. The parasite is eventually killed by asphyxia or as a result of the local production of toxic compounds in the course of melanization. The molecular control of the lamellocyte differentiation is currently under investigation, and has recently been shown to involve the gene *collier*, an orthologue of the mammalian gene encoding early B-cell factor (EBF) (Crozatier *et al.*, 2004). These results reveal the existence of new parallels between *Drosophila* and mammals in cellular immunity.

3.3. Recognition and phagocytosis by haemocytes

Plasmatocytes express dedicated phagocytic receptors, such as the CD36 homologue encoded by the gene *croquemort* for the phagocytosis of apoptotic cells (Franc *et al.*, 1999), the scavenger receptor CI (dSR-CI), that mediate binding to Gram-positive and Gram-negative bacteria (Ramet *et al.*, 2001), and PGRP-LC, involved in binding to Gram-negative bacteria (Ramet *et al.*, 2002). Recently published reports indicate that other receptors participate in the recognition and clearing of bacteria by haemocytes. The gene *eater* was isolated in an RNAi screen in a macrophage *Drosophila* cell line (Kocks *et al.*, 2005). This new phagocytic receptor characterized by several repeats of an EGF motif in its ectodomain, plays an important role in the phagocytosis of bacteria *in vitro* and *in vivo*. Another fascinating candidate phagocytic receptor is down syndrome cell adhesion molecule (DSCAM), a receptor initially characterized for its role in neuronal development. This gene is expressed in haemocytes and, through alternative splicing of three sets of highly interchangeable exons, could generate proteins binding bacteria with different specificities (Watson *et al.*, 2005).

Haemocytes also participate in the control of infection through production of secreted molecules (Irving *et al.*, 2005). These include thioester-containing proteins (TEPs), which are related to molecules of the α2-macroglobulin/complement factor C3 family (Blandin and Levashina,

2004b). The transcription of three of the six *Drosophila* genes encoding TEPs is increased after an immune challenge, and two of these genes, *tep2* and *tep4*, are mainly expressed in haemocytes (Lagueux *et al.*, 2000). Although the exact role of TEPs in the immune response in *Drosophila* is still unclear, studies in another insect, the malaria vector *A. gambiae*, indicate that TEPs act as opsonins to promote phagocytosis, in a C3-like manner (Levashina *et al.*, 2001). In particular, the haemocyte-specific protein TEP1 binds to and kills the malaria parasite *Plasmodium berghei*, and is a critical determinant of vectorial capacity of the mosquito (Blandin *et al.*, 2004). TEP3 and TEP4 were also recently shown to participate in the phagocytosis of bacteria in *Anopheles* (Moita *et al.*, 2005). The killing mechanism of TEP1, as well as the functions of other members of the family in different insect species, represents promising avenues of research for the coming years.

Apart from blood cells, cells from barrier epithelia found in the digestive, reproductive, and respiratory tracts, as well as the malpighian tubules, also participate to protect the host against infection. These cells produce, either constitutively or upon exposure to pathogens, a subset of antimicrobial peptides (Tzou *et al.*, 2000). The production of microbicidal reactive oxygen species was also recently shown to play a key role in *Drosophila* gut immunity (Ha *et al.*, 2005a; Ha *et al.*, 2005b). Similar local responses in epithelia have been demonstrated in *Anopheles* (e.g., Vlachou *et al.*, 2005), and are likely to exist in honey bees. Indeed, induction of antimicrobial peptide expression could be demonstrated upon *per os* infection of *A. mellifera* with *P. l. larvae* (Evans, 2004).

4. The response to viral infections

Apart from infections by bacteria, fungi and parasites, insects are also frequently exposed to viral infections, and in some cases can transmit viral diseases to mammalian hosts, including humans (Mackenzie *et al.*, 2004). Viral diseases are also a major problem in apiculture, causing serious economic losses. About 20 viruses, all with RNA genomes, have been identified in honey bees.

4.1. Evolutionary conserved antiviral defenses

Surprisingly little is known about how invertebrates control or eliminate viruses. One evolutionarily conserved mechanism used by hosts to counter viral infection is programmed cell death. The apoptotic response of cells to infection has drastic effects on virus replication and gene expression, and therefore results in a control of the infection. The impor-

tance of programmed cell death in the control of viral infections in insects is best illustrated in the case of the baculovirus AcMNPV, which encodes the apoptotic suppressor gene p35. Expression of p35 in infected cells prevents apoptosis and allows the virus to replicate to high titers. Infectivity of p35 mutant viruses is greatly reduced, thus revealing the importance of apoptosis in the control of viral infection in insects (Lee *et al.*, 1998). Blood cells have also been shown to play a role in the control of baculovirus expression by encapsulating virus infected tracheal cells (Trudeau *et al.*, 2001). Phenoloxydase secreted in the plasma of Lepidopteran insects has also been shown to have potent virucidal activity, at least against baculoviruses (Popham *et al.*, 2004).

Another ancient host-defense mechanism against viruses is RNA interference (Lecellier and Voinnet, 2004). This mechanism of RNA degradation triggered by double stranded RNA is indeed an efficient antiviral defense system in plants. Recent work in the worm *Caenorhabditis elegans* further point to the relevance of RNAi to control animal virus replication (Lu *et al.*, 2005; Wilkins *et al.*, 2005). The fact that some insect viruses, such as the Flock house virus for example, express suppressors of RNAi (Chao *et al.*, 2005), like many plant viruses, suggest that indeed RNA interference participates in the control of viral load in infected insects. However, at least in the case of *Drosophila*, RNAi is most probably cell autonomous (Roignant *et al.*, 2003), and a role in systemic antiviral response is unlikely. This may not necessarily be the case for all insects species, as indicated by the fact that injection of double stranded RNA in the body cavity of *Anopheles* mosquitoes is an efficient way to systematically shut down gene expression (Blandin *et al.*, 2002). It will be interesting to see if this is also the case for honey bees (Aronstein and Saldivar, 2005).

Interestingly, no general inducible antiviral mechanism had been reported in any invertebrate until recently. This question was recently addressed using *Drosophila* as a model. Few viruses have been isolated from the fruit-fly. Among these, one of the best characterized is probably the *Drosophila* C virus (DCV) (Cherry *et al.*, 2005; Cherry and Perrimon, 2004; Johnson and Christian, 1998; Jousset *et al.*, 1977).

4.2. The Dicistroviridae DCV: a model to study antiviral response in *Drosophila*.

DCV is a member of a family of insect-specific viruses, the Dicistroviridae. Some members of this family are frequently encountered in honey bees. DCV is a non-enveloped small (30 nm diameter for its icosaedric capsid) single stranded positive-strand RNA virus that resembles picornaviridae in several of its biophysical properties. In fact, DCV princi-

pally differs from picornaviruses in its specific genome organization, and the presence of two open reading frames (ORFs) (Johnson and Christian, 1998; Jousset *et al.*, 1977). These reading frames allow the synthesis of two polyproteins that undergo proteolytic maturation. The first ORF encodes an RNA helicase, a protease and an RNA-dependent RNA polymerase. The second ORF, which is preceded by an internal ribosomal entry site (IRES), encodes the four proteins of the capsid (VP1-4). *Drosophila* is a natural host for DCV, which is transmitted horizontally through contact or ingestion. Experimental infection can be achieved by intrathoracic injection of a viral suspension (Cherry and Perrimon, 2004; Sabatier *et al.*, 2003). This leads to infection of several tissues by DCV, with rapid accumulation of viral particles in the cytoplasm of cells from the fat body and, in female flies, the epithelial sheath surrounding the egg chamber. DCV particles can also be detected in cells from tracheae, muscles, and the digestive tract.

4.3. Pherokines: a new family of molecules related to odor/pheromone binding molecules induced by infection

In a first step to characterize the response of *Drosophila* to virus infection, MALDI-TOF mass spectrometry differential analysis between the haemolymph of DCV-injected versus buffer-injected flies was performed (Sabatier *et al.*, 2003). In sharp contrast with the response to bacterial or fungal infection, no induction of peptides in the 0-10 kDa size range could be observed. In fact, only one induced molecule of molecular mass 12.8 kDa could be detected. Purification and microsequencing of this molecule revealed that it corresponds to one of the three members of a small family of secreted proteins, referred to as pherokines (phk) (Sabatier *et al.*, 2003). Interestingly, the first member of this family, OS-D/A10 (Phk-1), is specifically expressed in the olfactory region of antennae in *Drosophila*, and was proposed to function as a pheromone/odor binding protein (McKenna *et al.*, 1994; Pikielny *et al.*, 1994). The two other members of the family are induced by infection: (i) Phk-2 corresponds to the molecule induced in the haemolymph upon infection with DCV; whereas (ii) *phk-3* transcripts are transiently upregulated in flies following infection with a mixture of Gram-positive and Gram-negative bacteria, and in tissue culture cells stimulated with crude Gram-negative bacterial extracts (Sabatier *et al.*, 2003).

Molecules related to Pherokines have been identified in the insect orders Diptera, Dictyoptera, Hymenoptera, Lepidoptera, Orthoptera, and Phasmatodea (Wanner *et al.*, 2004). They are characterized by four cysteines involved in two disulfide bridges and forming a characteristic $CX_6CX_{18}CX_2C$ signature motif. X-ray structure determination of one of

these molecules, the chemosensory protein CSPMbraA6 from the moth *Mamestra brassicae*, revealed a novel type of α-helical fold with 6 helices delineating a narrow channel (Lartigue *et al.*, 2002). This channel can accommodate aliphatic ligands. Molecules related to Pherokines have often been isolated from chemosensory organs. In some cases however, these molecules have been associated with functions other than olfaction/chemosensation. For example, the protein p10 from the cockroach *Periplaneta americana*, which is 50% identical to Phk-2, is strongly upregulated in the developing epidermis of regenerating legs in nymphae, suggesting a function in development (Kitabayashi *et al.*, 1998). In agreement with this finding, the *Drosophila* genes *phk-2* and *phk-3* are most highly expressed during metamorphosis, and are regulated by the molting steroid hormone ecdysone (Lee *et al.*, 2003; Sabatier *et al.*, 2003). These findings, together with the fact that ectopic overexpression of Phk-2 does not protect flies against a DCV challenge, suggest that rather than being directly involved in the immune control of infection, Phk-2 and -3 may be involved in tissue-remodeling, either in the course of normal development, or following damage induced by infection. Clarification of the exact physiological roles of pherokines now awaits the identification and characterization of *D. melanogaster* mutant lines.

Interestingly, global microarray (and proteomic) analysis of the transcriptional response to infection in *Drosophila* and *Anopheles* revealed that several putative odorant binding proteins (OBPs) are upregulated by infection (De Gregorio *et al.*, 2001; Dimopoulos *et al.*, 2002; Irving *et al.*, 2001; Levy *et al.*, 2004). The identification of structurally similar molecules in studies on chemoreception on one hand and immunity on the other, raises the question of the potential importance of communication between insects confronted to infection. This may be particularly important for social insects like honey bees. Indeed, it was shown that one social response of honey bees to infection is a brood comb colony fever (Starks *et al.*, 2000). Of note, this response appears to be generated in bees before the symptoms are visible in larvae, raising the possibility that larvae communicate the ingestion of the pathogen. Honey bees will be a powerful model to investigate the function of chemosensory proteins in the control of infection in social insects.

4.4. DCV triggers a JAK/STAT dependent transcriptional response in flies

To further characterize the response of flies to virus infections, the global transcriptional response to DCV infection was examined using high density DNA microarrays. A substantial up-regulation (by a factor of

two or more) of some 140 genes was observed in DCV infected flies when compared with buffer injected flies. Thirty-five per cent of these genes were also upregulated by other microbial infections (bacteria, fungi), indicating a more general response to stress and infection. Interestingly the other 65% seem to be specifically induced by DCV, indicating that *Drosophila* can recognize viral infection and trigger a dedicated response (Dostert *et al.*, 2005). Analysis of the list of genes induced by DCV did not provide clues concerning the signalling pathway(s) activated during the infection. In particular, the genes targeted by the Toll or Imd pathways are not significantly induced by DCV. A detailed analysis of one strongly induced gene, called *vir-1* (for *virus induced RNA-1*) was carried out (Fig. 4a). *vir-1* is not upregulated by different bacterial or fungal infections, nor by a number of different stresses (e.g. heat-shock, cold shock, mechanical pressure, dehydration). By contrast, *vir-1* is strongly induced upon infection with another insect virus, Flock House virus (FHV, a member of the Nodaviridae). These results indicate that *vir-1* expression is specifically triggered upon recognition of either the virus itself, or a specific aspect of viral pathogenesis.

The gene *vir-1* has been used as a read-out of the response to virus infection, and a virus-response element has been located in a 190bp fragment of the *vir-1* promoter. This fragment contains a consensus binding site for the transcription factor STAT92E (Hou *et al.*, 1996; Yan *et al.*, 1996). An inducible STAT binding activity could indeed be observed in nuclear extracts of DCV infected flies (Fig. 4b). STAT transcription factors are activated upon phosphorylation by the JAK tyrosine-kinases. Phosphorylation induces dimerization and nuclear translocation of STAT, thereby allowing transcriptional activation of target genes. The *Drosophila* genome encodes a single JAK kinase, Hopscotch, which regulates the factor STAT92E (Hombria and Brown, 2002; Hou *et al.*, 2002). In *hopscotch* mutant flies, *vir-1* induction by DCV is strongly reduced. The virus-dependent induction of several other genes is also impaired in this genetic background. The activity of Hopscotch is regulated by the plasma membrane receptor Domeless, a homologue of the gp130 signal transducing subunit of the mammalian interleukine-6 receptor. Domeless is a receptor for the cytokines of the Unpaired (Upd) family, which boasts three members in *Drosophila*. The JAK/STAT pathway also involves the negative regulators SOCS36E, dPIAS, and STAM, which control STAT92E activation (Fig. 4c). Overexpression of either a dominant-negative version of Domeless, or of dPIAS, affects induction of *vir-1* by DCV, confirming the importance of the JAK/STAT pathway in the antiviral response in *Drosophila* (Dostert *et al.*, 2005).

Interestingly, impaired induction of a subset of genes in *hopscotch*

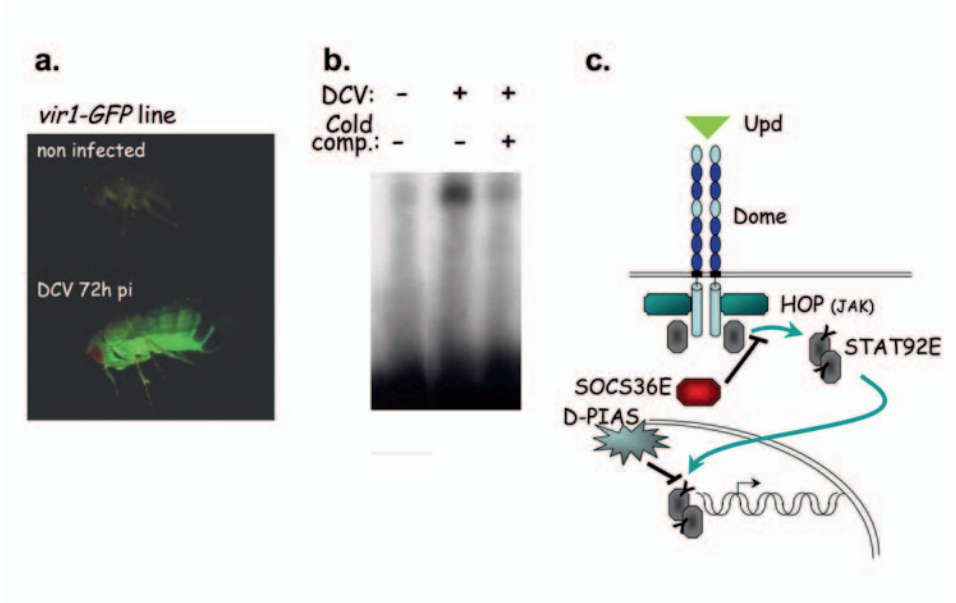


Figure 4. Induction of a transcriptional response by DCV infection.

a. A 2.5kb fragment of 5' regulatory sequences from the *vir-1* gene confers DCV-inducible expression to a GFP reporter gene. This fragment contains two consensus binding sites for the transcription factor STAT92E. b. Induction of a STAT DNA binding activity by DCV infection. Nuclear extracts from control and DCV infected flies were analyzed using an electrophoretic mobility shift assay with an optimal STAT binding site, in the presence or absence of a 50-fold excess of cold competitor. c. The *Drosophila* JAK/STAT pathway. The interleukin-6 gp130 related receptor Domeless (Dome) is activated by the cytokine Unpaired (Upd). This leads to activation of the JAK tyrosine kinase Hopscotch (Hop), which phosphorylates the transcription factor STAT92E. Phosphorylated STAT forms dimers and translocates to the nucleus.

mutant flies correlates with a significant increase in the viral load compared to wild-type flies, and to increased sensitivity to DCV infection. Altogether, these data indicate that the JAK/STAT pathway is necessary for the induction of a subpopulation of DCV regulated genes in *Drosophila*, and indicate that some of these genes participate in the control of the viral infection. The *hopscotch* gene therefore defines a virus susceptibility locus in *Drosophila* (Dostert *et al.*, 2005). This gene is conserved in *A. mellifera* (Tab. II), and it will be interesting to see if it participates in the control of bee viral infections. The JAK/STAT pathway was initially characterized in mammals for its role in signal transduction downstream of interferon receptors: our results therefore point to the evolutionary ancient origin of

the role of the JAK/STAT pathway in antiviral defences.

Curiously, some genes remain inducible to wild-type levels in *hopscotch* deficient flies. Interestingly, those genes do not have consensus STAT binding sites in their promoter region. These data further suggest that at least one other signalling cascade participates in the response to virus infection in *Drosophila* (Dostert *et al.*, 2005). Characterization of this pathway will reveal novel aspects of the innate antiviral response in insects, and may have relevance for our understanding of the antiviral response in mammals.

5. Concluding remarks

In summary, the picture emerging from studies on host-defence in insect models is more complex than anticipated. In particular, the experiments carried out in *Drosophila* clearly indicate that the responses to challenge with bacteria, fungi, large parasites or viruses are different. Therefore, there is some degree of specificity in the insect immune response, although of course not to the same exquisite degree as in the vertebrate adaptive immune system. The clear-cut, distinct, immune phenotypes of *Toll*, *imd* or *Hopscotch* mutant flies indicate that *Drosophila* can distinguish between fungi, bacteria and viruses, and mount a somewhat adapted immune response.

Another unexpected conclusion from this series of work is that the innate immune system in *Drosophila* relies on signalling pathways and genes that have been conserved through evolution, and have counterparts in mammals: the Toll pathway is composed of molecules which have homologues in the IL-1R/TLR pathway in mammals; the Imd pathway is reminiscent of the mammalian TNF pathway; and the JAK/STAT pathway that participates in the response to viral infection in flies is of course evocative of the interferon pathway in mammals. Hence the fruit-fly has emerged as a powerful model to study prototypic innate immunity. Incidentally, the conservation of important innate immunity signalling pathways between *Drosophila* and mammals indicates that studies on host-defence in flies may have relevance for other insect species, including honey bees.

In spite of these similarities, significant differences exist between host-defence in insects and mammals. These differences pertain to the different physiologies of the organisms, as well as to their different evolutions over time. For example, recognition of infectious non-self by soluble receptors that activate proteolytic cascades controlling either melanization or generation of an active cytokine ligand for the Toll receptor, ap-

pears best suited for insects, in which haemolymph bathes all organs by virtue of the open circulatory system. Other differences pertain to the nature of the receptors involved in the recognition of infective microorganisms. This task is mainly ascribed to PGRPs and βGRPs in insects, and to TLRs and Nods in mammals. Challenging questions for the future will address the comparison of infectious non-self recognition and activation of signalling by innate immunity receptors in insects and mammals. The nature of the receptors for virus or parasitic infections, as well as the characterization of the effector molecules induced, also represent promising avenues of research for the coming years.

References

Agaisse H., Petersen U. M., Boutros M., Mathey-Prevot B., Perrimon, N. (2003) Signaling role of hemocytes in *Drosophila* JAK/STAT-dependent response to septic injury, Dev. Cell 5, 441-450.

Akira S., Takeda K. (2004) Toll-like receptor signalling, Nat. Rev. Immunol. 4, 499-511.

Aronstein K., Saldivar E. (2005) Characterization of a honey bee Toll related receptor gene Am18w and its possible involvement in antimicrobial immune defense, Apidologie 36, 3-14.

Belvin M. P., Anderson K. V. (1996) A conserved signaling pathway: the *Drosophila* toll-dorsal pathway, Ann. Rev. Cell. Dev. Biol. 12, 393-416.

Beutler B. (2004) Inferences, questions and possibilities in Toll-like receptor signalling, Nature 430, 257-263.

Bian G., Shin S. W., Cheon H. M., Kokoza V., Raikhel A. S. (2005) Transgenic alteration of Toll immune pathway in the female mosquito *Aedes aegypti*, Proc. Natl. Acad. Sci. U S A 102, 13568-13573.

Bischoff V., Vignal C., Boneca I. G., Michel T., Hoffmann J. A., Royet J. (2004) Function of the *drosophila* pattern-recognition receptor PGRP-SD in the detection of Gram-positive bacteria, Nat. Immunol. 5, 1175-1180.

Blandin S., Levashina E. A. (2004a) Mosquito immune responses against malaria parasites, Curr. Opin. Immunol. 16, 16-20.

Blandin S., Levashina E. A. (2004b) Thioester-containing proteins and insect immunity, Mol. Immunol. 40, 903-908.

Blandin S., Moita L. F., Kocher T., Wilm M., Kafatos F. C., Levashina E. A. (2002) Reverse genetics in the mosquito *Anopheles gambiae*: targeted disruption of the Defensin gene, Embo Rep. 3, 852-856.

Blandin S., Shiao S. H., Moita L. F., Janse C. J., Waters A. P., Kafatos F. C., Levashina E. A. (2004) Complement-like protein TEP1 is a determinant of vectorial capacity in the malaria vector *Anopheles gambiae*, Cell 116, 661-670.

Boman H. (1995) Peptide antibiotics and their role in innate immunity, Annu. Rev. Immunol. 13, 61-92.

Braun A., Hoffmann J. A., Meister M. (1998) Analysis of the *Drosophila* host defense in domino mutant larvae, which are devoid of hemocytes, Proc. Natl. Acad. Sci. U S A 95, 14337-14342.

Bulet P., Dimarcq J., Hetru C., Lagueux M., Charlet M., Hegy G., Van Dorsselaer A., Hoffmann J. (1993) A novel inducible antibacterial peptide of *Drosophila* carries an O-glycosylated substitution, J. Biol. Chem. 268, 14893-14897.

Bulet P., Stöcklin R., Menin L. (2004) Anti-microbial peptides: from invertebrates to vertebrates, Immunol. Rev. 198, 169-184.

Casteels P., Ampe C., Jacobs F., Tempst P. (1993) Functional and chemical characterization of Hymenoptaecin, an antibacterial polypeptide that is infection-inducible in the honeybee (*Apis mellifera*), J. Biol. Chem. 268, 7044-7054.

Casteels P., Ampe C., Jacobs F., Vaeck M., Tempst P. (1989) Apidaecins: antibacterial peptides from honeybees, Embo J. 8, 2387-2391.

Casteels P., Ampe C., Riviere L., Van Damme J., Elicone C., Fleming M., Jacobs F., Tempst P. (1990) Isolation and characterization of abaecin, a major antibacterial response peptide in the honeybee (*Apis mellifera*), Eur. J. Biochem. 187, 381-386.

Casteels P., Tempst P. (1994) Apidaecin-type peptide antibiotics function through a non-poreforming mechanism involving stereospecificity, Biochem. Biophys. Res. Commun. 199, 339-345.

Casteels-Josson K., Capaci T., Casteels P., Tempst P. (1993) Apidaecin multipeptide precursor structure: a putative mechanism for amplification of the insect antibacterial response, Embo J. 12, 1569-1578.

Casteels-Josson K., Zhang W., Capaci T., Casteels P., Tempst P. (1994) Acute transcriptional response of the honeybee peptide-antibiotics gene repertoire and required post-translational conversion of the precursor structures, J. Biol. Chem. 269, 28569-28575.

Chang C. I., Ihara K., Chelliah Y., Mengin-Lecreulx D., Wakatsuki S., Deisenhofer J. (2005) Structure of the ectodomain of *Drosophila* peptidoglycan-recognition protein LCa suggests a molecular mechanism for pattern recognition, Proc. Natl. Acad. Sci. U S A 102, 10279-10284.

Chang C. I., Pili-Floury S., Herve M., Parquet C., Chelliah Y., Lemaitre B., Mengin-Lecreulx D., Deisenhofer J. (2004) A *Drosophila* pattern recognition receptor contains a peptidoglycan docking groove and unusual L,D-carboxypeptidase activity, PLoS Biol. 2, E277.

Chao J.A., Lee J.H., Chapados B.R., Debler E.W., Schneemann A., Williamson J.R. (2005) Dual modes of RNA-silencing suppression by Flock House virus protein B2, Nat. Struct. Mol. Biol 12, 952-957.

Charatsi I., Luschnig S., Bartoszewski S., Nusslein-Volhard C., Moussian B. (2003) Krapfen/dMyd88 is required for the establishment of dorsoventral pattern in the *Drosophila* embryo, Mech. Dev. 120, 219-226.

Cherry S., Doukas T., Armknecht S., Whelan S., Wang H., Sarnow P., Perrimon N. (2005) Genome-wide RNAi screen reveals a specific sensitivity of IRES-containing RNA viruses to host translation inhibition, Genes Dev. 19, 445-452.

Cherry, S., Perrimon, N. (2004) Entry is a rate-limiting step for viral infection in a *Drosophila* melanogaster model of pathogenesis, Nat. Immunol. 5, 81-87.

Chiang C., Beachy P. A. (1994) Expression of a novel Toll-like gene spans the parasegment boundary and contributes to hedgehog function in the adult eye of *Drosophila*, Mech. Dev. 47, 225-239.

Choe K. M., Werner T., Stoven S., Hultmark D., Anderson K. V. (2002) Requirement for a peptidoglycan recognition protein (PGRP) in Relish activation and antibacterial immune responses in *Drosophila*, Science 296, 359-362.

Crozatier M., Ubeda J. M., Vincent A., Meister M. (2004) Cellular immune response to parasitization in *Drosophila* requires the EBF orthologue collier, PLoS Biol. 2, E196.

De Gregorio E., Han S. J., Lee W. J., Baek M. J., Osaki T., Kawabata S., Lee B. L., Iwanaga S., Lemaitre B., Brey P. T. (2002) An immune-responsive Serpin regulates the melanization cascade in *Drosophila*, Dev. Cell 3, 581-592.

De Gregorio E., Spellman P. T., Rubin G. M., Lemaitre B. (2001) Genome-wide analysis of the *Drosophila* immune response by using oligonucleotide microarrays, Proc. Natl. Acad. Sci. U S A 98, 12590-12595.

DeLotto Y., DeLotto R. (1998) Proteolytic processing of the *Drosophila* Spatzle protein by easter generates a dimeric NGF-like molecule with ventralising activity, Mech. Dev. 72, 141-148.

Dimarcq J. L., Keppi E., Dunbar B., Lambert J., Reichhart J. M., Hoffmann D., Rankine S. M., Fothergill J. E., Hoffmann J. A. (1988) Insect immunity. Purification and characterization of a family of novel inducible antibacterial proteins from immunized larvae of the dipteran *Phormia terranovae* and complete amino-acid sequence of the predominant member, diptericin A, Eur. J. Biochem. 171, 17-22.

Dimopoulos G., Christophides G. K., Meister S., Schultz J., White K. P., Barillas-Mur, C., Kafatos F. C. (2002) Genome expression analysis of *Anopheles gambiae*: responses to injury, bacterial challenge, and malaria infection, Proc. Natl. Acad. Sci. U S A 99, 8814-8819.

Dostert C., Jouanguy E., Irving P., Troxler L., Galiana-Arnoux D., Hetru C., Hoffmann J. A., Imler J. L. (2005) The Jak-STAT signaling pathway is required but not sufficient for the antiviral response of *drosophila*, Nat. Immunol. 6, 946-953.

Eldon E., Kooyer S., D'Evelyn D., Duman M., Lawinge P., Botas J, Bellen H. (1994) The *Drosophila* 18 wheeler is required for morphogenesis and has striking similarities to Toll, Development 120, 885-899.

Elrod-Erickson M., Mishra S., Schneider D. (2000) Interactions between the cellular and humoral immune responses in *Drosophila*, Curr. Biol. 10, 781-784.

Evans C. J., Hartenstein V., Banerjee U. (2003) Thicker than blood: conserved mechanisms in *Drosophila* and vertebrate hematopoiesis, Dev. Cell 5, 673-690.

Evans J. D. (2004) Transcriptional immune responses by honey bee larvae during invasion by the bacterial pathogen, *Paenibacillus larvae*, J. Invertebr. Pathol. 85, 105-111.

Fehlbaum P., Bulet P., Michaut L., Lagueux M., Broeckaert W., Hetru C., Hoffmann J. (1994) Insect immunity: septic injury of *Drosophila* induces the synthesis of a potent antifungal peptide with sequence homology to plant antifungal peptides, J. Biol. Chem. 269, 33159-33163.

Ferrandon D., Imler J. L., Hoffmann J. A. (2004) Sensing infection in *Drosophila*: Toll and beyond, Semin. Immunol. 16, 43-53.

Foley E., O'Farrell P. H. (2003) Nitric oxide contributes to induction of innate immune responses to gram-negative bacteria in *Drosophila*, Genes Dev. 17, 115-125.

Fontana R., Mendes M. A., de Souza B. M., Konno K., Cesar L. M., Malaspina O., Palma M. S. (2004) Jelleines: a family of antimicrobial peptides from the Royal Jelly of honeybees (*Apis mellifera*), Peptides 25, 919-928.

Franc N. C., Heitzler P., Ezekowitz R. A., White K. (1999) Requirement for croquemort in phagocytosis of apoptotic cells in *Drosophila*, Science 284, 1991-1994.

Fujiwara S., Imai J., Fujiwara M., Yaeshima T., Kawashima T., Kobayashi K. (1990) A potent antibacterial protein in royal jelly. Purification and determination of the primary structure of royalisin, J. Biol. Chem. 265, 11333-11337.

Ganz T. (2003) Defensins: antimicrobial peptides of innate immunity, Nat. Rev. Immunol. 3, 710-720.

Georgel P., Naitza S., Kappler C., Ferrandon D., Zachary D., Swimmer C., Kopczynski C., Duyk G., Reichhart J. M., Hoffmann J. A. (2001) *Drosophila* immune deficiency (IMD) is a death domain protein that activates the antibacterial response and can promote apoptosis, Dev. Cell 1(4), 503-514.

Gesellchen V., Kuttenkeuler D., Steckel M., Pelte N., Boutros M. (2005) An RNA interference screen identifies Inhibitor of Apoptosis Protein 2 as a regulator of innate immune signalling in *Drosophila*, Embo Rep. 6, 979-984.

Geuking P., Narasimamurthy R., Basler K. (2005) A genetic screen targeting the TNF/Eiger signaling pathway: Identification of *Drosophila* TAB2 as a functionally conserved component, Genetics 171, 1683-1694.

Gobert V., Gottar M., Matskevich A. A., Rutschmann S., Royet J., Belvin M., Hoffmann J. A., Ferrandon D. (2003) Dual activation of the *Drosophila* toll pathway by two pattern recognition receptors, Science 302, 2126-2130.

Gottar M., Gobert V., Michel T., Belvin M., Duyk G., Hoffmann J. A., Ferrandon D., Royet J. (2002) The *Drosophila* immune response against Gram-negative bacteria is mediated by a peptidoglycan recognition protein, Nature 416, 640-644.

Guan R., Roychowdhury A., Ember B., Kumar S., Boons G. J., Mariuzza R. A. (2004), Structural basis for peptidoglycan binding by peptidoglycan recognition proteins, Proc; Natl; Acad; Sci; U S A 101, 17168-17173.

Ha E. M., Oh C. T., Bae Y. S., Lee W. J. (2005a) A direct role for dual oxidase in *Drosophila* gut immunity, Science 310, 847-850.

Ha E. M., Oh C. T., Ryu J. H., Bae Y. S., Kang S. W., Jang I. H., Brey P. T., Lee, W. J. (2005b) An antioxidant system required for host protection against gut infection in *Drosophila*, Dev. Cell. 8, 125-132.

Hedengren M., Asling B., Dushay M., Ando I., Ekengren S., Wihlborg M., Hultmark D. (1999) Relish, a Central Factor in the Control of Humoral but Not Cellular Immunity in *Drosophila*, Mol. Cell 4, 1-20.

Hoffmann J. (2003) The immune response of *Drosophila*, Nature 426, 33-38.

Hombria J. C., Brown S. (2002) The fertile field of *Drosophila* Jak/STAT signalling, Curr. Biol. 12, R569-575.

Horng., T., Medzhitov R. (2001) *Drosophila* MyD88 is an adapter in the Toll signaling pathway, Proc. Natl. Acad. Sci. U S A 98, 12654-12658.

Hou S. X., Zheng Z., Chen X., Perrimon N. (2002) The Jak/STAT pathway in model organisms: emerging roles in cell movement, Dev. Cell 3, 765-778.

Hou X. S., Melnick M. B., Perrimon N. (1996) Marelle acts downstream of the *Drosophila* HOP/JAK kinase and encodes a protein similar to the mammalian STATs, Cell 84, 411-419.

Hultmark D., Engstrom A., Andersson K., Steiner H., Bennich H., Boman H. G. (1983) Insect immunity. Attacins, a family of antibacterial proteins from *Hyalophora cecropia*, Embo J. 2, 571-576.

Irving P., Troxler L., Heuer T. S., Belvin M., Kopczynski C., Reichhart J. M., Hoffmann J. A., Hetru C. (2001) A genome-wide analysis of immune responses in *Drosophila*, Proc. Natl. Acad. Sci. U S A 98, 15119-15124.

Irving P., Ubeda J. M., Doucet D., Troxle L., Lagueux M., Zachary D., Hoffmann J. A., Hetru C., Meister M. (2005) New insights into *Drosophila* larval haemocyte functions through genome-wide analysis, Cell Microbiol. 7, 335-350.

Johnson K. N., Christian P. D. (1998) The novel genome organization of the insect picorna-like virus *Drosophila* C virus suggests this virus belongs to a previously undescribed virus family, J. Gen. Virol. 79, 191-203.

Jousset F. X., Bergoin M., Revet B. (1977) Characterization of the *Drosophila* C virus, J. Gen. Virol. 34, 269-283.

Kambris Z., Hoffmann J. A., Imler J. L., Capovilla M. (2002) Tissue and stage-specific expression of the Tolls in *Drosophila* embryos, Gene Expr. Patterns 2, 311-317.

Kaneko T., Goldman W. E., Mellroth P., Steiner H., Fukase K., Kusumoto S., Harley W., Fox A., Golenbock D., Silverman N. (2004) Monomeric and polymeric gram-negative peptidoglycan but not purified LPS stimulate the *Drosophila* IMD pathway, Immunity 20, 637-649.

Kanost M. R., Jiang H., Yu X. Q. (2004) Innate immune responses of a lepidopteran insect, *Manduca sexta,* Immunol. Rev. 198, 97-105.

Kitabayashi A. N., Arai T., Kubo T., Natori S. (1998) Molecular cloning of cDNA for p10, a novel protein that increases in the regenerating legs of *Periplaneta americana* (American cockroach), Insect Biochem. Mol. Biol. *28,* 785-790.

Klaudiny J., Albert S., Bachanova K., Kopernicky J., Simuth J. (2005) Two structurally different defensin genes, one of them encoding a novel defensin isoform, are expressed in honeybee *Apis mellifera,* Insect Biochem. Mol. Biol. 35, 11-22.

Kleino A., Valanne S., Ulvila J., Kallio J., Myllymaki H., Enwald H., Stoven S., Poidevin M., Ueda R., Hultmark D., Lemaitre B., Ramet M. (2005) Inhibitor of apoptosis 2 and TAK1-binding protein are components of the *Drosophila* Imd pathway, Embo J. 24, 3423-3434.

Kocks C., Cho J.H., Nehme N., Ulvila J., Pearson A.M., Meister M., Strom C., Conto S.L., Hetru C., Stuart L.M., Stehle T., Hoffmann J.A., Reichhart J.M., Ferrandon D., Ramet M., Ezekowitz R.A. (2005) Eater, a transmembrane protein mediating phagocytosis of bacterial pathogens in *Drosophila*, Cell 123, 335-346.

Kurz C. L., Ewbank J. J. (2003) *Caenorhabditis elegans*: an emerging genetic model for the study of innate immunity, Nat. Rev. Genet. 4, 380-390.

Lagueux M., Perrodou E., Levashina E. A., Capovilla M., Hoffmann J. A. (2000) Constitutive expression of a complement-like protein in toll and JAK gain-of-function mutants of *Drosophila*, Proc. Natl. Acad. Sci. U S A 97, 11427-11432.

Lambert J., Keppi E., Dimarcq J. L., Wicke C., Reichhart J. M., Dunbar B., Lepage P., Van Dorsselae A., Hoffmann J., Fothergill J., Hoffmann D. (1989) Insect immunity: isolation from immune blood of the dipteran *Phormia terranovae* of two insect antibacterial peptides with sequence homology to rabbit lung macrophage bactericidal peptides. Proc Natl Acad Sci U S A 86, 262-266.

Lanot R., Zachary D., Holder F., Meister M. (2001) Postembryonic hematopoiesis in *Drosophila*, Dev. Biol. 230, 243-257.

Lartigue A., Campanacci V., Roussel A., Larsson,A. M., Jones T. A., Tegoni M., Cambillau C. (2002) X-ray structure and ligand binding study of a moth chemosensory protein, J. Biol. Chem. 277, 32094-32098.

Lecellier C. H., Voinnet O. (2004) RNA silencing: no mercy for viruses? Immunol. Rev. 198, 285-303.

Lee C. Y., Clough E. A., Yellon P., Teslovich T. M., Stephan D. A., Baehrecke E. H. (2003) Genome-wide analyses of steroid- and radiation-triggered programmed cell death in *Drosophila*, Curr. Biol. 13, 350-357.

Lee J. C., Chen H. H., Chao Y. C. (1998) Persistent baculovirus infection results from deletion of the apoptotic suppressor gene p35, J. Virol. 72, 9157-9165.

Lemaitre B., Kromer-Metzger E., Michaut L., Nicolas E., Meister M., Georgel P., Reichhart J., Hoffmann J. (1995) A recessive mutation, immune deficiency (imd), defines two distinct control pathways in the *Drosophila* host defense, Proc. Natl. Acad. Sci. U S A 92, 9365-9469.

Lemaitre B., Nicolas E., Michaut L., Reichhart J., Hoffmann J. (1996) The dorsoventral regulatory gene cassette spätzle/Toll/cactus controls the potent antifungal response in *Drosophila* adults, Cell *86*, 973-983.

Leulier F., Parquet C., Pili-Floury S., Ryu J. H., Caroff M., Lee W. J., Mengin-Lecreulx D., Lemaitre B. (2003) The *Drosophila* immune system detects bacteria through specific peptidoglycan recognition, Nat. Immunol. 4, 478-484.

Leulier F., Rodriguez A., Khush R. S., Abrams J. M., Lemaitre B. (2000) The *Drosophila* caspase Dredd is required to resist Gram-negative bacterial infection, Embo Rep. 1, 353-358.

Leulier F., Vidal S., Saigo K., Ueda R., Lemaitre B. (2002) Inducible expression of double-stranded RNA reveals a role for dFADD in the regulation of the antibacterial response in *Drosophila* adults, Curr. Biol. 12, 996-1000.

Levashina E., Ohresser S., Bulet P., Reichhart J., Hetru C., Hoffmann J. (1995) Metchnikowin, a novel immune-inducible proline-rich peptide from *Drosophila* with antibacterial and antifungal properties, Eur. J. Biochem. 233, 694-700.

Levashina E. A., Moita L. F., Blandin S., Vriend G., Lagueux M., Kafatos F. C. (2001) Conserved role of a complement-like protein in phagocytosis revealed by dsRNA knockout in cultured cells of the mosquito, *Anopheles gambiae*, Cell 104, 709-718.

Levy F., Bulet P., Ehret-Sabatier L. (2004) Proteomic analysis of the systemic immune response of *Drosophila*, Mol. Cell Proteomics 3, 156-166.

Ligoxygakis P., Bulet P., Reichhart J. M. (2002a) Critical evaluation of the role of the Toll-like receptor 18-Wheeler in the host defense of *Drosophila*, Embo Rep. 3, 666-673.

Ligoxygakis P., Pelte N., Ji C., Leclerc V., Duvic B., Belvin M., Jiang H., Hoffmann J. A., Reichhart J. M. (2002b) A serpin mutant links Toll activation to melanization in the host defence of *Drosophila*, Embo J. 21, 6330-6337.

Lu R., Maduro M., Li F., Li H. W., Broitman-Maduro G., Li W. X., Ding S. W. (2005) Animal virus replication and RNAi-mediated antiviral silencing in *Caenorhabditis elegans*, Nature 436, 1040-1043.

Lu Y., Wu L. P., Anderson K. V. (2001) The antibacterial arm of the *drosophila* innate immune response requires an IkappaB kinase, Genes Dev. 15, 104-110.

Mackenzie J. S., Gubler D. J., Petersen L. R. (2004) Emerging flaviviruses: the spread and resurgence of Japanese encephalitis, West Nile and dengue viruses, Nat. Med. 10, S98-S109.

McKenna M. P., Hekmat-Scafe D. S., Gaines P., Carlson J. R. (1994) Putative *Drosophila* pheromone-binding proteins expressed in a subregion of the olfactory system, J. Biol. Chem. 269, 16340-16347.

Medzhitov R., Janeway C. A., Jr. (1997) Innate immunity: the virtues of a nonclonal system of recognition, Cell 91, 295-298.

Medzhitov R., Preston-Hurlburt P., Janeway C. (1997) A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity, Nature 388, 394-397.

Meister M. (2004) Blood cells of *Drosophila*: cell lineages and role in host defence, Curr. Opin. Immunol. 16, 10-15.

Meister S., Kanzok S. M., Zheng X. L., Luna C., Li T. R., Hoa N. T., Clayton J. R., White K. P., Kafatos F. C., Christophides G. K., Zheng L. (2005) Immune signaling pathways regulating bacterial and malaria parasite infection of the mosquito *Anopheles gambiae,* Proc. Natl. Acad. Sci. U S A 102, 11420-11425.

Mellroth P., Karlsson J., Steiner H. (2003) A scavenger function for a *Drosophila* peptidoglycan recognition protein, J. Biol. Chem. 278, 7059-7064.

Michel T., Reichhart J. M., Hoffmann J. A., Royet J. (2001) *Drosophila* Toll is activated by Gram-positive bacteria through a circulating peptidoglycan recognition protein, Nature 414, 756-759.

Moita L. F., Wang-Sattler R., Michel K., Zimmermann T., Blandin S., Levashina E. A., Kafatos F. C. (2005) *In vivo* identification of novel regulators and conserved pathways of phagocytosis in *A. gambiae*, Immunity 23, 65-73.

Morisato D., Anderson K. (1994) The spätzle gene encodes a component of the extracellular signaling pathway establishing the dorsal-ventral pattern of the *Drosophila* embryo, Cell 76, 677-688.

Naitza S., Rosse C., Kappler C., Georgel P., Belvin M., Gubb D., Camonis J., Hoffmann J. A., Reichhart J. M. (2002) The *Drosophila* immune defence against Gram-negative infection requires the death domain protein dFADD, Immunity 17, 575-581.

Nimchuk Z., Eulgem T., Holt B.F. 3rd, Dangl J.L. (2003) Recognition and response in the plant immune system, Annu. Rev. Genet. 37, 579-609.

Pikielny C. W., Hasan G., Rouyer F., Rosbash M. (1994) Members of a family of *Drosophila* putative odorant-binding proteins are expressed in different subsets of olfactory hairs, Neuron 12, 35-49.

Poltorak A., He X., Smirnova I., Liu M.Y., Van H.C., Du X., Birdwell D., Alejos E., Silva M., Galanos C., Freudenberg M., Ricciardi-Castagnoli P., Layton B., Beutler B. (1998) Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene, Science 282, 2085-2088.

Popham H. J., Shelby K. S., Brandt S. L., Coudron T. A. (2004) Potent virucidal activity in larval Heliothis virescens plasma against Helicoverpa zea single capsid nucleopolyhedrovirus, J. Gen. Virol. 85, 2255-2261.

Rabel D., Charlet M., Ehret-Sabatier L., Cavicchioli L., Cudic M., Otvos L. Jr., Bulet P. (2004) Primary structure and in vitro antibacterial properties of the *Drosophila* melanogaster attacin C Pro-domain, J. Biol. Chem. 279, 14853-14859.

Ramet M., Manfruelli P., Pearson A., Mathey-Prevot B., Ezekowitz R. A. (2002) Functional genomic analysis of phagocytosis and identification of a *Drosophila* receptor for *E. coli,* Nature 416, 644-648.

Ramet M., Pearson A., Manfruelli P., Li X., Koziel H., Gobel V., Chung E., Krieger M., Ezekowitz R. A. (2001) *Drosophila* scavenger receptor CI is a pattern recognition receptor for bacteria, Immunity 15, 1027-1038.

Reiser J. B., Teyton L., Wilson I. A. (2004) Crystal structure of the *Drosophila* peptidoglycan recognition protein (PGRP)-SA at 1.56 A resolution, J. Mol. Biol. 340, 909-917.

Rock F., Hardiman G., Timans J., Kastelein R., Bazan J. (1998) A family of human receptors structurally related to *Drosophila* Toll, Proc. Natl. Acad. Sci. U S A 95, 588-593.

Roignant J. Y., Carre C., Mugat B., Szymczak D., Lepesant J. A., Antoniewski C. (2003) Absence of transitive and systemic pathways allows cell-specific and isoform-specific RNAi in *Drosophila*, Rna 9, 299-308.

Rubin G. M., Lewis E. B. (2000) A brief history of Drosophila's contributions to genome research, Science 287, 2216-2218.

Rutschmann S., Jung A., Hetru C., Reichhart J., Hoffmann J., Ferrandon D. (2000a) The Rel protein DIF mediates the Toll-dependent antifungal response in *Drosophila*, Immunity 12, 569-580.

Rutschmann S., Jung A., Zhou R., Silverman N., Hoffmann J. A., Ferrandon D. (2000b) Role of *Drosophila* IKKγ in a Toll-independent antibacterial immune response, Nature Immunol. 1, 342-347.

Sabatier L., Jouanguy E., Dostert C., Zachary D., Dimarcq J. L., Bulet P., Imler J. L. (2003) Pherokine-2 and -3: Two *Drosophila* molecules related to pheromone/odor-binding proteins induced by viral and bacterial infections, Eur. J. Biochem. 270, 3398-3407.

Seppo A., Matani P., Sharrow M., Tiemeyer M. (2003) Induction of neuron-specific glycosylation by Tollo/Toll-8, a *Drosophila* Toll-like receptor expressed in non-neural cells, Development 130, 1439-1448.

Shen M., Cui L., Ostiguy N., Cox-Foster D. (2005) Intricate transmission routes and interactions between picorna-like viruses (Kashmir bee virus and sacbrood virus) with the honeybee host and the parasitic varroa mite, J. Gen. Virol. 86, 2281-2289.

Shimanuki H., Knox D. A. (1997) Bee health and international trade, Rev. Sci. Tech. Off. Int. Epizoot. 16, 172-176.

Shin S. W., Kokoza V., Bian G., Cheon H. M., Kim Y. J., Raikhel A. S. (2005) REL1, a homologue of *Drosophila* dorsal, regulates toll antifungal immune pathway in the female mosquito *Aedes aegypti*, J. Biol. Chem. 280, 16499-16507.

Shin S. W., Kokoza V., Lobkov I., Raikhel A. S. (2003) Relish-mediated immune deficiency in the transgenic mosquito *Aedes aegypti*, Proc. Natl. Acad. Sci. U S A 100, 2616-2621.

Silverman N., Zhou R., Stoven S., Pandey N., Hultmark D., Maniatis T. (2000) A *Drosophila* IkappaB kinase complex required for Relish cleavage and antibacterial immunity? Genes Dev. 14, 2461-2471.

Starks P. T., Blackie C. A., Seeley T. D. (2000) Fever in honeybee colonies. Naturwissenschaften 87, 229-231.

Steiner H. (2004) Peptidoglycan recognition proteins: on and off switches for innate immunity, Immunol. Rev. 198, 83-96.

Steiner H., Hultmark D., Engstrom A., Bennich H., Boman H. G. (1981) Sequence and specificity of two antibacterial proteins involved in insect immunity, Nature 292, 246-248.

Stoven S., Silverman N., Junell A., Hedengren-Olcott M., Erturk D., Engstrom Y., Maniatis T., Hultmark D. (2003) Caspase-mediated processing of the *Drosophila* NF-kappaB factor Relish, Proc. Natl. Acad. Sci. U S A 100, 5991-5996.

Sun H., Bristow B. N., Qu G., Wasserman S. A. (2002) A heterotrimeric death domain complex in Toll signaling, Proc. Natl. Acad. Sci. U S A 99, 12871-12876.

Sun X., Chen S., Li S., Yan H., Fan Y., Mi H. (2005) Deletion of two C-terminal Gln residues of 12-26-residue fragment of melittin improves its antimicrobial activity, Peptides 26, 369-375.

Takehana A., Yano T., Mita S., Kotani A., Oshima Y., Kurata S. (2004) Peptidoglycan recognition protein (PGRP)-LE and PGRP-LC act synergistically in *Drosophila* immunity, Embo J. 23, 4690-4700.

Tauszig S., Jouanguy E., Hoffmann J. A., Imler, J. L. (2000) Toll-related receptors and the control of antimicrobial peptide expression in *drosophila*, Proc. Natl. Acad. Sci. U S A 97, 10520-10525.

Tauszig-Delamasure S., Bilak H., Capovilla M., Hoffmann J. A., Imler J. L. (2002) *Drosophila* MyD88 is required for the response to fungal and Gram-positive bacterial infections, Nat. Immunol. 3, 91-97.

Theopold U., Schmidt O., Soderhall K., Dushay M. S. (2004) Coagulation in arthropods: defence, wound closure and healing, Trends Immunol. 25, 289-294.

Trudeau D., Washburn J. O., Volkman L. E. (2001) Central role of hemocytes in *Autographa californica* M nucleopolyhedrovirus pathogenesis in *Heliothis virescens* and *Helicoverpa zea*, J. Virol. 75, 996-1003.

Tzou P., De Gregorio E., Lemaitre B. (2002) How *Drosophila* combats microbial infection: a model to study innate immunity and host-pathogen interactions, Curr. Opin. Microbiol. 5, 102-110.

Tzou P., Ohresser S., Ferrandon D., Capovilla M., Reichhart J. M., Lemaitre B., Hoffmann J. A., Imler J. L. (2000) Tissue-specific inducible expression of antimicrobial peptide genes in *Drosophila* surface epithelia, Immunity 13, 737-748.

Vidal S., Khush R. S., Leulier F., Tzou P., Nakamura M., Lemaitre B. (2001) Mutations in the *Drosophila* dTAK1 gene reveal a conserved function for MAPKKKs in the control of rel/NF-kappaB-dependent innate immune responses, Genes Dev. *15,* 1900-1912.

Vlachou D., Schlegelmilch T., Christophides G. K., Kafatos F. C. (2005) Functional genomic analysis of midgut epithelial responses in *Anopheles* during Plasmodium invasion, Curr. Biol. 15, 1185-1195.

Wanner K. W., Willis L. G., Theilmann D. A., Isman M. B., Feng Q., Plettner E. (2004) Analysis of the insect os-d-like gene family, J. Chem. Ecol. 30, 889-911.

Watson F. L., Puttmann-Holgado R., Thomas F., Lamar D. L., Hughes M., Kondo M., Rebel V. I., Schmucker D. (2005) Extensive diversity of Ig-superfamily proteins in the immune system of insects, Science 309, 1874-1878.

Weber A. N., Tauszig-Delamasure S., Hoffmann J. A., Lelievre E., Gascan H., Ray K. P., Morse M. A., Imler J. L., Gay N. J. (2003) Binding of the *Drosophila* cytokine Spatzle to Toll is direct and establishes signaling, Nat. Immunol. 4, 794-800.

Werner T., Liu G., Kang D., Ekengren S., Steiner H., Hultmark D. (2000) A family of peptidoglycan recognition proteins in the fruit fly *Drosophila* melanogaster, Proc. Natl. Acad. Sci. U S A 97, 13772-13777.

Wilkins C., Dishongh R., Moore S. C., Whitt M. A., Chow M., Machaca K. (2005) RNA interference is an antiviral defence mechanism in *Caenorhabditis elegans,* Nature 436, 1044-1047.

Yan R., Small S., Desplan C., Dearolf C. R., Darnell J. E. Jr. (1996) Identification of a Stat gene that functions in *Drosophila* development, Cell 84, 421-430.

Yang X., Cox-Foster, D. L. (2005) Impact of an ectoparasite on the immunity and pathology of an invertebrate: evidence for host immunosuppression and viral amplification, Proc. Natl. Acad. Sci. U S A 102, 7470-7475.

Chapter 8

HONEY BEE GENOMICS AND BREEDING FOR RESISTANCE TO VIRUS INFECTIONS

Robin F.A. MORITZ and Jay D. EVANS

Table of Contents

1. Introduction

Chemotherapy of viral diseases of honey bees is currently not possible and selection for honey bee stock that resists or tolerates viruses and viral vectors seems the only feasible solution to mitigate the impact of viral diseases on honey bees. Selection programmes for disease resistance in honey bees in the past had only mixed success, primarily because the testing and selection of the phenotype of honey bee colonies is extremely time consuming (usually two years per generation) and tools for swift selection of disease resistance are currently lacking. Since queens and drones mate in flight, mating control is difficult unless highly skilled and laborious instrumental insemination of queens is used. At the same time Europe, Africa, and Western Asia harbour a huge wealth of various indigenous honey bee subspecies of *Apis mellifera*, with a vast potential to reveal mechanisms for disease resistance. We here suggest the development of both swift molecular tools for confirming mating control and selection of resistant colonies, based on target genes which control specific disease resistance in honey bees. This is possible because the complete honey bee genome (*Apis mellifera*) has become available, establishing this economically and ecologically essential organism as a model system for genomic research.

2. Classical tools of honey bee breeding

Bee breeding, like animal breeding in general, aims at developing selection tools to improve the quality of honey bees for bee management. While poultry, swine and cattle breeding has led to the development of domesticated strains and agriculturally highly tuned organisms, the honey bee is by no means domesticated. For one, man kept colonies can readily swarm off into the wild with perfect survival capability and wild or feral populations of *Apis mellifera* exist on all continents but Antarctica (Moritz *et al.*, 2005). How can it be that an animal as popular and successful as the honey bee, has resisted standard selection attempts for so long ?

Things were difficult with honey bee breeding for a long time in history. Beekeepers were ignorant of the mating biology of honey bees for thousands of years, until the work of Anton Janscha from Slovenia at the end of the 18th century (Janscha, 1771). This ignorance certainly did no good to selection progress in the past, and is a key reason why selection by breeding has been slow compared to breeding success in other livestock. Even today selective breeding in honey bees is difficult and again it is Janscha's finding that queens and drones mate in flight which cannot be

controlled by the beekeeper and is a major obstacle for stock improvement and maintenance of desired stock.

2.1. Mating control

Today the mating behaviour of queens and drones is well documented. Queens and drones mate in flight at drone congregation areas about 20 m above ground (Winston, 1987) which typically prevents effective mating control. Bee breeders employ great effort to achieve controlled matings by transporting both drone producing colonies and virgin queens to offshore islands that lack honey bees (Harris *et al.*, 2002) or other remote regions they assume to be free of other honey bee colonies (Laidlaw and Page, 1997). Some recent studies (Neumann *et al.*, 1999) suggest that even on islands unplanned matings can occur leaving a certain amount of uncertainty in mating control. The development of instrumental insemination as a routine technique (Mackensen, 1947) overcame this problem. However, the technique is rather elaborate (Moritz and Kühnert, 1984) and is rarely used in large-scale breeding programs because it requires specific skills and equipment. Instrumental insemination did however established itself as an indispensable and frequently used tool in studies on honey bee genetics.

2.2. The colony phenotype

Another major problem in bee breeding however may stem from honey bees living in colonies. A colony trait results from a colonial phenotype where thousands of individual animals collaborate to achieve a collective trait, in contrast to the single individual with a single genotype targeted by classical animal breeding (Moritz and Southwick, 1987). Both natural selection and artificial (human-driven) selection primarily operate at the level of the colony (Fuchs and Moritz, 1999) and less so on the level of the individual. Because the colonial phenotype is a crucial component it is rather difficult to determine the breeding value of an individual queen or drone (Bienefeld and Pirchner, 1991; Boecking *et al.*, 2000). In addition, testing the performance of a colony in temperate climates usually requires more than one year, making honey bee selection and breeding both a slow and expensive procedure with regard to improving the colonial phenotype.

2.3. Selection for disease resistance

Selection for disease resistance is even more laborious because it requires colonies being infected under standardized conditions. In many countries this is legally not possible, which prevents systematic selection for disease resistant stock. There are only few examples of successful breeding for disease resistance. The classical example is that of hygienic

behaviour first detected by Rothenbuhler (1964). He found that workers uncap brood and remove larvae that are infected by *Paenibacillus larvae larvae* (American foulbrood). He postulated a two-locus system to determine uncapping and removing, which indeed became a textbook classic of behavioural genetics. Later work however showed that the genetics are probably more complex: at least seven loci are involved in hygienic behaviour (Moritz, 1988; Lapidge *et al.*, 2002). Nevertheless, it seems clear that hygienic behaviour strongly depends on gene effects and can thus readily be selected (Spivak, 2001). The impact of hygienic behaviour is far reaching because it directly or indirectly affects almost all brood diseases, including virus infections. A good example of the breadth of hygienic behaviours is the SMR (suppressed mite reproduction) line developed by Harbo and Harris (2005). They observed that highly hygienic honey bees could efficiently suppress *Varroa destructor* infestations by removing mites from cells containing developing bees.

A few other examples of successful breeding also relate to *V. destructor* infestations, where after a few generations of strict colony level selection the susceptibility to *V. destructor* could be greatly reduced (Büchler, 2000; Kulinčević *et al.*, 1992; Fries *et al.*, 2003, 2005; Lodesani *et al.*, 2002). In Fries *et al.*'s work, bee breeding for disease tolerance was mainly driven by natural mortality rather than stock improvement *per se*. Non-surviving colonies died because of the infestation or the resulting secondary infections and not because the bee breeder excluded them from further matings. This approach has been frequently termed as the "Bond experiment" (*Live and Let Die*) to address the difference from classical selective breeding. Clearly this more or less stochastic approach is an unsatisfactory strategy given the current need to address an increasing impact of viral diseases in honey bees. Lodesani *et al.* (2002) applied a bidirectional selection over three generations using controlled artificial infestation of honey bee with *V. destructor*. This allowed for a more systematic estimation of the underlying quantitative genetic parameters for *V. destructor* resistance. Nevertheless, even Lodesani *et al.* (2002) could not find a biological mechanism driving the resistance of colonies to *V. destructor*. They found that colonies with less brood had also less mites, which explained only 36% of the observed covariance. In addition the observed reduction in brood in the resistant line was correlated with a reduction in honey performance, showing the difficulties in using a multifaceted colony level selection approach. It would certainly be desirable to employ approaches selecting for actual disease resistance mechanisms rather than composite colonial phenotypes to better predict selection response and develop a system that overcomes selection approaches taking three to four years. Moreover, one has to keep in mind

that, even given that a resistant strain has been identified, it still needs to be propagated to the beekeeper community. Since uncontrolled matings in subsequent generations can destroy any selective advantage, one has to constantly maintain the specific desired line. This can easily generate problems at the population level, including inbreeding and homozygosity at the sex determining locus. Clearly it would be extremely helpful to have a swift reliable tool to select alleles directly responsible for disease resistance from an otherwise diverse population of bees.

While behavioural resistance mechanisms can help defend colonies against existing disease, individual defenses are likely to be an equally strong mechanism for avoiding or moderating disease. We feel that such individual defenses have been understudied to date. Therefore, it is of great practical relevance to identify immunity mechanisms and other individual defenses which can help prevent the spread of disease in the colonies (Chen *et al.*, 2000; Evans, 2004). The genetic basis of the insect immune system is well studied in the fruit fly *Drosophila melanogaster* (Hultmark, 2003; Imler and Hoffmann, 2003; see Chapter 7 for a comprehensive review) and various mosquitoes that vector human disease agents and various genes controlling immune reactions have been identified (Dimopoulos, 2003; Levashina, 2004). With this knowledge from classical genetic model systems, the swift advance of DNA techniques, and the availability of the honey bee genome, unexpectedly novel breeding concepts seem to be in reach. New approaches target on gene function rather than classical breeding or marker assisted procedures, resulting in a major conceptual leap in honey bee breeding and genetics. Selection can now be based on genomics by screening the expression of genes and the presence or absence of target genes in the genome rather than testing phenotypes of honey bee colonies over many years.

3. Molecular DNA tools

Molecular-genetic tools revolutionized genetics and also honey bee genetics (Gadau *et al.*, 2000). With the development of precise genetic maps with markers saturating the entire genome (Arechavaleta-Velasco and Hunt, 2004; Solignac *et al.*, 2004) it became possible to quantitatively determine the genetic components behind complex behavioural and physiological traits.

3.1. Gene identification

Thanks to the unravelling of the *Drosophila* genome (Adams *et al.*, 2000), the search for analogous genes in *Apis* was greatly facilitated and

a suite of genes (Whitfield *et al.*, 2002, Nunes *et al.*, 2004) could be identified and their function studied in classical expression studies (Evans and Wheeler, 2001, Whitfield *et al.*, 2003). The identification of honey bee specific genes, which are not already known from other insect systems is more difficult, requiring labour intensive, painstaking and time consuming work. For example the identification of the sex locus (*csd*) of the honey bee required more than seven years research with classical mapping and chromosome walking (Beye *et al.*, 1996, 1999, 2003). In the course of the project, the latest technology in functional genetics (RNA interference) was adapted to the honey bee system (Beye *et al.*, 2002) in order to silence *csd* expression in female tissues and confirm the function of *csd* in the sex determination process.

With the characterization of the honey bee genome (Box 1) the possibilities for genomic research in honey bees have dramatically improved. With the aid of about 2000 microsatellite loci (Solignac *et al.*, 2004) it was possible to align the sequenced honey bee genome. Genes already known from other organisms (e.g. *Drosophila*) can now be identified by standard bio-informatics. The availability of the sequence also greatly facilitates the search for further honey bee specific genes. Although these studies still re-

Box 1. The Honey Bee Genome Project.

Following the sequencing of the genome of the fruit fly *Drosophila melanogaster*, a century-old model for genetics research, various communities of insect researchers realized that such a project was both desirable and feasible for their favourite species. For bees there were soon worldwide discussions of how to accomplish a genome sequence. It was apparent that such a project would 1) involve collaborative efforts across many groups, and 2) would require funding and expertise from outside the bee community. In the US, an attempt was made in November 2001 to secure funding for a draft bee genomic sequence under the auspicious of the US National Institutes of Health National Human Genome Research Initiative competition for 'Non-Mammalian Models'. While this proposal failed, the momentum generated was perfectly timed for the start of an additional NIH-NHGRI program, the so-called 'excess capacity' sequencing competition. The following February, a proposal to this body was rated well and, with volunteered support from the Baylor College of Medicine Human Genome sequencing Center (BCM), the project was underway later in 2002.

The draft genome and assembly has been published in Nature (Honey Bee Genome Sequencing Consortium, 2006) by a consortium including many members of the worldwide bee research community in collaboration with scientists from BCM and the broad genomics community.

quire mapping and gene expression work, these are greatly facilitated with the complete sequence at hand. For example the gene "thelytoky" (=female producing parthenogenesis), *th*, was mapped down to 20 cM within a few months (Lattorff *et al.*, 2004 and 2006) once the mapping population had been established. These two genes, *csd* and *th*, are currently the only two honey bee specific single locus systems which are mapped and *csd* the only identified (*th* still pending). This may seem to be low in comparison to *Drosophila*, but reflects the difficulty in finding complex traits of interest that are strongly regulated by a single gene, especially in the absence of a precise tool for generating mutant phenotypes.

3.2. Quantitative trait loci

Most phenotypes will vary quantitatively and not qualitatively. For some of these traits, a few major genes can be identified with primary influence on the phenotype. These quantitative trait loci (QTL) can be mapped by testing linkage with segregation of a large number of variable markers (e.g. with Randomly Amplified Polymorphic DNA (RAPD) or microsatellite markers) which saturate the genome. In honey bees QTL have been identified for traits linked to phenotypes of individual bees. For example pollen collecting of workers is controlled by three major QTL: *pln 1, pln2* and *pln 3* (Hunt *et al.*, 1995; Page *et al.*, 2000). They have been shown to interact with other loci (Humphries *et al.*, 2005, Rüppel *et al.*, 2004a and 2004b) which adds to the complexity of genetic control of pollen foraging. Other QTL have been detected for defence behaviour (Guzman-Novoa *et al.*, 2002; Hunt *et al.*, 1998; Lobo *et al.*, 2003, Arechavaleta-Velasco and Hunt, 2004), body size (Hunt *et al.*, 1998), learning behaviour (Chandra *et al.*, 2001) and hygienic behaviour (Lapidge *et al.*, 2002). Markers which are linked to the QTL can be used in Marker Assisted Selection (MAS, Lande and Thomson, 1990), which has been shown to be useful in animal breeding. Unfortunately, it is often difficult to find suitable makers in genomes with high recombination rates and the honey bee has the highest recombination rate of all genomically studied organisms (Solignac *et al.*, 2004). The recombination rate is less than 50kb/cM (Beye *et al.*, 1999), 10-fold higher than in *Drosophila* (Merriam *et al.*, 1991). Marker assisted mapping will therefore only be possible if physically very closely linked makers are developed. In addition, mapping studies require much larger numbers of marker loci to saturate the genome than in other animal systems. With the availability of the sequence (90%) and over 2000 microsatellite markers the genome could now be assigned to 16 linkage groups reflecting the 16 chromosomal set (Solignac *et al.*, 2004). However, the high recombination

rate can be a true advantage for gene identification, because linked markers can be physically very close to a target gene and still segregate at measurable levels. Once a genomic region with a QTL has been identified, the availability of the sequence allows for the saturation of this target region with a large number of novel highly variable microsatellite markers for fine mapping. These markers can then be used to pinpoint specific genes in the genome just through mapping alone allowing for the identification of a gene even before testing its actual function (Lattorff *et al.*, 2006).

3.3. Direct tests of gene function

Given a set of candidate genes generated by mapping or gene-expression studies, the next step is to confirm the importance of these genes for honey bee health. Two molecular-genetic techniques are widely used to do this. The 'gold standard' for determining gene function is to eliminate or enhance the activity of individual genes through genetic transformation of individuals or cell lines. Such transgenic techniques are technically difficult, subject to tight environmental regulation, and dependent on genomes which readily accept inserted sections of DNA. Honey bees are problematic for each of these reasons, and transgenic bees have been cultivated only rarely (Robinson *et al.*, 2000).

A powerful alternative method for gene validation takes advantage of the temporary silencing of genes via a process termed RNA interference (RNAi) (Fire *et al.*, 1998). This process is widespread in eukaryotes and appears to be robust in honey bees of different life stages (Amdam *et al.*, 2003; Aronstein and Saldivar, 2005; Beye *et al.*, 2003; Farooqui *et al.*, 2004; Gatehouse *et al.*, 2004; Guidugli *et al.*, 2005). Indeed, the effects of RNAi in bees appear to be systemic, such that double-stranded RNA injected to silence a specific gene can have a persistent and expanded effect. In this sense RNAi is a more powerful tool in bees than in the canonical genetic model, the fruit fly, a species for which RNAi works less well at the organism level (Roignant *et al.*, 2003; Friedman and Perrimon, 2004). To date, RNAi studies have been used to confirm or negate the roles of several genes implicated in honey bee development, immunity, and learning and it is clear that this technique will continue to play a central role in defining disease-related genes in honey bees.

4. Breeding for individual resistance traits

Honey bee breeding will have to be adapted to take advantage of these novel developments in molecular tools. Artificial insemination will

continue to play an important role in bee breeding, and will be aided by the ability to pre-screen potential drones and queens with genetic tests prior to insemination. In general, the breeding procedure will depend on the sex of the target individuals. If traits in females are to be tested the procedure is much more elaborate than when haploid drones can be used.

4.1. Using drones for selection

Although honey bees live in colonies, which greatly complicates selection and breeding of disease resistant stock, they provide at the same time the genetically simplest organism for selection: haploid drones. Honey bee breeding has never used this fundamental advantage for selection programmes. The reason is obvious: drones do not engage in any particular tasks whose enhancements may be desirable colony traits. They do not forage for nectar, do not pollinate, do not guard, do not built combs and in the beekeepers eye they do not seem to do anything useful but mating. However, this argument may fall short as soon as it comes to resistance to diseases and to viral diseases in particular. Viruses require individual organisms for replication and clearly if all individuals in a colony were resistant, the entire colony would be resistant. Virus loads are typically determined in workers, however, drones also can be attacked by viruses and they therefore are a prime tool for selection for disease resistance. For example the black queen cell virus was recently detected in drone brood (Siede and Büchler, 2003) causing severe larval damage and death of infected individuals. Bee breeders selecting for disease resistance should therefore not only look at worker or queen phenotypes but also include drones into their consideration. For viruses associated with *V. destructor* infestation, drones may even be the most important hosts (Fuchs 1990), whose rates of parasitism dictate the course of infection within a colony and, ultimately, colony death. Screening for virus resistance in drones can follow the same procedure as those developed for workers (see Chapter 1). Infections of animals with virus particles can be done in the larval, pupal or adult stage. Because the males are haploid, gene expression is not blurred by allelic interactions and the genetic value of a drone should be very closely linked to its phenotype.

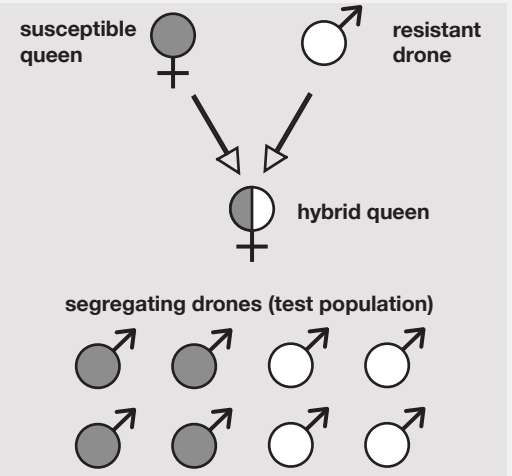
The breeding procedure is straight forward, once a resistant or susceptible strain has been identified. At first the resistance or the susceptibility has to be reconfirmed for each specific strain by testing individual drones with an appropriate bioassay for the specific virus (e.g. inoculation of individual drone larvae reared under controlled conditions on an artificial diet in incubator). Once the drone phenotypes confirm the colonial phenotype, hybrid queens must be produced which carry both resistance

and susceptibility alleles. This is achieved by rearing offspring queens produced by a queen from the low line which has been inseminated with semen of a single drone from the high resistance strain or vice versa. These hybrid queens are introduced into a strong colony but remain unmated and are induced to lay unfertilised eggs (with a CO_2 treatment) so that they exclusively produce drone offspring (Box 2). Drone brood offspring can then be individually tested for resistance.

In addition to the tests on individual drones, one will also need to perform classical colony evaluation, to make sure that resistance of the individuals correlates with resistance at the colony level. Using this procedure, genome regions, which are linked to susceptibility or resistance, can be initially screened with microsatellite markers using "bulk segregant analysis" (Michelmore *et al.*, 1991) where individual drones of either phenotype are pooled to search for cosegregating loci (Lattorff *et al.*, 2006). These roughly mapped regions can be located in the honey bee genome sequence defining the range in which the putative QTL is located. In a next step new microsatellite markers can be developed for fine scale mapping in these regions by just taking the information from the genome sequence. A mapping population of 200 drones can be sufficient to identify a single open reading frame (Solignac *et al.*, 2004) representing the one gene in question. Using the sequence information of this gene one can then screen for Single Nucleotide Polymorphisms (SNPs) characterizing the resistant

Box 2. Breeding scheme for producing the test drones.

A hybrid queen is reared from offspring produced by a queen of a susceptible line (grey), which was inseminated by a drone from a resistant strain (white). These hybrid queens are experimentally induced to produce only drone offspring (CO_2-treatment) and large numbers of drones (several hundreds) can be used for mapping and testing purposes.



and susceptible alleles. This will provide the final tool to allow for specific target gene selection. The advantage of SNPs over MAS is obvious: the gene itself is the marker and recombination cannot interfere with the identification of the genotype.

4.2. Using females for selection

When females are used, the breeding scheme becomes substantially more elaborate. Again hybrid queens need to be produced but these now need to be back crossed with one drone each from the high and low line. The double cross is necessary because we do not know in advance which allele will be dominant. The queen's female offspring will thus be sired by either father drone. The first step is to determine which father carries the dominant allele, as reflected by constancy in the phenotypes of workers from that patriline. Next, segregation in the offspring of the other father can generate information on the impact of the maternal alleles on the offspring phenotype. QTL analyses software (e.g. QTL cartographer, Wang *et al.*, 2005) can now be used to search for major loci controlling the trait in question. In case of a single locus, Box 3 shows the expected segregation pattern.

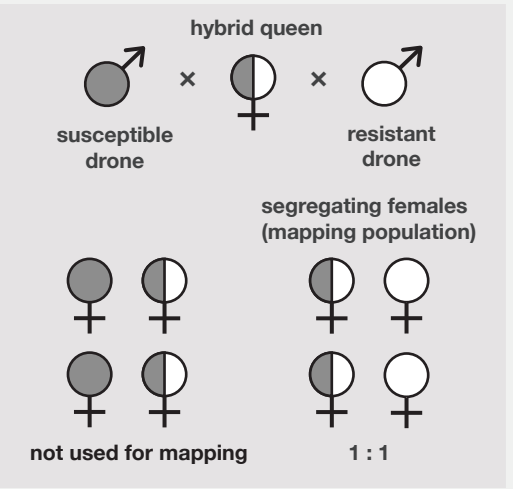
4.3. Colony traits?

One should be aware that both approaches, selection on drones and selection on workers, leave out complex resistance mechanisms at the

Box 3. Breeding scheme for producing the test females.

A hybrid queen is mated to both a drone from the high and the low line.

The female offspring produced by these queens can be used for mapping and testing purposes.



colony level, where many workers interact to perform collective tasks facilitating disease resistance. Such colony characteristics have been shown to be extremely important for disease prevention and resistance. For example, hygienic behaviour of workers is a classical composite task, where various workers uncap brood cells and a set of other workers remove the diseased brood from the cell (Spivak, 2001). However, although some of these behaviours appear to have a genetic background, it is highly unlikely that it will be possible to tackle such composite colony traits in the near future at the functional genomic level. Not only are there interactions between alleles in the genome, but also we need to consider interactions among workers, between workers and brood, and reactions of the entire colony towards external environmental variability (Moritz, 1986). Although the prominent example on pollen foraging showed that this approach can be feasible (Page *et al.*, 2000), this may remain an exception. Indeed, pollen handling is perhaps a more individualistic task: The same worker which collects the pollen in the field is also the one which deposits the pollen in the cell in the colony. This contrasts with composite tasks such as nectar foraging, where the nectar forager transfers its crop load to hive bees, which then further distribute the nectar in the colony. These composite tasks are typical for many colonial traits. If colonial honey bee disease resistance mechanisms are based on such complex interactions among workers, then they are not easy to be dealt with at the functional genomics level. We may need to await yet another methodological jump, in order to embark on developing selection schemes for disease resistance mechanisms that operate at the colony level. Again, there is a good likelihood that such strategies will be aided by the haplodiploid genetic system of bees.

5. Genomic tools

5.1. Gene expression studies

At any given time, only a fraction of the genes in a genome are active (expressed). An understanding of changes in gene expression in response to new circumstances, such as infection with a virus, can provide strong inference for the functional roles of genes. Gene expression studies can be carried out in massively parallel arrays (microarrays) consisting of thousands of candidate genes tested against a single life stage or tissue from honey bees. Microarrays tend to be more economical than single-gene studies. They also allow for numerous internal controls against the activity levels of genes, improving the chances of capturing genes whose

activity is dependent on a specific circumstance. The genes placed onto microarrays are generally sequenced products derived from cDNA libraries (Expressed Sequence Tags, or EST's). While EST microarrays for the brain are available (~3000 genes, Whitfield *et al.*, 2002), existing microarray studies have focused on fundamental issues related to caste determination (Evans and Wheeler, 2001) and division of labour (Whitfield *et al.*, 2003) rather than disease resistance. Gene expression studies are currently not employed to identify the function of novel honey bee specific genes but concentrate on comparisons with known genes from the *Drosophila* genome sequence. One fortunate offshoot of the honey bee genome project will be the production and dissemination of microarrays that include all of the confirmed and predicted genes in the bee genome (> 15 000). It will also be possible to design such arrays that are tailored to genes implicated in specific traits of interest, including disease responsiveness. Studies based on these arrays will offer excellent opportunities to improve breeding and management of bees with respect to viral disease. Along with providing information on differing responses of bees from different stocks and life stages toward viruses, array studies can directly assess how viruses impact bee health. More generally, array studies can be used to study bee nutrition, queen production, worker and queen longevity, and tolerance toward both intended and incidental exposure to chemicals. The identification and verification of genes whose products are indicators of bee health can be used to better understand the diversity of challenges facing bee colony members. Along with uses for improving bee breeding, this information can clarify which practices in beekeeping are truly beneficial, and which could be changed in order to improve bee health.

5.2. Comparative genomics

In order to be useful for biological inferences, newly identified genes in honey bees will have to be placed into robust molecular-genetic pathways and/or grouped on the basis of common biological processes, including the immune response. A secure prediction of genetic function will depend on years of experimental verification using RNAi and breeding studies. Nevertheless, function of many genes in the bee genome can be inferred on the basis of sequence homology to genes found in other organisms. Therefore, comparative approaches on immune genes between bees and better-studied organisms such as *Drosophila melanogaster* and the malaria mosquito *Anopheles gambiae* will be especially important for predicting which honey bee genes might be involved with disease. Knowledge of antiviral responses in *Drosophila* has advanced rapidly in the past year (Dostert *et al.*, 2005; Ip, 2005), providing a baseline for understanding

viral responses in bees. Resources to make such comparisons are evolving in parallel with the arrival of honey bee genome resources and are listed below. Bees, in turn, are likely to inform genetic studies in other organisms. This is perhaps especially true for genes involved with disease resistance and tolerance. Thanks to a wealth of parasites and pathogens, alongside numerous studies of the impacts of bee disease, honey bees will likely prove to be an excellent model for genetic mechanisms involved in disease responses to bacteria, viruses, fungi, and other widespread pathogens. For bee viruses, a better picture is emerging of both pathologies and distribution (e.g., Chen *et al.*, 2005) and of possible immune responses to viruses or their arthropod vectors (Gregory *et al.*, 2005; Yang and Cox-Foster, 2005). This picture will only become more clear with the newly discovered immunity candidates present in the bee genome, and with the breeding and screening techniques described here.

The annotation of the honey bee genome sequence has revealed many expected components of insect immune systems as well as a number of surprises. Bees seem to have more or less intact immune pathways when compared to the well-studied fruit fly (Royet *et al.*, 2005; Tanji and Ip 2005; see Chapter 7). Nevertheless, there remain strong differences between bees and flies with respect to predicted immune genes. Interestingly, it appears that most immune-gene families have fewer members in honey bees when compared to fruit flies and mosquitoes. With respect to these 'missing' genes, honey bees seem to have a lower diversity of proteins used to both recognize pathogens and, in the end, to attack pathogens directly. For example, bees have only four proteins in a group linked to recognition of gram-positive bacteria and fungi (PGRP's, Choe *et al.*, 2002; Gottar *et al.* 2002), while fruit flies and mosquitoes have 12 and 14 members of this group respectively. In terms of immune end products, the genome sequence has uncovered only one additional antimicrobial peptide in bees, leaving a total of six, sharply lower than the 20+ such proteins in flies (Christophides *et al.*, 2004). If the low numbers of bee immune players hold up, this will somewhat simplify efforts to validate these genes and, ultimately, incorporate desired alleles at these genes in to breeding population. In contrast, should bees have a completely novel immune response or components, this will require some years of research to correctly identify and validate members involved in fighting off disease.

Some proteins identified in honey bees do appear to play roles in fighting disease. The honey bee peptide abaecin shows antibacterial properties (Casteels *et al.*, 1990) and levels of the gene encoding this peptide are correlated with susceptibility at the colony level toward an important bacterial pathogen, *Paenibacillus larvae,* cause of American foulbrood

(Evans and Pettis, 2005). Another antimicrobial peptide from bees, apidaecin, is produced as a series of apparently interchangeable parts ('cassettes') that may enhance the abilities of bees to dramatically upregulate their immune response (Casteels *et al.*, 1989; Casteels-Josson *et al.*, 1994) or, more interestingly, could allow bees to tailor a response more precisely to specific pathogens. This discovery preceded recent work showing an unexpected complexity in the immune responses of a diverse set of invertebrates. Invertebrates now appear to have both finely tuned immune systems as well as plausible mechanisms for amplifying and 'learning' an immune response to specific pathogens (Schmid-Hempel, 2005; Watson *et al.*, 2005). In this way, insect immunity looks even more analogous to that shown by humans and other vertebrates.

Searches for genes important for honey bee disease will be aided by the fact that there are ongoing genome projects in a total of 16 insect species from five orders. Given this diversity, it will be possible to predict novel immune players shared by bees and other insects besides the ubiquitous fruit fly. In the end, there will be integrated databases aimed at connecting honey bee information with data from other insects and the metazoa more generally. Similar databases are needed for genome sequences and functional information from many of the important bee viruses, as well as for ongoing genome projects involving pathogens such as *Paenibacillus larvae* and *Ascosphaera apis*.

5.3. Future directions in honey bee genomics – bee management

So where do we go from here ? The density of bee colonies in many European countries is currently suspected to be far below natural levels in spite of beekeeping (Moritz *et al.*, 2006). The destruction of natural habitats and landscapes has been identified as the main reason for the bee decline in general (Kevan and Phillips, 2001) but clearly the increasing impact of colony losses due to honey bee diseases also contributes to the dramatic decline. Moreover, there is an alarming loss of apiaries, where beekeepers close their operations because they do not want to regularly treat their colonies with acaricides, antibiotics and other chemicals just to keep their colonies alive. Last but not least, honey contaminations with chemical residues destroy consumer trust and markets, causing a further decline of beekeeping. Therefore, much is at stake, and the consequences of obtaining disease resistant honey bees are reaching far beyond the beekeeper community and cut deep into environmental and societal issues. At the same time there is valid optimism that new techniques and insights from genomics and molecular biology will fit well into standard breeding schemes as a way to improve bee health. Microarrays and other emerging

technologies can be designed with an aim towards honey bee selection, truly revolutionizing selection and breeding of disease resistant honey bees. We are clearly at the brink of a new age in bee breeding, which is dearly needed to cope with the urgent demands laid out in apiculture.

5.4. Internet resources

Genomic information is enticing and there are many sites now, established from within and outside the bee research community, which are presenting genome information in a unique and helpful way. These sites are public, free, and those maintaining them are open to suggestions for how to improve their offerings. Here are a few that promise to provide helpful bee genomic information for the long term.

- *Baylor College of Medicine* http://www.hgsc.bcm.tmc.edu/projects/
- *Honey Bee Genome Resource Guide* http://www.ncbi.nlm.nih.gov/genome/guide/bee/
- *BeeBase*: http://racerx00.tamu.edu/bee_resources.html
- *ENSEMBL-Bee:* : http://www.ensembl.org/Apis_mellifera/
- *Beespace*: http://www.igb.uiuc.edu/beespace/
- *QTL cartographer*: http://statgen.ncsu.edu/qtlcart/WQTLCart.htm

References

Adams M.D., Celniker S.E., Holt R.A., Evans C.A., Gocayne J.D., Amanatides P.G., Scherer S.E., Li P.W., Hoskins R.A., Galle R.F., George R.A., Lewis S.E., Richards S., Ashburner M., Henderson S.N., Sutton G.G., Wortman J.R., Yandell M.D., Zhang Q., Chen L.X., Brandon R.C., Rogers Y.H.C., Blazej R.G., Champe M., Pfeiffer B.D., Wan K.H., Doyle C., Baxter E.G., Helt G., Nelson C.R., Miklos G.L.G., Abril J.F., Agbayani A., An H.J., Andrews-Pfannkoch C., Baldwin D., Ballew R.M., Basu A., Baxendale J., Bayraktaroglu L., Beasley E.M., Beeson K.Y., Benos P.V., Berman B.P., Bhandari D., Bolshakov S., Borkova D., Botchan M.R., Bouck J., Brokstein P., Brottier P., Burtis K.C., Busam D.A., Butler H., Cadieu E., Center A., Chandra I., Cherry J.M., Cawley S., Dahlke C., Davenport L.B., Davies A., de Pablos B., Delcher A., Deng Z.M., Mays A.D., Dew I., Dietz S.M., Dodson K., Doup L.E., Downes M., Dugan-Rocha S., Dunkov B.C., Dunn P., Durbin K.J., Evangelista C.C., Ferraz C., Ferriera S., Fleischmann W., Fosler C., Gabrielian A.E., Garg N.S., Gelbart W.M., Glasser K., Glodek A., Gong F.C., Gorrell J.H., Gu Z.P., Guan P., Harris M., Harris N.L., Harvey D., Heiman T.J., Hernandez J.R., Houck J., Hostin D., Houston D.A., Howland T.J., Wei M.H., Ibegwam C., Jalali M., Kalush F., Karpen G.H., Ke Z.X., Kennison J.A., Ketchum K.A., Kimmel B.E., Kodira C.D., Kraft

C., Kravitz S., Kulp D., Lai Z.W., Lasko P., Lei Y.D., Levitsky A.A., Li J.Y., Li Z.Y., Liang Y., Lin X.Y., Liu X.J., Mattei B., McIntosh T.C., McLeod M.P., McPherson D., Merkulov G., Milshina N.V., Mobarry C., Morris J., Moshrefi A., Mount S.M., Moy M., Murphy B., Murphy L., Muzny D.M., Nelson D.L., Nelson D.R., Nelson K.A., Nixon K., Nusskern D.R., Pacleb J.M., Palazzolo M., Pittman G.S., Pan S., Pollard J., Puri V., Reese M.G., Reinert K., Remington K., Saunders R.D.C., Scheeler F., Shen H., Shue B.C., Siden-Kiamos I., Simpson M., Skupski M.P., Smith T., Spier E., Spradling A.C., Stapleton M., Strong R., Sun E., Svirskas R., Tector C., Turner R., Venter E., Wang A.H.H., Wang X., Wang Z.Y., Wassarman D.A., Weinstock G.M., Weissenbach J., Williams S.M., Woodage T., Worley K.C., Wu D., Yang S., Yao Q.A., Ye J., Yeh R.F., Zaveri J.S., Zhan M., Zhang G.G., Zhao Q., Zheng L.S., Zheng X.Q.H., Zhong F.N., Zhong W.Y., Zhou X.J., Zhu S.P., Zhu X.H., Smith H.O., Gibbs R.A., Myers E.W., Rubin G.M., Venter J.C. (2000) The genome sequence of *Drosophila melanogaster*. Science 287, 2185-2195.

Amdam G.V., Simões Z.L., Guidugli K.R., Norberg K., Omholt S.W. (2003) Disruption of vitellogenin gene function in adult honeybees by intra-abdominal injection of double-stranded RNA. BMC Biotech. 3, 1.

Arechavaleta-Velasco M.E., Hunt G.J. (2004) Binary trait loci that influence honey bee (Hymenoptera: Apidae) guarding behaviour. Ann. Entomol. Soc. Amer. 97, 177-183.

Aronstein K., Saldivar E. (2005) Characterization of a honey bee Toll related receptor gene Am18w and its potential involvement in antimicrobial immune defense. Apidologie 36, 3-14.

Beye M., Härtel S., Hagen A., Hasselmann M., Omholt S.W. (2002) Specific developmental gene silencing in the honey bee using a homeobox motif. Insect. Mol. Biol. 11, 527-532.

Beye M., Hasselmann M., Fondrk M.K., Page R.E., Omholt S.W. (2003) The gene *csd* is the primary signal for sexual development in the honeybee and encodes an SR-type protein. Cell 114, 419-429.

Beye M., Hunt G.J., Page R.E., Fondrk M.K., Grohmann L., Moritz R.F.A. (1999) Unusually high recombination rate detected in the sex locus region of the honey bee (*Apis mellifera*). Genetics 153, 1701-1708.

Beye M., Moritz R.F.A., Crozier R.H., Crozier Y.C. (1996) Mapping the sex locus of the honeybee (*Apis mellifera*). Naturwissenschaften 83, 424-426.

Bienefeld K., Pirchner F. (1991) Genetic correlations among several colony characters in the honey-bee (Hymenoptera, Apidae) taking queen and worker effects into account. Ann. Entomol. Soc. Amer. 84, 324-331.

Boecking O., Bienefeld K., Drescher W. (2000) Heritability of the *Varroa*-specific hygienic behaviour in honey bees (Hymenoptera: Apidae). J. Anim. Breed. Genet. 117, 417-424.

Büchler R. (2000) Design and success of a German breeding program for *Varroa* tolerance. Am Bee J. 140, 662-665.

Casteels P., Ampe C., Jacobs F., Vaeck M., Tempst P. (1989) Apidaecins: Antibacterial peptides from honeybees. EMBO Journal 8, 2387-2391.

Casteels P., Ampe C., Riviere L., Van Damme J., Ellicone C., Fleming M., Jacobs F., Tempst P. (1990) Isolation and characterization of abaecin, a major antibacterial response peptide in the honeybee (*Apis mellifera*). Europ. J. Biochem. 187, 381-386.

Casteels-Josson K., Zhang W., Capaci T., Casteels P., Tempst P. (1994) Acute transcriptional response of the honeybee peptide-antibiotics gene repertoire and required post-translational conversion of the precursor structures. J Biol. Chem. 269, 28569-28575.

Chandra S.B., Hunt G.J., Cobey S., Smith B.H. (2001) Quantitative trait loci associated with reversal learning and latent inhibition in honeybees (*Apis mellifera*). Behav. Genet. 31, 275-285.

Chen Y.W., Wang C.H., An J., Ho K.K. (2000) Susceptibility of the Asian honeybee, *Apis cerana*, to American foulbrood, *Paenibacillus larvae larvae*. J. Apic. Res. 39, 169-175.

Chen, Y.P., Feldlaufer, M.F., Higgins, J.A. (2005) Quantitative real-time reverse transcription-PCR analysis of deformed wing virus infection in the honeybee (*Apis mellifera* L.). Appl. Environm. Microbiol. 71, 436-441.

Choe K.M., Werner T., Stöven S., Hultmark D., Anderson K.V. (2002) Requirement for a peptidoglycan recognition protein (PGRP) in relish activation and antibacterial immune responses in *Drosophila*. Science 296, 359-362.

Christophides G.K., Vlachou D., Kafatos F.C. (2004) Comparative and functional genomics of the innate immune system in the malaria vector *Anopheles gambiae*. Immunol. Rev. 198, 127-148.

Dimopoulos G. (2003) Insect immunity and its implication in mosquito-malaria interactions. Cell Microbiol. 5, 3-14.

Dostert C., Jouanguy E., Irving P., Troxler L., Galiana-Arnoux D., Hetru C., Hoffmann J.A., Imler J.L. (2005) The Jak-STAT signaling pathway is required but not sufficient for the antiviral response of drosophila. Nature Immunol. 6, 946-953.

Evans J.D., Wheeler D.E. (2001) Expression profiles during honeybee caste determination. Genome Biol. 2, research0001.1-0001.6.

Evans J.D. (2004) Transcriptional immune responses by honey bee larvae during invasion by the bacterial pathogen, *Paenibacillus larvae*. J. Invert. Pathol. 85, 105-111.

Evans J.D., Pettis J.S. (2005) Colony-level effects of immune responsiveness in honey bees, *Apis mellifera*. Evolution 59, 2270-2274.

Farooqui T., Smith B.H., Vaessin H. (2004) Octopamine receptors in the honeybee (*Apis mellifera*) brain and their disruption by RNA-mediated interference. J. Insect Physiol. 50, 701-713.

Fire A., Xu S., Montgomery M., Kostas S., Driver S., Mello C. (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 391, 806-811.

Friedman A., Perrimon N. (2004) Genome-wide high-throughput screens in functional genomics. Curr. Opin. Genet. Dev. 14, 470-476.

Fries I., Hansen H., Imdorf A., Rosenkranz P. (2003) Swarming in honey bees (*Apis mellifera*) and Varroa mite (*Varroa destructor*) population development in Sweden. Apidologie 34, 1-9.

Fries I., Imdorf A., Rosenkranz P. (2005) Survival of mite (*Varroa destructor*) infested honey bee (*Apis mellifera*) colonies in a Nordic climate. Apidologie, in prep.

Fuchs S. (1990) Preferences for drone brood cells by *Varroa jacobsoni* in colonies of *Apis mellifera carnica*. Apidologie 21, 193-199.

Fuchs S., Moritz R.F.A. (1999) Evolution of extreme polyandry in the honeybee *Apis mellifera* L. Behav. Ecol. Sociobiol. 45, 269-275.

Gadau J., Page R.E., Werren J.H., Schmid-Hempel P. (2000) Genome organization and social evolution in Hymenoptera. Naturwissenschaften 87, 87-89.

Gatehouse H.S., Gatehouse L.N., Hodges S., Malone L.A., Tregidga E., Todd J. (2004) Amylase activity in honey bee hypopharyngeal glands reduced by RNA interference. J. Apic. Res. 43, 9-13.

Gottar M., Gobert V., Michel T., Belvin M., Duyk G., Hoffmann J.A., Ferrandon D., Royet J. (2002) The *Drosophila* immune response against Gram-negative bacteria is mediated by a peptidoglycan recognition protein. Nature, 416, 640-644.

Gregory P.G., Evans J.D., Rinderer T., and De Guzman L. (2005) Conditional immune-gene suppression of honeybees parasitized by Varroa mites. J. Insect Sci. **5,** 1-5.

Guidugli, K.R., Barchuk, A.R., Nascimento, A.M., Simões, Z.L.P., Amdam, G.V., Omholt, S., Hartfelder, K. (2005) Vitellogenin regulates hormonal dynamics in the worker caste of a eusocial insect. FEBS Lett. 579, 4961-4965.

Guzman-Novoa E., Hunt G.J., Uribe J.L., Smith C., Arechavaleta-Velasco M.E. (2002) Confirmation of QTL effects and evidence of genetic dominance of honeybee defensive behavior: Results of colony and individual behavioral assays. Behav. Genet. 32, 95-102.

Harbo J.R., Harris J.W. (2005) Suppressed mite reproduction explained by the behaviour of adult bees. J. Apic. Res. 44, 21-23.

Harris J., Rinderer T., Kuznetsov V., Danka R., Delatte G., De Guzman L., Villa J. (2002) Imported Russian honey bees: Quarantine and initial selection for Varroa resistance. Am. Bee J. 142, 591-596.

Honey Bee Genome Sequencing Consortium (2006) Complete genome sequence and annotation for a social insect, the honey bee *Apis mellifera*. Nature, in press.

Hultmark D. (2003) *Drosophila* immunity: paths and patterns. Curr. Opin. Immunol. 15, 12-19.

Humphries M.A., Fondrk M.K., Page R.E. (2005) Locomotion and the pollen hoarding behavioral syndrome of the honeybee (*Apis mellifera* L.). J. Comp. Physiol. A 191, 669-674.

Hunt G.J., Guzmán-Novoa E., Fondrk M.K., Page R.E. (1998) Quantitative trait loci for honey bee stinging behavior and body size. Genetics 148, 1203-1213.

Hunt G.J., Page R.E., Fondrk M.K., Dullum C.J. (1995) Major quantitative trait loci affecting bee foraging behavior. Genetics 141, 1203-1213.

Imler J.L., Hoffmann J. (2003) Toll signalling: the TIReless quest for specify. Nature Immunol. 4, 7-8.

Ip Y.T. (2005) Drosophila innate immunity goes viral. Nature Immunol. 6, 863-864.

Janscha A. (1771) Abhandlungen vom Schwärmen der Bienen. Joseph Kurzböck, Vienna.

Kevan P.G., Phillips T.P. (2001) The economic impacts of pollinator declines: an approach to assessing the consequences. Conserv. Ecol. 5, 8.

Kulinčević J.M., Rinderer T.E., MladjanV.J., Buco S.M. (1992) Five years of bidirectional genetic selection for honey-bees resistant and susceptible to *Varroa jacobsoni*. Apidologie 23, 443-452.

Laidlaw H.H.J., Page R.E. (1997) Queen rearing and bee breeding. Wicwas Press, Cheshire, CT.

Lande R., Thomson R. (1990) Efficiency of marker-assisted selection in the improvement of quantitative traits. Genetics 124, 743-756.

Lapidge K.L., Oldroyd B.P., Spivak M. (2002) Seven suggestive quantitative trait loci influence hygienic behavior of honey bees. Naturwissenschaften 89, 565-568.

Lattorff H.M., Moritz R.F.A., Fuchs S. (2004) A single locus determines thelytokous parthenogenesis of laying honeybee workers (*Apis mellifera capensis).* Heredity 94, 533-537.

Lattorff H.M.G., Moritz R.F.A., Solignac M. (2006) Fine mapping of the pleiotropic *thelytoky* gene in the honeybee. Genome, in prep.

Levashina, E.A. (2004) Immune responses in *Anopheles gambiae*. Insect. Biochem. Mol. Biol. 34, 673-678.

Lobo N.F., Hill C.A., Romero-Severson J., Hunt G.J., Collins F.H., Ton L.Q., Emore C. (2003) Genomic analysis in the sting-2 quantitative trait locus for defensive behavior in the honey bee, *Apis mellifera*. Genome Res. 13, 2588-2593.

Lodesani M., Crailsheim K., Moritz R.F.A. (2002) Effect of some characters on the population growth of mite *Varroa jacobsoni* in *Apis mellifera* L colonies and results of a bi-directional selection. J. Appl. Entomol. 126, 130-137.

Mackensen O. (1947) Effect of carbon dioxide on initial oviposition of artificially inseminated and virgin queen bees. J. Econ. Entomol. 40, 344-349.

Merriam J., Ashburner M., Hartl D.L., Kafatos F.C. (1991) Toward cloning and mapping the genome of *Drosophila*. Science 254, 221-225.

Michelmore R.W., Paran I., Kesseli R.V. (1991) Identification of markers linked to disease-resistance genes by bulked segregant analysis: A rapid method to detect markers in specific genomic regions by using segregating populations. Proc. Natl. Acad. Sci. USA 88, 9828-9832.

Moritz R.F.A. (1986) Estimating the genetic variance of group characters: Social behavior of honeybees (*Apis mellifera*). Theor. Appl. Genet. 72, 513-517.

Moritz R.F.A. (1988) A re-evaluation of the two-locus model for hygienic behavior in honeybees *Apis mellifera* L. J. Heredity 79, 257-262.

Moritz R.F.A., Kühnert M. (1984) Seasonal effects on artificial insemination of honeybee queens, *Apis mellifera*. Apidologie 15, 223-231.

Moritz R.F.A., Southwick E.E. (1987) Phenotype interactions in group behavior of honey bee workers *Apis mellifera* L. Behav. Ecol. Sociobiol. 21, 53-58.

Moritz R.F.A., Haertel S., Neumann P. (2005) Global invasions of the western honeybee (*Apis mellifera* L.) and the consequences for biodiversity. Ecosci. 12, 289-301.

Moritz R.F.A., Kraus B., Kryger P., Crewe R.M. (2006) The size of wild honeybee populations (*Apis mellifera scutellata*) in Africa: implications for the conservation of honeybees in Europe. Conserv. Genet., in prep.

Neumann P., Moritz R.F.A., Van Praagh J.P., Dustmann J.H. (1999) Testing reliability of a potential island mating apiary using DNA microsatellites. Apidologie 30, 257-276.

Nunes F.M.F., Valente V., Sousa J.F., Cunha M.A.V., Pinheiro D.G., Maia R.M., Araujo D.D., Costa M.C.R., Martins W.K., Carvalho A.F., Monesi N., Nascimento A.M., Peixoto P.M.V., Silva M.F.R., Ramos R.G.P., Reis L.F.L., Dias-Neto E., Souza S.J., Simpson A.J.G., Zago M.A., Soares A.E.E., Bitondi M.M.G., Espreafico E.M., Espindola F.S., Paco-Larson M.L., Simoes Z.L.P., Hartfelder K., Silva W.A. (2004) The use of open reading frame ESTs (ORESTES) for analysis of the honey bee transcriptome. BMC Genomics 5, 84.

Page R.E., Fondrk M.K., Hunt G.J., Guzman-Novoa E., Humphries M.A., Nguyen K., Greene A.S. (2000) Genetic dissection of honeybee foraging behavior. J. Heredity 91, 474-479.

Robinson K.O., Ferguson H.J., Cobey S., Vaessin H., Smith B.H. (2000) Sperm-mediated transformation of the honey bee, *Apis mellifera*. Insect Mol. Biol. 9, 625-634.

Roignant J.Y., Carre C., Mugat B., Szymczak D., Lepesant J.A., Antoniewski C. (2003) Absence of transitive and systemic pathways allows cell-specific and isoform-specific RNAi in Drosophila. RNA 9, 299-308.

Rothenbuhler W.C. (1964) Behavior genetics of nest cleaning in honey bees. IV. Responses of F1 and backcross generations to disease-killed brood. Amer. Zool. 4, 111-123.

Royet J., Reichhart J.M., Hoffmann J.A. (2005) Sensing and signalling during infection in *Drosophila*. Curr. Opin. Immunol. 17, 11-17.

Rüppel O., Pankiw T., Nielsen D.I., Beye M., Page R.E. (2004a) The genetic architecture of the behavioural ontogeny of foraging in honeybee workers. Genetics 167, 1767-1779.

Rüppel O., Pankiw T., Page R.E. (2004b) The genetic architecture of honeybee foraging behavior. J. Hered. 95, 481-491.

Schmid-Hempel P. (2005) Evolutionary ecology of insect immune defenses. Ann. Rev. Entomol. 50, 529-551.

Siede R., Büchler R. (2003) Symptomatic Black Queen Cell Virus Infection of drone brood on Hessian bee yards. Berlin. München. Tierärtzl. Wochenschr. 116, 130-133.

Solignac M., Vautrin D., Baudry E., Mougel F., Loiseau A., Cornuet J.M. (2004) A microsatellite-based genetic linkage map of the honeybee, *Apis mellifera* L. Genetics 167, 253-262.

Spivak M. (2001) Mechanisms of American Foulbrood resistance and current research on hygienic behaviour, in Moritz R.F.A. (Ed.) Molecular Mechanisms of Disease Tolerance in Honeybees, BRI Dol, Czech Republic, pp. 155-174.

Tanji T., Ip Y.T. (2005) Regulators of the Toll and Imd pathways in the *Drosophila* innate immune response. Trends Immunol 26, 193-198.

Wang S., Basten C.J., Zeng Z.B. (2005) Windows QTL Cartographer 2.5. Dept. Statistics, North Carolina State University, Raleigh, NC. (http://statgen.ncsu.edu/qtlcart/WQTLCart.htm).

Watson F.L., Puttmann-Holgado R., Thomas F., Lamar D.L., Hughes M., Kondo M., Rebel V.I., Schmucker D. (2005) Extensive diversity of Ig-superfamily proteins in the immune system of insects. Science 309, 1874-1878.

Whitfield C.W., Band M.R., Bonaldo M.F., Kumar C.G., Liu L., Pardinas J.R., Robertson H.M., Soares M.B., Robinson G.E. (2002) Annotated expressed sequence tags and cDNA microarrays for studies of brain and behaviour in the honey bee. Genome Res. 12, 555-566.

Whitfield C.W., Cziko A.M., Robinson G.E. (2003) Gene expression profiles in the brain predict behavior in individual honey bees. Science 302, 296-299.

Winston M.L. (1987) The Biology of the Honey Bee. Harvard University Press, Cambridge MA, 281 pp.

Yang X., Cox-Foster D.L. (2005) Impact of an ectoparasite on the immunity and pathology of an invertebrate: Evidence for host immunosuppression and viral amplification. Proc. Nat. Acad. Sci. USA 102, 7470-7475.

Chapter 9

OVERVIEW OF THE REGULATORY FRAMEWORK FOR APICULTURE

Michael BROWN

Table of Contents

1. Introduction

A myriad of rules, laws and regulations impinge on all of our lives all of the time, thousands upon thousands of them, at both local to international level that for the most part we are only vaguely familiar. There has to be a law somewhere for just about anything, from those that are more frivolous relating to cosmetic perfection of fruit or the definition of chocolate and the acceptable shapes of bananas to the more serious such as speed limits and road safety, emissions, anti pollution and environmental protection laws, general law and order, climate change and the definitions and compositions of food products.

Apiculture has a diverse set of laws and regulations that govern its activities. As the risks and threats to apiculture increase through globalisation it has become ever more important to have appropriate and sensible regulations in place, with rolling supporting research and development programmes, inspection and extension services in place to assist with protecting apiculture from new pests and diseases and the measures in place to ensure safe food production for the customers and consumers of honey and hive products. It is equally important for beekeepers to be aware of them, try to keep up with new legislation and respect them to prevent or reduce the chances of anything going seriously wrong.

2. Drivers for regulation

The legislation governing apiculture is wide ranging and as well as covering the obvious aspects such as control of bee pests and diseases also deals with quality, hive product composition, origin, authenticity, traceability, food hygiene, food safety, labelling and so forth (Brown, 2004; EUROPA, 2005; FSA, 2005; Jukes, 2005; Martin, 2003; TRACE, 2005). The principal drivers for regulation of apiculture around the world are Animal Health (protection of honey bees and the environment where the main beneficiary is the beekeeper) and Food Safety (protection of the consumer who is main beneficiary). There can be a conflict of interest here with particularly with the beekeeper's emphasis on production and disease control to make a living tackling an increasing range of pests and diseases with an ever decreasing arsenal of products at his disposal and on the other hand the consumer whose prime concern is the quality, nutritional value and authenticy of the food. The use of veterinary medicines can leave residues, and honey has recently been in the spotlight where its quality image has been tarnished by the finding of significant residues of veterinary medicines requiring the identified honey removed under statutory product recall from shop shelves

(FSA, 2005). It is essential to retain the quality image and reputation of the market and the confidence of the consumer to maintain profitability.

2.1. Biodiversity and alien species

A third driver is biodiversity, which may not at first glance seem relevant to apiculture. Bio security is the exclusion, eradication or effective management of the risks posed by pests and disease to the economy, environment and human health (MAF, 2005). Many countries have surveillance and bio security programmes in place to provide the necessary data and appropriate and adequate levels of protection for the country from threats to ecosystems and biodiversity. Biological invasions are a widespread and significant component of human caused global environmental change. All over the world, animals and plants that evolved somewhere else are turning up where they are not wanted, having been transported by us in ships, trains, planes and automobiles, inadvertently or intentionally, and in a small percentage of cases causing irreparable damage to the environment and ecosystems so there could be enrichment or impoverishment (Davis, 2003; McGrath, 2005; Mooney *et al.*, 2005). Our appetite for the exotic can fuel this problem, by importing and using plants in gardens (there are over 50,000 exotic plants species in UK gardens (Baker *et al.*, 2005)) or purchasing animals through the pet stores and releasing them into the wild once they become too difficult or they become unwanted (Rixon *et al.*, 2005). This is why there are established populations of Burmese pythons in Florida (McGrath, 2005). The probability of importing new pests into geographical areas via commercial pathways has increased with the greater volume, speed and frequency of international trade (MacLeod *et al.*, 2002). Invasive species are considered the second most important threat to global biodiversity after habitat loss. It is been suggested by some scientists that these may be a leading cause of animal extinctions (Clavero and Garcia-Berthou, 2005). There is nothing new in this phenomenon (Elton, 1958), but what has changed is the accelerated scale of introductions through world trade with huge costs and environmental and human health consequences (GISP, 2005; Hulme, 2003; ISSG, 2005; Juliano and Lounibus, 2005; Mack and Lonsdale, 2001; Manchester and Bullock, 2000; Marris, 2005; McKinney and Lockwood, 1999; McGrath 2005; Mooney *et al.*, 2005; NISC, 2005; Pimentel et al., 2000; Pimentel *et al.*, 2001; Simberloff, 2004; Van Driesche and Van Driesche; 2000; Vitousek *et al.*, 1997). Increasing globalisation and the liberalisation of trade will inevitably spread more invasive pests and organisms including potentially dangerous disease causing micro organisms for human health (WHO, 2005).

The Convention on Biological Diversity (CBD, 2005; Fasham and

Trumper, 2001) established in 1992 placed obligations on members to prevent the introduction of, control or eradicate alien species which threaten ecosystems, habitats or species. The Bern Convention requires each contracting party to strictly control the introduction of non-native species.

The International Plant Protection Convention (IPPC) first signed in 1951 and revised and updated since then is designed to protect plant health (to secure common and effective action to prevent the spread and introduction of pests of plants and plant products). It has over 100 governments as Contracting Parties. It was extensively revised in 1997 to reflect the provision of the World Trade Organisation Sanitary and Phytosanitary Agreement (WTO SPS) including the requirement for Pest Risk Analysis, (see paragraph 5 below). In 2001 the IPPC term 'pest' was extended to include organisms that:

- Directly affect uncultivated/unmanaged plants with few commercial consequences e.g. Dutch elm disease
- Indirectly affect plants by other processes such as competition as weeds both in crops and uncultivated/unmanaged plants, for example, Japanese knotweed
- Indirectly affect plants through effects on other organisms that are beneficial to plants e.g. pollinators and detrivores like earthworms.

Non-native species policies and management practices to reduce the risks are becoming an integral part of government policy in the UK (Defra, 2003; Fasham and Trumper, 2001; Baker *et al.*, 2005). What this means therefore is that honey bee pests (affecting a major pollinator) are also included in international plant health legislation as well as Animal health legislation.

The relevance of invasive species and biodiversity for apiculture is clear. With increased movement of honey bees and the associated pests and diseases around the world the impact is a decline in health of the honey bee of the major available pollinator of cultivated crops and wild flora (Manchester and Bullock, 2000). The impact is perhaps not on the same scale of say the brown tree snake (*Boiga irregularis*) on the island of Guam which has wiped out nine of the 12 forest dwelling bird species and two of the eleven native lizards, zebra mussels (*Driessena polymorpha*) in North American waterways, the comb jellyfish (*Mnemiopsis leidyi*) introduced via ship's ballast water into the Blacksea, the Asian longhorn beetle (*Anoplophora glabripennis*) or the aggressive plant invaders (e.g. Japanese knotweed (*Fallopia japonica*), water hyacinth (*Eichhornia crassipes*) and Kudzu (*Pueraria montana var. lobata*) to name just a few of them (GISP, 2005; ISSG, 2005; NISC, 2005). An A-Z of the world's current most wanted

list of 100 can be consulted at leisure on the global invasive species internet website, and makes fascinating but disturbing reading (ISSG, 2005). An ironic twist is that some of these non-native plant species turn out to be useful for apiculture.

Undesirable bee subspecies (Africanised honey bees and the Cape bee) and bee pests like *Varroa destructor* and the small hive beetle (*Aethina tumida*) can be categorised as invasive. Africanised honey bees have spread up through South America into the USA since the 1950s, have displaced the European honey bees, are a public health hazard in certain circumstances and compete with native bees for available forage (Villanueva *et al.*, 2005). They also make the business of managing honey bee colonies extremely difficult because of their behavioural characteristics, notably the defensive behaviour. *Varroa destructor* is now a global pest, causing millions of dollars of costs and damage each year to apiculture (with the exception of Australia). It has caused huge losses of honey bee colonies throughout the world where it has established and continues to do so. The small hive beetle (*Aethina tumida*, also known as the SHB) was first confirmed outside its sub Saharan native range in Eastern United States in 1998 (first suspected in 1996). It is now present or has been recorded in over 30 US States demonstrating its potential to spread rapidly (Hood, 2004; Neumann and Elzen, 2004). The SHB is an "out of the blue" new pest and as well as the economic damage it has already caused the apiculture industry could affect biodiversity by attacking the nests of other species of bees in Europe and elsewhere, e.g. bumble bees, solitary bees or potentially Asian honey bees if transported to Asia. Experiments in the laboratory have shown that the beetle is capable of reproducing successfully in bumblebee nests (Stanghellini *et al.*, 2000).

All of this coupled with the potential impacts of climate change (making it easier for invasive species to establish) in certain geographical areas makes for a potentially worrying future indeed. As temperatures rise, areas of Europe suitable for the establishment of pests are predicted to increase (UKCIP, 2005).

3. Why protect bees?

The main justification for government intervention to protect honey bee health is the importance of managed bees as pollinators of agricultural and horticultural crops, as essential contributors to sustainable agriculture and biodiversity. Honey bees also play an important pollination role in respect of many wild species of flora, the value of which is difficult

to estimate but is certainly substantial (Temple *et al.*, 2001). Barclay and Moffett, (1984) estimated the contribution of honey bees to wild plants that were major food sources to wildlife in the US. The commercial value of honey and hive products although important is outstripped by the pollination contribution. In a number countries the fragmented nature of the apiculture sector, with large numbers of small scale beekeepers, a low number of commercial operators and as a consequence a failure to address bee health issues means that centralised management is still needed to maintain standards of enforcement and disease control, therefore in the interests of the wider community and the environment (Temple *et al.*, 2001).

The honey bee (*Apis mellifera*) plays a dominant role, being the major managed pollinator available to provide this service. Honey bees of course are not the only important pollinators (Corbet *et al.*, 1991; Kemp, 2000). Bombiculture as an industry has grown significantly in the last decade (Koppert, 2005; BIO-BEE, 2005; BIOBEST, 2005), and solitary bee production on a large scale is still in its in infancy (Allen, 2003; Bosch and Kemp, 2002). For both of these, there are limited or no trade regulations or pest and disease structures in place.

3.1. Contribution to society

The ecological service of pollination carried out by a range of animals (mostly bats, bees, beetles, birds, butterflies, flies, moths and wasps) provides almost incalculable economic and ecological benefits to humans, flowering plants and wildlife. Within this valuable array, bees come out as the worlds dominant pollinators, as the approximately 17,000 known bee species (Michener, 2000) collectively interact with most of the planet's quarter million angiosperm species (Buchmann and Ascher, 2005). Pollination by bees and other insects is the first step in the flowering/fruiting process resulting in the production of vegetables and fruits containing viable seeds that are essential nutrition comprising approximately 35% of the human diet (Buchmann and Nabhan, 1997). In short without pollinating insects the world would fall apart. Honey bees play an essential part of this function as managed pollinators. Pollination is the most important contribution bees make to human economies and the value of honey and beeswax pales in comparison to the value of fruits, vegetables, seeds, oils and fibres whose yields are optimised by pollinating bees (Delaplane and Mayer, 2000; Free, 1993; Gibb, 1991). There are many examples of studies and economic analyses that have estimated the value of honey bees as pollinators to agriculture. In the USA Robinson *et al.* (1989a, b) estimated this at $9.3 billion annually, Morse & Calderone (2000) revised this figure to $14 billion annually. In Europe, Borneck and Merle (1989), estimated that

of European crops valued at a total market value of 65 million ECUs, honey bees benefited 85% of these. Corbet *et al.* (1991) estimated the economic value of the increase in yield as 4250 million ECUs. Carreck and Williams (1998) estimated the economic value of bees (honey bees and bumblebees) as pollinators of major selected commercially grown insect pollinated crops in the UK to exceed £200 million. Estimates for agricultural and horticultural crops grown commercially in the UK that benefit from honey bee pollination are in the region of £120-£200 million p.a. while the value of honey production UK fluctuates between £10 million and £35 million per annum (Temple et al, 2001). Gibbs & Muirhead (1998) quote $1.2 billion as the value of crop pollination provided by honey bees in Australia (commercial and feral bees) and Gordon & Davis (2003) quote $1.7 billion.

The EU apiculture sector comprises approximately 12 million colonies managed by more than half a million beekeepers. Although the EU is the world's third largest honey producer, it is not self-sufficient and depends on the external market to meet consumer demand (COM, 2001, 2004; Jones, 2004). Apiculture is an integral part of the Community's rural economy and a reduction in available pollinators due to the adverse impact of endemic pests and diseases including exotic pests could seriously affect the sustainability and economic prosperity of apiculture as well as impacting on agriculture, horticulture and the environment, causing disruption to essential and valuable pollination services provided by honey bees. Lack of bees could leave a pollinator vacuum leading to economic consequences. There are a few estimates that attempt to quantify the impact of pests (*V. destructor* in particular) on the pollinating efficiency of honey bee colonies and therefore the impact of allowing an exotic pest to establish. The New Zealand Ministry of Agriculture and Forestry (MAF) estimated that the effect of the loss of pollination through the loss of managed and feral pollinators and the increased costs of beekeeping would cost New Zealand between $400 and $900 million NZ dollars over next 35 years (Goodwin, 2004; MAF, 2000, 2002a; Simpson, 2002). These economic impact assessments illustrate the potential costs to society of allowing the establishment of exotic pests and the justification for spending money on appropriate surveillance and extension programmes.

Significant colony losses can occur when the pest or disease damage is uncontrolled, the consequence being a reduction in bees available for essential pollination activities as well as economic damage for the beekeeper. This can put the sustainability of industry at risk. Honey bee losses of up to 50% in some areas of the USA in the winter of 2004/2005 required imports of package bees from Australia to make up the shortfall of bees needed specifically for almond pollination, a multimillion dollar industry

entirely reliant on the provision of a million colonies of bees in the spring (California Almond Board, 2005; Eischen *et al.*, 2005). Additionally, up to 40 000 colonies were moved from Florida right across the states specifically for almond pollination (Lumpkin, 2005). Varroa was thought to have contributed significantly to the colony demise (Farrelly, 2005). Plans are in place to reduce the risks of shortfall next year, with beekeepers being asked to take care not to move Fire Ants at the same time (CDFA, 2005; Traynor, 2005). Estimated losses caused by the impact of the newly arrived small hive beetle in Florida alone in 1998 were $3 million (Hood, 2004). Annual losses in the United States caused by American foulbrood brood are in the order of $5 million or more annually (Shimanuki, 1997). In recent years colony losses experienced throughout Europe have also been high (EU, 2004), attributed in part to the continued impact of varroa and its associated pathogens. Additionally, the numbers of feral colonies have declined particularly where *V. destructor* has become established; meaning the reliance on managed pollinators to fulfil the pollinator role has increased. All of these examples illustrate the importance of maintaining bee health for the good of any nations economy.

4. Animal health

The broad objectives of any Animal health framework for honey bees will be to protect honey bee populations and to support the sustainability of the apiculture industry. The objective of EU animal health legislation is to protect and raise the health status and condition of animals in the Community, in particular food-producing animals, whilst permitting intra-Community trade and imports of animals and animal products in accordance with the appropriate health standards and international obligations.

This is done by controlling serious, endemic diseases and pests so as to minimise the economic and environmental impact as much as possible, advising beekeepers on the recognition and control of serious diseases and pests, and minimising the risk of importation of exotic pests and diseases into the country and managing the risk should serious exotic organisms be discovered. In a nutshell to protect the honey bee whilst permitting trade and imports of bees and their products in accordance with the appropriate health standards and international obligations.

4.1. Historic perspective

Much of the legislation for control of bee diseases (control orders, acts, importation laws and so forth) has been in place in countries for

many years. These laws list the pests and diseases that are controlled and those that are exotic or undesirable, how this control will be done, the beekeepers statutory obligations including notification of the presence of suspect disease/pests to inspectors and veterinarians. In many countries this means registration of the beekeepers' apiaries with the appropriate authorities (Crane, 1990). The initial drivers for the introduction of these laws were either high levels of disease that required action or social need in terms of generating food. The high incidence of American foulbrood disease in the UK was a major factor in the introduction of the first foulbrood control order in 1942 together with the necessity to produce honey as a substitute for sugar. At the time there were 500 000 colonies and a lot more people kept them and managed them than nowadays. In the US the Honey bee Act of 1922 was introduced and was also designed to protect the industry from new pests and diseases, particularly in response to threat of the tracheal mite *Acarapis woodi*.

Prior to 1982 imports of honey bees into the UK were permitted from anywhere. As *V. destructor* spread and was confirmed established in countries geographically closer to the UK the Importations of Bees Order was drawn up. It was introduced in 1982 to reduce the risks of introduction and delay its inevitable arrival. The Order effectively prohibited the import of bees (packages, queens, nucleus and full size colonies) except under licences from countries with high bee health status, specifically this meant where *V. destructor* was thought to be absent. Of course, *V. destructor* arrived into the UK but perhaps later than would otherwise have occurred without these protective measures.

Legislation once introduced should not be static and remain a tablet of stone, but be flexible and evolve as circumstances change to take account of new threats on the horizon. It is the responsibility of beekeepers and scientists (particularly those with regulatory responsibilities) to flag up new concerns, and make sure their governments and for Europe; the Commission are informed of issues and emerging threats through effective dialogue.

A recent example of strengthening legislation and how this worked well is the EU legislation introduced in 2003, which makes the small hive beetle (*Aethina tumida*) and *Tropilaelaps* spp. mites notifiable throughout the Community.

4.2. EU framework

Both EU and national legislation govern control of bee pests and diseases in Member States. European Community legislation consists of Directives, Regulations, and Decisions, which are then transposed

and implemented into Member States domestic legislation (i.e. English, French, Italian law and so forth). The Member State legislation consists of measures to implement the directives and regulations and so forth. This legislation provides the framework under which diseases are controlled, contingency plans set up and invoked to deal with any exotic pest or disease outbreaks. Inspection and surveillance programmes should be designed to at least give a chance at eradication once new pests detected.

4.3. Principal EU legislation

EU animal health legislation requires that consignments of bees moved within and into the Community must be accompanied by a health certificate issued by a competent authority confirming freedom from specified notifiable diseases. The "Veterinary Checks" Directives (90/425, 91/496) place obligations on Member States to exercise control over movements of animals and animal products, including honey bees, either between Member States, or into the Community from third countries.

Specifically:

(i) Council Directive 92/65/EEC (also known as the Balai directive) lays down animal health requirements governing trade within and imports into the Community of certain animals (including bees). It lists American foulbrood, *Aethina tumida* (the small hive beetle) and *Tropilaelaps* spp. (parasitic mites), as notifiable diseases of honey bees throughout the Community. The directive also allows for Member States to set up national control programmes for certain diseases such as European foulbrood, varroosis and acarapisosis. It also lays down a model health certificate for bees, which must be completed by the competent authority to signify that the health conditions as laid down in the Directive are met. This certificate must accompany consignments of bees when they enter intra-community trade within the EU.

(ii) Commission Decision 2003/881/EC (as amended by Commission Decision 2005/60/EC) sets out the health certification requirements for the importation of both honey bees and bumble bees from third countries. In the case of honey bees, imports of queen bees will be authorised only from those third countries listed in Part 1 of Annex 2 to Council Decision 79/542/EEC. Currently, Council Directive 82/894/EEC requires Member States are required to inform the European Commission (EC) and the OIE if an outbreak of the small hive beetle (SHB) or *Tropilaelaps* occurs in their country (via CVO). It is possible that after such notification, the EC would consider establishing measures

to deal with the outbreak on a EU-wide basis. Such measures would be introduced through the European Commission's Standing Committee on the Food Chain and Animal Health (SCOFCAH). Member countries of the OIE are also required to inform that Organisation of outbreaks of any bee pests or diseases listed in its Terrestrial Animal Health Code.

(iii) Council Directive 90/425/EEC sets out the procedures for veterinary and zootechnical checks applicable in intra-Community trade in certain live animals and products.

(iv) Council Directive 91/496/EEC lays down the principles governing the organisation of veterinary checks on animals entering the Community from third countries.

Imports of honey bees are permitted into the EU provided the exporting third country can meet all the requirements of Commission Decision 2003/881/EC. Imports are currently restricted to queen bees and attendant workers only. There is also an obligation on importers that in addition to the attendant workers, they must submit the queen cages, packaging and any other material that accompanies the queen to a laboratory for examination for exotic pests and diseases. Bee imports must enter the European Community through designated Veterinary Border Inspection Posts (BIPs).

Post import inspectors or veterinarians under the competent authority may also carry out spot checks on consignments of honey bees from other Member States.

4.4. Plant health legislation

Bees also have the potential to spread plant pathogens and the Plant health regulations have been put in place to minimise this risk. Commission Directive 2003/116/EEC introduces more stringent measures to specifically prevent the spread of *Erwinia amylovora* (fireblight) within the European Community. Fireblight is a serious notifiable disease of apples, pears and related trees and shrubs in the Rosaceae family. Bee hives have been identified as an important factor in the spread of fireblight. The Directive provides for special measures regarding the movement of bee hives to fireblight 'protected zones' within the EU. Between 15 March and 30 June, beehives may only be moved into or within these zones provided that documentary evidence accompanying the hives indicates that one of the specific conditions have been met. The risk analysis process under the IPPC framework may also flag up the possibility of spread of other exotic plant pathogens of quarantine significance by honey bees (Sansford, 2001).

5. International framework

International trade in honey bees and their products is governed by relatively new international laws, which provide the framework for protection but also the framework for legitimate certified trade.

5.1. Trade legislation

The GATT-WTO Uruguay agreement (General Agreements on Tariffs and Trade – World Trade Organisation) on the application of Sanitary and Phytosanitary Measures (Annex I - the SPS agreement; Council Decision 94/800/EC, Official Journal EU L336, 1994) came into force on 1 January 1995 with the establishment of the World Trade Organization (WTO, 2002; SPS, 2002). This was a step towards world trade liberalisation. 148 (July 2005) countries are members of the WTO and are therefore bound by WTO International trade obligations.

Imports from third countries are governed by the SPS agreement. Under the agreement the OIE (Office International des Épizooties: Annex II) is recognised as the relevant international organisation for developing standards, guidelines and recommendations relating to animal health (OIE, 2005a). The aim of this agreement is to ensure that Sanitary and Phytosanitary measures are applied only to the extent necessary to protect human, animal or plant life or health, i.e. they do not constitute a disguised restriction to international trade. The agreement is intended to ensure continued protection of bee health whilst liberalising bee trade, and permitting it to be completed carefully and safely.

Under these arrangements an import ban for hive products or bees cannot be justified if measures can be introduced to prevent entry of disease. These measures (e.g. quarantine) must be based on proven science and must be technically justifiable and defensible. These measures including risk assessment based on international standards where they exist and developed by international organisations. If there is a scientific justification for why these measures do not provide an appropriate level of protection, then additional measures that provide a higher level can be applied. Trade is now based on managed or acceptable risk, rather than *zero* risk, and these agreements ought to permit it to be done safely. This is a balance between the individual countries' sovereign rights to set health protection measures whilst facilitating trade (AQIS, 1999; Brown *et al.*, 2002; Flanders, 1999; Matheson, 1995a, 1997, 2000; Murray, 2002, Ogden and Reid, 1996; Wooldridge, 1996, 2000).

5.2. Terrestrial Animal Health Code

The international standard for animal health in relation to bees is the Terrestrial Animal Health Code (OIE, 2005b). The aim of the Terrestrial Animal Health Code is to assure the sanitary safety of international trade in terrestrial animals and their products. This is achieved through the detailing of health measures to be used by the veterinary authorities of importing and exporting countries to avoid the transfer of agents pathogenic for animals or humans, while avoiding unjustified sanitary barriers. The OIE Terrestrial Code is a reference document for use by Veterinary Authorities, import/export services, epidemiologists and all those involved in international trade.

The health measures in the Terrestrial Code (in the form of standards, guidelines and recommendations) are formally adopted by the OIE International Committee and the general assembly of all Delegates of OIE Member Countries. The code incorporates guidance on: general provisions in Part 1, Obligations and ethics in trade, risk analysis, import and export procedures. Part 2 covers the OIE listed diseases and for bees (Chapter 2.9) these are currently Acarapisosis, American foulbrood, European foulbrood, Varroosis and *Tropilaelaps* spp. mites. It is anticipated that the small hive beetle will be added to this list. Part 3 Appendices provides guidance on the principles of diagnostic tests, health control and hygiene, quarantine, general principles and surveillance systems for specific diseases and model veterinary certificates.

The Terrestrial Code is the result of consensus among the veterinary authorities of OIE Member Countries, and constitutes a reference within the World Trade Organization (WTO) Agreement on the Application of Sanitary and Phytosanitary Measures (SPS Agreement) as an international standard for animal health and zoonoses. The code is updated regularly to take account of new developments, and in Europe the OIE consult Member States Veterinary authorities via the Commission (and vice versa). The OIE Terrestrial Manual of diagnostic tests for the listed pests and diseases in the Terrestrial code is likewise regularly updated (OIE, 2005a).

5.3. Import Risk Analysis

Risk analysis is a systematic aid to decision-making and policy (Annex III). It has been used routinely for many years to examine the risks associated with nuclear power, chemical and industrial processes, environmental issues and space exploration, but relatively recently to the veterinary sphere. An assessment of the risks of trading in a particular

commodity is known as risk analysis. It is the formal, systematic method for examining the probability of introduction of an exotic disease or pest, and the seriousness of such an outcome (AQIS, 1998; FAO, 1996; Murray, 2002; OIE, 2004pas cité dans biblio, 2005; WTO, 2005). Disease or pest risk analyses cover animal or plant health risks from pests and diseases or human health risks from disease carried by animals, plants or their products or human health risks arising from the pests.

Any importation of animals and animal products necessarily involves a degree of risk to the importing country. Risk analysis provides importing countries with an objective, repeatable and defensible method of assessing the risks posed by a particular proposed import of animals, animal products, animal genetic material, foodstuffs, biological products or pathological material. Formal methodologies have been developed and transparent processes emerged, although this is still an evolving discipline (Murray, 2002). Risk analyses for honey bee genetic material and hive products have recently been published (Brown, 1999; Flanders, 1999; Pharo *et al.*, 2004; Wheling, 2000).

The approach of these analyses is to consider the OIE listed diseases and pests plus those commonly considered as *significant* for international trade to the importing country that could be transported in bees or hive products (known as the commodities). Analysis of the risks is based on information provided by the veterinary services of the proposed exporting country of origin, consideration of the scientific literature and professional and scientific opinion from experts around the world. In the Plant health arena, pest risk analyses are completed under the guidance embodied in the IPPC (EPPO 2002, FAO, 1996). Important parallel risk analysis should also examine the potential of honey bees to move significant plant pathogens.

5.4. Surveillance standards

These should provide the data on which to base the decisions on risk. However, lack of data may hamper this. Some countries publish comprehensive surveillance standards, for example New Zealand (MAF, 2002b). The results of the pest management programmes and strategies together with the results of the apicultural exotic disease and pests surveys are also published (McMillan, 2001, 2003). This provides more confidence in the data that risk assessors can use to make their assessments, ie answers to the questions on the status and impact of particular problems and data on the point a pest changes from being exotic or apparently absent to present.

5.5. Apiary surveillance

Surveillance is an essential component of claims for freedom from disease or infection and provides data to support the import risk analysis process. Surveillance means the continuous investigation of a given population to detect the occurrence of disease for control purposes, and involves testing and regular sampling of a part of the population of beehives. Regular and routine colony examination is essential for early detection. Earliest warning depends on vigilance by both the beekeeper and inspectors. At the very least, early detection would allow control action to be targeted promptly where it is most needed to reduce the spread of these pests throughout the country. The objective of surveillance for exotic pests or diseases is to detect them before they have had the time to spread significantly.

Surveillance programmes and sampling protocols should be designed according to OIE principles (Chapter 1.3.and Appendix 3.4.2 of the Terrestrial Animal Health Code) so as to maximise the probability of early detection of any exotic pest or disease and allow for eradication to be attempted. Components of the system would include:

- Scientific based surveys (representative inspection coverage of the bee colony populations in the country)
- Ability to undertake effective disease investigation and reporting
- Laboratory diagnostic capabilities
- Training programmes for bee inspectors and industry stake holders

The inspection programmes should be risk based. Apiary datasets and Geographic Information Systems (GIS) are integral to the system and can be used to identify and prioritise inspections of "At risk" apiaries (ARAs). Identified ARAs, for example near ports or freight terminals, are targeted and regularly inspected. Each apiary has a 'risk score' calculated mathematically from its proximity to risk sources. Surveillance is targeted at high scoring apiaries and large numbers of these apiaries need to be inspected annually. If an exotic pest is detected/suspected, then apiary inspections should concentrate in the area around the apiary, and search patterns adjusted using GIS and tracings information.

It is recognised, however, that selection of and inspection of ARAs, is based on current understanding of the most likely routes for entry, but despite this, it may mean that the surveillance programme may miss unexpected introductions (Goodwin, 2004). The programmes need to be adjusted to take account of improved knowledge of the means of spread and dispersal of these pests, and designed to provide a chance at eradication.

5.6. Level of world trade

Millions of tons of commodities are moved around the globe annually, an increase of 50% since 1990. New trading partners are now emerging which will contribute to further increases, e.g. China and India. Growth is predicted to increase substantially in the next years with passenger and cargo freight growing annually at 4.8 and 6.2% respectively (ACI, 2005; Boeing, 2005). Worldwide sea borne cargo has doubled from 1.8 billion tons in 1980 to 3.6 billion tons in 2004. In 1980 containerised traffic represented 6.3% of world traffic. By 2004 this has risen to 23.8%. This is expected to rise with the main catalyst for growth coming from China. In 2004, the amount of cargo moving into and out of the UK reached 555 million tons.

5.7. Why are honey bees traded?

Thousands of bees: queens, colonies, nucleus colonies, package bees are sold worldwide to meet an increasing demand from beekeepers wanting to quickly establish productive colonies or obtain new genetic stock. Package bees are despatched by the pallet load air cargo and often used to start new colonies as a method of rapid stock increase, provide replacements for losses in the spring or used as disposable pollination units. When delivered the bees are usually placed directly into pre-prepared hives. Bees from the southern hemisphere in particular provide early replacement before bees are available in quantity from other EU Member States (Apiservices, 2005; Hini, 2004, Gibbs and Muirhead, 1988; Malka Queens, 2005; Pacific Queens, 2005). Replacements will be in high demand when high colony losses occur. Demand in the US increased in 2005 (Lumpkin, 2005; Harrison, 2005a, 2005b) and in the UK in 2005 imports of queens bees have tripled from to compensate for the high losses and poor over-wintering.

Trade in bees and bee products have been a commercial activity ever since man has kept bees (Crane, 1990). This was mostly local level with little movement of the products so the risk of spread of disease was lower than it is today (Fléché, 1997). In our modern world, trade has increased for all produce and the volume increased markedly. Southern hemisphere countries such as Australia, New Zealand, Chile, and Argentina, can hit an early spring market in Europe at a time when beekeepers need high numbers of replacement stocks. This trade is substantial and this includes both inter community (from outside the EU) and intra-Community (within the EU) (Crane, 1990; Lodesani and Costa, 2003, Büchler and Pechhacker, 2005). What has changed is the speed and rate of exchange of commercial materials. The world has gone global, and apiculture is no exception. The risk therefore of spread of pests and disease had markedly increased.

5.8. Other bee pollinators

Both bombiculture and the production of other solitary bees for pollination services are relatively new and developing industries in relation to apiculture. They have however grown substantially in the last decade (BIOBEST, 2005; Logan Bee Laboratory 2005; Oxford Bee Company, 2005). These bees are also a valuable and exploitable pollinator resource. There is little or no international framework governing their sales and distribution around the world. There is of course the potential to import non-indigenous bee species into other countries and also the potential to spread pests and pathogens. The need for a regulatory framework as theses industries grow should be explored further. Bumble bees have most recently been included in EU Decision 2003/881 as they could be a potential vector for small hive beetle spread, although this association has only been to date demonstrated in the laboratory (Stanghellini, 2000).

6. Food safety

Another major regulatory area that impinges on apiculture is food safety. The production, hygienic and safe processing, distribution, retail, packaging and labelling of foodstuffs are governed by a mass of food safety acts, laws, regulations, codes of practice, food safety management systems (eg Hazard Analysis Critical Control Point HACCP) and supporting guidance. The EU as with governments around the world is placing much greater emphasis on human health and its improvement, supported by a considerable research and development budget. Regulating the contaminants in the food its citizens eat is an important part of this, and the avoidance of unnecessary or potentially harmful residues in products such as honey is therefore desirable. Recent food 'scares' are a common event nowadays: BSE, dioxins, Foot and Mouth disease, genetically modified crops, food colourings have been subject to powerful publicity by influential media including television, radio, newspapers. Food scares are potentially very harmful to sales and the "natural" image of honey as a quality product. The public tend to believe in risk versus no risk, rather than various levels of risk from unacceptable to manageable, acceptable or negligible, which makes the job of the scientist and risk assessor difficult. It also makes the policy maker's stance therefore much more understandable. The European Union is very sensitive to consumer demands and risk perceptions and its policy makers therefore tend to be naturally conservative when interpreting such risks. Data on residues in honey are available on line through published reports and the rapid alert system. The central

goal of the European Commission's food safety policy is to ensure a high level of protection of human health and consumers' interests in relation to food, taking into account diversity, including traditional products, whilst ensuring the effective functioning of the internal market (EUROPA, 2005). Similar objectives are in place elsewhere, with just about every country having its food safety legislation and organisations involved (CFIA, 2005; FDA, 2005).

The Commission's guiding principle, which was set out in its White Paper on Food Safety is to apply an integrated approach from farm to table covering all sectors of the food chain, including feed production, primary production, food processing, storage, transport and retail sale (COM, 1999). Consumers look for products of the highest quality, and thus the conditions in which the products are produced, processed and placed on the market are of great importance to their quality and value, and it is essential to introduce uniform standards, ensuring compliance with public health requirements. The Commission, in its role as guardian of the European Community Treaties, is responsible for ensuring that Community legislation on food safety, animal health, plant health and animal welfare is properly implemented and enforced.

6.1. The Food and Veterinary Office (FVO)

As a Commission service, the Food and Veterinary Office (FVO, 2005) plays an important role in fulfilling this task. The mission of the FVO is, through its evaluations, to:

- Promote effective control systems in the food safety and quality, veterinary and plant health sectors;
- Check on compliance with the requirements of EU food safety and quality, veterinary and plant health legislation within the European Union and in third countries exporting to the EU;
- Contribute to the development of EU policy in the food safety and quality, veterinary and plant health sectors,
- And to inform stakeholders of the outcome of evaluations.

6.2. World trade in honey and hive products

World wide trade in honey and hive products is significant. Honey production is the most important commodity of around 1.25 million tons (COM 2001; COM, 2004); FAO 2003) and exports account for about 360,000 tons. The major exporting countries are China, Argentina, Mexico, the USA and Canada. In 2002, the EU (15 Member States) produced 112 000 tons, but also imported about 158 000 (44% of the global imports

in 2001). The most important importers of honey in the EU are Germany (92 000 tons) and the UK (25 000 tons). Trade in the other hive products, pollen, royal jelly, beeswax is also significant, with 2 000 tons of royal jelly is produced each year in China much of it destined for export. This trade could bring with it risks of contamination, residues or disease and pests transmission.

6.3. The EU Regulatory Framework for monitoring of food safety

In the EU each Member State must also now have in place formal and statutory veterinary drug monitoring and surveillance programmes, agreed with the European Commission (Council Directive 97/747 EC), and the rapidly increasing level of sophistication and sensitivity of analytical methods makes discovery of residues, however, small, much more likely (e.g., LC-MS/MS).

Honey forms part of the large number of foodstuffs that must be monitored for residues in the EU given its status as a commonly used basic commodity. It is also exceptionally vulnerable to food scare stories and 'bad press' in the event of the discovery of a residue issue since it is perceived by the consumer to be a pure, entirely natural product.

All food is subject to monitoring for its safety to the consumer. A large number of organisations are responsible for food safety and monitoring around the world (General references: AFSSA, 2005; BFR, 2005; CFIA, 2005; EFSA, 2005; EMEA, 2005; EUROPA, 2005; FDA, 2005; FSA, 2004, 2005, FSIS, 2005; FSANZ, 2005; FVO, 2005; SNFA, 2005). These institutes are responsible for commissioning research, risk assessment, provision of advice and guidance.

Within the EU designated national reference laboratory (NRL) in each member state has responsibility for the statutory monitoring of nationally produced and imported food (including honey) as well as the non-statutory veterinary residue testing of imported food including honey from retail outlets (CSL, 2005). It also covers import controls for Port Health Authorities.

The veterinary residues surveillance programmes play a central role in ensuring that the consumer is protected against potentially harmful residues of veterinary medicines in food. In Europe and around the world there are powers under Residues Regulations and various Food Safety Acts to remove from the food chain products containing residues at levels, which represent a danger to human health (this was recently done in the UK for imported honey from China (FSA, 2005)).

Producers of animal and animal products commonly use veterinary

medicines across the world. Residues of these drugs can carry over into foods including processed and unprocessed meat, fish, dairy products and honey. Concerns exist about the direct toxicity of these residues to consumers, and also the indirect effects on immune responses from long-term exposure. To safeguard the food chain, strict EU regulations governing veterinary medicine residues are in force. There are a multitude of regulations governing the use and approval of veterinary medicines (APVMA, 2005; EMEA, 2005; EUROPA, 2005; VMD, 2005), food production, packaging and sale to protect the EU consumer in terms of consistency, value for money and food quality. For the beekeeper or honey packer with an eye to selling honey in the EU, whether exporters or home producers, the most important in terms of quality standards are Directives 2377/90/EC, 96/23/EC and Decision 97/747/EC. Decision 97/747 fixes the frequencies and types of residue sampling required for certain substances in milk, eggs, rabbit meat, wild and farmed game and honey (Piro and Mutinelli, 2003). The basic definitions used in residue monitoring within the legislation are contained in Annex IV.

6.4. Directive 2377/90/EC

This is the Community procedure for the establishment of Maximum Residue Limits of veterinary medicinal products in foodstuffs of animal origin. It consists of four annexes that define the categories of substances and how they should be dealt with (Annex IV).

6.5. Directive 96/23/EC

Directive 96/23 describes measures to monitor certain substances and residues in live animals and animal products and the action to be taken if positive samples are detected. Honey is defined as a product of animal origin. It lays out the conditions through which 2377/90 is 'policed'. In summary the Directive:

- Demands Monitoring Plans from each Member State that must fit expected criteria. These must be approved by the European Commission
- Specifies criteria such as sampling levels and frequencies of sampling
- Demands that random checks are conducted to ensure that that producers comply
- Sets the requirements for approved National Reference Laboratories that are required to meet certain minimum standards

This directive specifies certain categories of substances that *must* be monitored (Annex IV).

The range of sophisticated instrumentation employed includes TLC, HPLC-Fl/UV, AMS, Biosensor and LC-MS/MS, and the screening and confirmatory methods used are well defined by EU Directives (e.g., Commission Decision 2002/657/EC)

6.6. Directive 178/2002/EC

Another important Directive to be aware of is Directive 178/2002/EC that lays down the general requirements of food law, established the European Food Safety Authority (EFSA, 2005) and sets up a robust cross-community communication network involving three levels of immediacy:

- Alert Notification – The highest level of rapid communication, usually involving high-risk discovery concerning residues in food that will affect more than one member state. The Commission may take action to remove products from sale and return them at cost to the producer
- Notification for information – No immediate action may be required, but information passed to other member states for consideration
- News only – no action necessary

Therefore the impact of a significant discovery of residues involving honey can have rapid, costly (for producers in cash terms and honey in terms of public confidence) and potentially damaging consequences across Europe. The problem of chloramphenicol (a banned substance in the EU) in Chinese honeys in recent years was a good example. Both the Directorate General (Trade) and Food and Veterinary Office (FVO, 2005) are very keen to send experts to assist developing countries in setting up adequate monitoring programmes and analytical techniques as well as advising on production methods to minimise residues. This is done with the aim in mind of encouraging such countries to export into the European consumer market.

6.7. An evolving framework

Regulations may change or be amended. Monitor EU regulations regularly (or seek advice from the Commission) to ensure compliance is met through the Internet Portal to European Law (EURLEX, 2005). It is also worth mentioning that there is some heated discussion in the EU even now about the best methods to be used to measure residues and their metabolites and how the level of risk should be interpreted. This is an area where scientists must play an important role in the future to enable policy makers in the European Community to interpret risk (from residues) sensibly. More 'background' work still remains to be done. In the UK, for example, the Central Science Laboratory (CSL, 2005), is being

funded by the Department for Environment Food and Rural affairs (Defra) to produce a 'library' of incurred tissue residues for access by others. Incurred residues is simply a way of measuring the likely residues you will find in various animal tissues given the application of various doses to the animal beforehand. This enables producers to estimate risk on the basis of the level of drug application. CSL is involved in a 3-year study to examine incurred drug residues in honey as part of the programme of work. CSL is also examining husbandry methods of disease control (e.g., European foulbrood) for use by UK beekeepers to remove current dependency on oxytetracyline and develop husbandry-based methods for control of the disease (Thompson *et al.*, 2005, 2006; Waite *et al.*, 2003).

6.8. Import legislation: Honey and Apiculture Products

The 'Balai' Directive 92/118/EEC (as amended) lays down the animal health and public health requirements governing trade in and imports into the European Community for a number of products of animal origin, including honey and apiculture products.

6.8.1. Honey

Commission Decision 2003/812/EC (as amended) provides for a list of third countries from which Member States can authorise imports of certain products, subject to Council Directive 92/118/EEC. This includes honey and royal jelly intended for human consumption. It is a requirement that Member States authorise imports of honey only from those third countries that have EC approved residue plans in place, that can guarantee monitoring of groups of residues and substances referred to in Annex 1 of Council Directive 96/23/EC.

Commission Decision 2004/432/EC details the list of third countries with approved residue plans and from which imports of honey can be permitted. This list of countries is subject to amendment and readers wishing to comply with legislation may therefore wish to consult with their own legal advisers or go through to the copies of legislation that are held on the European Union's legislation website (EURLEX, 2005).

6.8.2. Apiculture Products

Council Directive (EC) No. 1774/2002 lays down health rules concerning animal by-products not intended for human consumption. Annex VIII, Chapter IX lays down the requirements for apiculture products intended for use in apiculture. In the case of imports, apiculture products must come from third countries listed in Part 1 of Annex II to Council Decision 79/542/EC as last amended by Commission Decision (EC) No. 212/2004.

Apiculture products means honey, beeswax, royal jelly, propolis or pollen, not intended for human consumption or for industrial use, and in Great Britain this is implemented by the Products of Animal Origin (Third Country Imports Regulations).

7. Discussion and perspectives

Having completed a tour of the main regulatory framework and drivers for trade in bees and hive products, to conclude a few thoughts or poetic licence on the main issues facing apiculture today.

7.1. Decline in world bee health and hive product quality

Disease and pests threats can quickly become global rather than as in the past local or regional phenomena. In recent decades overall world bee health has declined with the continued and inexorable spread of pests and diseases across the planet (Fléché, 1997; Matheson, 1993, 1995b, 1996, 1997; Sanford, 2000). This now means in many instances beekeepers across the world now have to face common problems. Obvious examples include Varroosis, *Tropilaelaps* mites, foulbrood and the small hive beetle.

And so today beekeepers are facing increased problems with bee diseases and pests due to large-scale movements of colonies, package bees and queens from country to country as world trade increases with barriers to trade becoming less protectionist.

This in turn means that beekeepers must balance the need to control an increasing number of pests and diseases with producing a product, mainly honey, that is of high quality and clear of any treatment residues. This is a difficult task to engineer whilst fulfilling all the requirements of ever increasing and complex legislation.

A decline in product quality is most definitely a risk, and the evidence from the surveillance programmes suggests that residues are quite frequent in honey (Jamieson *et al.*, 2003; Faucon *et al.*, 2001; Martel and Zeggane, 2002, 2003; Martin, 2004; MAVIS, 2005). The use of medicines even through controlled systems has the potential to create significant residues in hive products (Thompson *et al.*, 2005). Maintaining product quality is essential to a thriving apiculture industry and there needs to be improved and targeted advice on the correct use of medicines. Beekeepers must become more aware of the risks they run by using any old unauthorised concoctions in their hives, despite the economic incentives to search for cheaper alternatives to deal with pests and diseases. The temptation through desperation is to resort to using anything that might work. This

drive is of course understandable given the small margins but not to the extent it compromises the product the consumer will buy. There is increasing demand for higher levels of food surveillance to ensure compliance with quality standards that are being demanded by consumers and tougher and tougher regulations being imposed to ensure this demand is met. This further increases the risk of non-compliance being revealed, especially since beekeepers in the West are increasingly facing the same problems such as *V. destructor* and small hive beetle, once only associated with beekeeping in Asia and Africa respectively.

Increasing trade volumes (e.g. package bees), due to greater demand for bees to compensate for increased losses (e.g. due to *V. destructor* resistance or other problems like poor winter survival) or the search for better genetic stock/productivity increases risks. With this rocketing demand, industry could therefore take greater risks to preserve profitability. Beekeepers have much easier access to the world market and the Internet is an easy source for online ordering. As a consequence of the increasing difficulties encountered in controlling pests and diseases (less efficient arsenal of medicaments), beekeepers may also be willing to take higher risks to look for the better bee, and step outside the framework that permits trade to be done safely. What is needed to halt decline in bee health is the continued shift of emphasis to work for selection of better disease tolerant or resistant stocks of bees better controls using a range of methods such as Integrated Pest Managment (IPM) (US Environmental Protection Agency, 2007) that reduce the dependence on medicaments and the chemical treadmill, coupled with good extension programmes providing advice on their application and on the early identification of pests and diseases as early recognition and action is the key to dealing with them, management systems that reduce the level of residues and improve the quality of the final hive products thus maintaining consumer confidence and the viability of the industry.

7.2. Developed vs developing world

Depending on which part of the world you are from will obviously mean different priorities. In many of the *developing* countries the main difficulties are producing honey to enhance incomes and survive, finding the resources necessary to set up cooperatives and networks and infrastructure to be able to export a quality product to markets such as the EU, and to understand the complex legislation and obtain advice on meeting the requirements (Jiwa, 2003; Saha, 2003; Ogaba, 2003). There are many organisations involved in helping with these aims (Apimondia, 2005; Bees Abroad, 2005; Bees for Development, 2005; Bradbear *et al.*, 2002; Brad-

bear, 2003; GTZ, 2005). Being able to export honey to the EU requires conformity with Commission Decision 2003/812/EC (as amended) which provides for a list of Third countries from which Member States can authorise imports of certain products, subject to Council Directive 92/118/EEC. This includes honey and royal jelly intended for human consumption. It is a requirement that Member States authorise imports of honey only from those third countries that have EC approved residue plans in place, that can guarantee monitoring of groups of residues and substances referred to in Annex 1 of Council Directive 96/23/EC. Commission Decision 2004/432/EC details the list of third countries with approved residue plans and from which imports of honey can be permitted. This list of countries is subject to amendment and readers wishing to comply with this legislation may therefore wish to consult with their own legal advisers for further advice. Copies of the legislation are held on the European Union's legislation website (EURLEX, 2005).

Other pressing concerns are mainly sustainable livelihoods and access to markets (ARC-PPRI, 2005). Beekeepers producing honey from species other than *Apis mellifera* have difficulties in having the product recognised and defined as honey in international markets. The definitions of honey vary, e.g. The EC Honey Directive (Council Directive 2001/110 EC) stipulates that honey is produced from *Apis mellifera* colonies therefore excluding all other bee species. The Codex Alimentarius definition includes honey from any bee (CODEX, 2005). This inconsistency is a problem for producers wishing to expand their markets.

And the difficulties of keeping and managing bees profitably may be exacerbated by the serious affects of pesticides (the impact of which is declining overall in Europe), which cause more honey bee colony mortality than any pest or disease.

Environmental contaminants will also impact colony productivity. Other factors include reducing the risks of introductions of pests and pathogens that are non indigenous to these areas also. We in Europe think of trade our way rather than the other way around, but introductions of *Apis mellifera* and the pest and disease organisms they carry have caused a number of serious problems over the years, this is how varroa reached North Africa for example.

7.3. Improving available data

Pests and diseases have spread far and wide. And with globalisation the potential for spread of these has increased, including new threats (alien species) outside their normal environment, e.g. the small hive beetle, *Aethina tumida*. For many of these pests and diseases their geograph-

ical incidence is well documented, as are the details of the surveillance systems/inspections programmes in place that support these data. Import risk analyses rely on up to date data on the incidence of particular pests and pathogens, and this in turn relies on the capabilities and level of the surveillance programmes in place in exporting countries. And even with the best surveillance programmes it is impossible to guarantee freedom with absolute confidence. It is therefore extremely difficult to arrive at conclusions involving risk. Risk Analyses are only as up to date as the available data, and are relatively easy to complete if your country already has the pests or pathogens being assessed present in the destination country, but when there is uncertainty this is a different matter. Two examples immediately come to mind. Based on the data available prior to 2000, risk analyses would have concluded that there was no risk of introducing *V. destructor* from New Zealand into Europe, and likewise prior to 2003, no risk of introducing the small hive beetle from Australia: however the reality was different. The ensuing conclusions of risk were incorrect, and this emphasise the difficulties and potential pitfalls in risk analysis if the data is lacking. Risk assessors have to make assessments/decisions that can turn out to be wrong. However, this formal process is still one of the best ways available for studying and measuring in depth the risks of importing a commodity. Information is available through the OIE web pages, through scientific opinion, and through various scientific publications, but you have to look hard for them and the process can take months of work. Information on disease incidence is not readily available and could definitely be improved. The Handistatus pages (OIE central repository) provide information on the disease incidence and distribution (if submitted by the member countries). There are many gaps that need to be filled. The health certificates will then be worth the paper they are written on. Regulators and risk assessors must have access to up to date information, otherwise it is difficult to arrive at decisions on a sound technical basis. There will always be elements of "judgement calls" that risk assessors have to make, but the more data the better to increase the confidence in the robustness of the data and the decisions that follow. Liberalisation of trade is all very well but there are definite risks!

7.4. Honey bee viruses and the bee trade

This book brings the science and current data on honey bee viruses together for the first time. For the most part in terms of regulatory control honey bee viruses do not currently form part of any statutory disease control programme and they are not part of the OIE Terrestrial Animal Health Code. Should they be included or not? This is not a straightforward

question to answer. Although the information on their distribution and impact is emerging all the time, there is still scope for considerable improvement to fill in the knowledge and capability gaps and available data on for example: host range, economic impact both on their own and in conjunction with other bee pests and pathogens, geographical distribution which is important. To take an example, up until recently Kashmir Bee Virus (a virus that has had its share of controversy in terms on international trade over the years) was considered to be absent (exotic) in Europe and to the UK prior to 2004. It has recently been detected in Spain, France Germany and Luxembourg (Büchler, 2003; Siede, 2005; Tentcheva *et al.*, 2004). Rapid molecular based diagnostic technologies such as real-time PCR provide the opportunity to rapidly complete large-scale surveys. KBV was detected in 2004 in the UK and has also been shown to be present at least since 1992 (by examining stored mites collected from infested apiaries in Devon in that year), when *V. destructor* was first confirmed in the UK. This technology can throw up new data on bee virus incidence through rapid screening, and can be used to build up a more comprehensive picture (Chantawannakul *et al.*, 2006). So the available data can change with advancing technologies and diagnostic techniques.

7.5. Surveillance systems

To improve the available data it is absolutely necessary to have improved surveillance systems in place employing up-to-date technologies such as GIS and diagnostic methods (PCR based methods for large scale surveys) in as many countries as possible and where resources permit, to set up the infrastructures and organisations and implement robust targeted surveillance. Inspection programmes should be designed to at least give a chance at eradication. Only through good systems providing robust information will we have a chance at early detection and any chance for consideration of eradication of exotic pests should they appear and have the information on which to produce well rounded risk assessments.

7.6. Extension and advice

Keeping up to date on the range of issues that affect apiculture and changes is difficult for all stakeholders as we live in a rapidly changing and complex world: for example keeping abreast of data on new and emerging threats and their risks, new and improved control methods and possibilities, new legislation in particular. The arms of the legislation have a long reach and ignorance of the law unfortunately is no defence. There is the continual need to make sure beekeepers are aware of the potential risks of contamination of products, the risks of moving pests and diseases around

the globe and the potential disastrous consequences. There is a framework that in theory should allow trade of honey bees and products to be done carefully and safely, with the flexibility to add new threats if they emerge through the auspices in Europe of Member States, the Commission and the OIE. However, if beekeepers are not aware of these and significant trade occurs outside this framework then it will only be a matter of time before the next important exotic pest arrives. As we know, honey bees (or hive products) can carry disease organisms over great distances; so it would only take one individual beekeepers management or decisions to import could directly affect the majority of the beekeeping profession. The idea that the "grass is greener on the other side of the fence" means the temptation to import what they perceive as a better bee particularly given the business pressures they are under. Don't do it. Any import of a new pest rather than through the proper certification processes would have serious consequences. We need to reduce unregulated otherwise known as illegal trade in bees. Prevention is surely better than cure. Unfortunately there is little you can do except vehemently discourage irresponsible and illegal trade from unknown uncertified sources and heighten awareness of the risks. For scientists and regulators and beekeepers, i.e. those involved in training must educate beekeepers to the existence of the complex regulatory frameworks so that this at least gives the possibility of safe trade and safe products. Good extension programmes need also to be in place to provide technical assistance to beekeepers on the correct use of treatments for disease control. Prophylactic or excessive treatment of hives (bucket chemistry), where medicines are just poured into hives contributes to residues in the final products and also propagates poor beekeeping husbandry practices, as it is so easy to do. The temptation is not to check what is actually going on in the hives and whether treatment is required. High use also accelerates the appearance of resistance to the drugs in the organisms you're trying to control.

Biosecurity education and well as the biosecurity strategy is firmly embedded in Australian and New Zealand culture (Animal Health Australia 2005; Biosecurity Australia, 2005; Protect New Zealand, 2005). Biosecurity New Zealand for example is active in promoting and educating citizens to the dangers, for example through the "know the enemy" active education and awareness campaigns. The campaign provides the information to arm New Zealanders with the information to identify harmful organisms and report them to Biosecurity New Zealand. By comparison in terms of awareness I think we are still catching up, and this approach could be developed further in Europe, even starting at an early age in schools. It is only very recently that a Code of practice for the import of

non-indigenous plants had been introduced in the UK (Defra, 2005).

Perhaps consideration should be given to the level of fines currently in place for flaunting import regulations. Are they sufficient? If for example a single person deliberately imports bees outside this framework introduces a pest that has serious and lasting economic consequences for the rest of the industry costing millions of Euros, should the levels of fines/ punishment handed out be more commensurate with the offence? In New Zealand offences under the Biosecurity Act 1993, eg illegal imports of bees or bee products do indeed risk large fines and/or terms of imprisonment (Harmon, 1999). Not in Europe where small fines if any are followed by just a "slap on the wrist" letter. Would these deterrents be enough and appropriate for Europe and would they gain support from beekeepers and stakeholders even though they are more draconian? What is proportionate and fair?

7.7. Declining funding base and Government involvement

It is ironic that at a time when beekeeping is facing greater threats and has need for continued support, funding for apiculture in general is being reduced across many of its disciplines (research, extension, inspection) in many parts of the globe. This has come about through a shift in philosophy, with the responsibility for disease control gradually shifting from government authorities to industry itself. In some parts of the world this has already happened, notably in New Zealand where there is industry management of pests under the Biosecurity Act 1993, e.g. the Pest Management Strategy for AFB (Reid, 2002) and for Varroa (Biosecurity New Zealand, 2005). The commercial structure of their industry permits this to work. There is nothing wrong with this philosophy in theory, and beekeeping associations have always recognised that they are responsible for managing their livestock. However, the reality may be different with industry lacking the structures to organise this themselves, which is particularly relevant for disparate or smaller industries. Therefore viable services need to be in place to support the sustainability of industry. Protection against market failure is one of the main reasons for maintaining bee health programmes in some countries (Temple *et al.*, 2001). Has to be a checks and balances on expenditure but there has to be control of disease. There is no doubt therefore in the future be an increased need for self policing and industry surveillance in parallel with this reducing expenditure on protection of the honey bee/ e.g. reductions in finance for both R&D and inspection and extension services. European beekeepers may need to think about a similar strategy, and get their act together to counteract the reduced support in the future.

The threat of the small hive beetle was brought vividly to life in Europe by the interception of SHB larvae in queens imported from Texas (Murilhas, 2004). There are no control methods yet available to provide adequate control to tackle this hive pest except emergency short-term treatments of limited efficacy. It is necessary to have contingency facing research and development programmes to find control solutions before this new pest arrives and contingency plans to deal with the eventual arrival of any new threat as well as continuing to work towards solutions for endemic pests such as *V. destructor* and foulbrood which continue to cause havoc. Strong beekeeping lobby groups are needed to maintain the pressure, conduct awareness campaigns for apiculture and continue to emphasise its vital role in sustainable food production and its contribution to the environment through pollination of crops and wild flowers. This needs to be kept in the forefront of politicians and regulators minds. Scientists and beekeepers must also make sure that regulators and risk managers have at their disposal the essential information to be able to assess the risks and adapt the legislation as necessary. In other words no surprises when it hits the fan.

7.8. Better Regulation

If the laws are sensible, rational measured appropriate and agreed then they should work well, sounds like Utopia. What sort of regulation do we need? I know the type we don't want, and that is the knee jerk irrational and unmeasured responses to try to deal with a problem. The long arms of the legislation reach far but the last thing anybody in business needs is regulation that makes no sense and interferes with the business of making a living by implementing ineffective measures such as for example an eradication programme with no chance of success. One of the worst cases of poor regulation is what took place in the United States during the early 1980's when the tracheal mite first entered the country (Miksha, 2004). Many colonies were killed in an attempt to eradicate what was seen afterwards to be a well-established pest with no hope of eradication, particularly with the migratory nature of North American beekeeping. Needless hardship was caused and high costs incurred. Scientists and researchers must make sure that the information reaches the regulators, the outcome being a sensible stakeholder consensus on the approach to take. So we need flexible legislation that is able to respond rapidly to changes. For this to work well, this requires good communication links along the chain from the regulators through to producers with all stakeholders interests covered. For example, in my view consideration could be given to including undesirable races of mellifera: the Africanized honey bees and

their hybrids (*Apis mellifera scutellata)* and *Apis mellifera capensis* within the framework of EU honey bees legislation. This would bring it into line with the requirements of a number of Third countries who are trading partners, where these are included on their lists of statutory undesirable organisms.

7.9. Recommendations from BRAVE

During the BRAVE meeting (BRAVE, 2005), discussions on trade and import regulations specifically for honey bee viruses were pretty wide ranging. It was clear that there are a number of capability and data gaps to plug. The main conclusions and questions to answer that came out of the meeting were as follows and could be taken forward for consideration in any future research plans

1. No recommendations currently to include honey bee viruses as statutory bee diseases within the OIE Terrestrial Animal health codes or EU legislation. Fundamental information required, there is not enough data, either on their impact or distribution to justify additional legislation beyond the formal risk analysis process that individual countries may complete.
2. Recommendation to conduct delimiting surveys and monitoring programmes using modern diagnostic technologies and standard diagnostic methods and protocols to assist with: improving information on distribution and status for recognised bee viruses and assessing and identifying emerging or potential risks. In this way it will be possible to get a "handle" on what we may viruses we may be dealing with, what their actual distribution is and what the economic and disease impact might actually be.
3. Establish harmonised diagnostic methods to measure pathological effects, impact in general including economic impacts, and set up studies and models to fill in identified gaps in knowledge.
4. More detailed data on disease agents; epidemiology of honey bee virus infections is required together with their interactions with other pests and pathogens, to provide better information both to policy personnel at Commission and Member State level and to researchers and beekeepers. To include definitions and descriptions of the diseases, tests used, causative agents.
5. Provide better training and education for beekeepers to identify disease problems, provide information on good husbandry, and information on international trade rules. This may help to reduce the risks of illegal or uncertified trade in honey bees and any introduc-

tions of exotic pests.

6. Complete rapid investigations of the causes of significant losses of honey bee colonies as and when they occur, and this could be through collaborative efforts across Europe for example. This improved collaboration and the information obtained could be placed on a EU-wide bee pathology interactive website. A network of specialists is important. Colleagues/scientists in Europe and overseas can assist with control methods and providing information on how to tackle new pests based on their R&D and experience.
7. Continue to horizon scan for other pests and diseases. Many thought that *Varroa* was the last great threat to world apiculture but then *Aethina* came to dinner. We must not take our eyes off the ball. The huge level of colony losses in Spain reportedly caused by *Nosema ceranae* is another example. Who would have predicted these?
8. Join all of the elements of Apiculture to form strong industry with a strong voice: through from regulation to research to training and extension to the producers. Joining up research to look at the big picture is an element of this approach and is beginning to happen and a welcome development (BRAVE, 2005; EurBee, 2004).

7.10. Closing comments

Whether we like it or not, global trade in bees and their commodities is a reality. Through experience we know movement of bees and products can introduce new pest and disease problems. I am sure most of us have heard about illegal imports of uncertified trade, queen bees coming into the country in suitcases or pockets. However, imports do not have to (or the risks are reduced), as long as the trade and certification framework is followed, backed up by adequate surveillance and inspection systems to reduce the risks as far as practicable and improve the data available on which to make risk management decisions (Matheson, 2000). We all depend on trade for our prosperity and you can't pull up the drawbridge and say no. The risks both in terms of animal health, food safety and biodiversity can be reduced with better compliance from industry (by concentrating on keeping pests out because with the best surveillance programmes in the world eradication is extremely difficult (Smith *et al.*, 2005), and education and extension programmes in partnership with beekeeping organisations can play a major role in this area.

Rocketing global trade has increased the numbers of exotic insects, plants and animals that hitch a ride on imported goods, in ship ballast water, in cargo holds, in airfreight, i.e. trade liberalisation and a less protec-

tionist stance has led to more problems than people bargained for. So in the meantime we can get prepared to deal with the next "out of the blue" exotic pest incursion, which I fear, is only a matter of time.

ANNEXES

I. Key principles of the sanitary and phytosanitary agreement (sps) (94/800/ec)

The SPS agreement is the WTO agreement on the application of sanitary and phytosanitary measures, with rules on how countries can protect the health of their people, animals and plants, while facilitating trade.

The importation of animals and animal products involves a degree of disease risk to the importing country. One or several diseases or infections may represent this risk. The principal aim of import risk analysis is to provide importing countries with an objective and defensible method of assessing the disease risks associated with the importation of animals, animal products, animal genetic material, feedstuffs, biological products and pathological material. The analysis should be transparent. This is necessary so that the exporting country is provided with clear reasons for the imposition of import conditions or refusal to import (SPS, 2005).

I.1. Justification of measures

Sanitary and phytosanitary measures are applied for no other purpose than that of protecting the health of animal, plant or human populations, and import measures should be based on sound science and risk assessment principles and should not form disguised barriers to trade. Standards should not arbitrarily or unjustifiably discriminate between countries where identical or similar conditions prevail. However, members may use measures, which result in higher standards if there is scientific justification. They can also set higher standards based on appropriate analysis of risks.

I.2. Equivalence

Member countries must accept the SPS measures (disease control & surveillance) of other member countries as equivalent if the exporting country can objectively demonstrate that they achieve the importing members appropriate level of protection, i.e. if the exporting country's controls & tests achieve the same results.

I.3. International Standards

Countries must establish SPS measures on the basis of an assessment of the actual risks involved, and do this using international standards, guidelines and recommendations where they exist to complete risk analysis, developed by the relevant international organisations. This is the OIE for animal diseases. The basis of risk analysis is information; available scientific evidence, relevant inspection, sampling and testing methods, prevalence of diseases or pests, and existence of disease-free or pest free areas. Relevant economic factors must also be taken into account, i.e. potential damage in terms of loss of production, establishment and spread of disease, the costs of control and the relative cost-effectiveness of alternative approaches to limiting the risks.

I.4. Transparency

All risk analysis documents are to be made available. Risk analysis should be transparent to provide the exporting country with a clear and documented decision on the conditions imposed for importation, or refusal of importation.

II. Office International des Épizooties (OIE)

This inter-governmental organisation (website address: www.oie.int) was set up in 1924 and is the world organisation for animal health matters. Its main aims are the development, standardisation and harmonisation of disease control standards and regulations for the purposes of international trade. Risk analysis, collection and dissemination of information on epizootic diseases, and promotion and co-ordination of research on contagious diseases of livestock are part of this process. In 1995, the creation of the WTO brought the OIE a new international role. The agreement on the application of sanitary and phytosanitary measures (SPS agreement) was among the set of agreements that established the WTO and designated the OIE as the relevant international organisation for animal health matters, to set international standards, guidelines and recommendations in the field of animal health and zoonoses. The two standards applicable to honey bees are the Terrestrial Animal Health Code and the Manual of Standard Diagnostic Tests and Vaccines for Terrestrial Animals. Both are used as the basis for and veterinary inspections, surveillance and disease control. The Terrestrial Code is designed to facilitate trade, whilst at the same time reducing the risk of spreading animal diseases (Ishibashi and Wilson, 2005; OIE, 2005).

III. Glossary of risk analysis terms

Risk Analysis

Risk analysis is a systematic process by which the appropriate authority (often a government body) considers trade in commodities, which could be potentially harmful to human, animal or plant health. It is defined as the process, which comprises hazard identification, risk assessment, risk management and risk communication.

Hazard

A hazard is something (element or event) which is potentially harmful to humans, other animals, plants or the environment, and that may produce an adverse event or outcome (e.g., pathogenic organisms in bee packages). If no hazard is identified, then there is no known risk.

Risk

This is the likelihood and magnitude of an adverse event, such as a disease organism, causing damage. It requires the existence of a hazard and implies that there may be several outcomes.

Agent

An organism, which causes or contributes to the development of a disease (normally an OIE Listed disease).

Commodity

Animals, animal products, animal genetic material, feedstuffs, biological products and pathological material.

Risk Assessment

This is the process of identifying and evaluating the risk resulting from a hazard, completed by a risk assessor. It can be categorised as qualitative, quantitative or both. Qualitative risk assessment uses terminology such as "relatively high", "negligible", or "low" to evaluate risk. Quantitative assessments use modelling to estimate mathematical probabilities of the amount of damage, and the likelihood of its occurrence. Risk assessment examines the effects, and takes into account the prevalence, of the pathogens in the source population and the probability of them surviving the pathway. The "pathway" is essentially the means by which a pathogen could be introduced, i.e. mode of transport of the commodity, the probability of any pathogen coming into contact with local bee population and the seriousness of that contact.

Risk assessment has two major steps:

Extensive information gathering and an analysis of all available scientific data, including an on-line search to assist with identification of the hazards, such as diseases and exotic pests. Collecting and assessing the in-

formation is often the most difficult and time-consuming part of risk assessment. Data may be difficult to find, difficult to obtain, or even non-existent. It also requires consultation with experts both in the UK/importing country and overseas (Wooldridge, pers. comm.). Evaluation of the risk of establishment will include an economic analysis if feasible (e.g., the cost of controlling the disease or pest and the loss of markets). The information on which decisions are based should be available for objective evaluation.

The process is sub-divided into:

Hazard Identification

This involves identifying the risk-producing agents and the conditions under which they may produce consequences. The hazard identification in this particular risk analysis examines the aetiology & epidemiology of each disease agent in detail to determine whether the commodities under consideration could be considered a means for their introduction into the importing country. Where the hazard identification concludes that bee packages are a potential hazard for introduction for a particular disease, then this is considered in further detail in the risk assessment. If the disease agent is not considered to represent a hazard then no risk assessment is completed.

Furthermore, if a disease is identified as a hazard, i.e. transportable and transmissible in the commodity, but is ubiquitous in the importing country, and there is no evidence that new exotic strains of a particular pathogen are present in the exporting country, and there is no official control or eradication programme in the UK, then no risk assessment is completed.

Release Assessment

For a risk to exist a hazard must be released in some way. Risk assessment is the process of estimating the potential for a risk source, either bees or hive products, genetic material, to introduce disease into a country.

Exposure Assessment

This step describes the possible exposure to the hazard released from the risk source of susceptible hosts in the importing country. Exposure assessment considers the likelihood of effective contact between the imported commodities and honey bees in the importing country.

Consequence Assessment

This is the process of describing the economic and health consequences of exposure to the risk agents. If the release assessment concludes that a disease agent is a potential hazard, then the consequences of its introduction and establishment need to be assessed. Consequences in human terms might be sickness, death, and for the environment it could be pollution.

Risk Estimation

This involves compiling and summarising the results from the above

steps. It involves a decision as to whether safeguards are necessary for a particular disease.

Risk Management

Risk management is the process of identifying and implementing measures that can be applied before, during or after import to reduce the risks and their consequences to an acceptable and manageable level. This is the formulation of risk mitigation measures (safeguards), which are considered appropriate for the identified hazards.

For honey bees this can include quarantine (assuming facilities exist and the funding is available to run them)

Risk Communication

The process of communicating the results to the appropriate authority and other interested parties, such as industry and the general public.

General references

Ahl *et al.*, 1993; AQIS, 1998, 1999; Covello and Merkoher, 1993; MAF, 2002; MAFF, 2000; Murray, 2002; OIE, 1993, 2001; Wooldridge, 1996, 2000; WTO, 2002.

IV. Basic Definitions within EU food monitoring legislation (EUROPA, 2005)

IV.1. Directive 2377/90/EC

MRL: Maximum Residue Limit for permitted substances. The maximum concentration of residue resulting for the use of a veterinary medicinal product (VMP) – You should note that there are none for honey. This limit takes into account the safety limits based on average daily consumption and toxicology

MRPL: Minimum Required Performance Limits – This covers analytical criteria only. In other words it is an artificial limit set for laboratories that cannot meet the highest standards of analysis

CCα: Decision limit – The limit at which it can be concluded with an error probability of α that a sample is non-compliant (i.e. positive).

CCβ: Detection capability - used to determine the "false negative" rate for screening assays

For permitted substances: e.g., Maximum Residue Limit (MRL) for permitted substances – (none current for honey): MRL < CCα < CCβ

For banned substances: e.g., MRPL for chloramphenicol in honey, CCα < CCβ < MRPL

A positive sample is any confirmed residue with a concentration of > CCα (α = 5% for a permitted substance and 1% for a banned substance).

Annex I: Substances for which an MRL can be set following evaluation of toxicological risk to human health
Annex II: Substances needing no MRL. Considered safe
Annex III: Substances which lack the scientific data needed to set MRL but may be given provisional MRL for defined period until data available
Annex IV: Substances for which no MRL can be set due to high risk to human health however small the quantity involved

Table I lists the active ingredients commonly used in beekeeping that have been considered and evaluated by the European Medicines Agency (EMEA, 2005). All such evaluations are tissue specific. In other words EMEA may approve an MRL for oxytetracycline in various animal tissues such as kidney or red meat, but if no such evaluation has been conducted for honey specifically then existing animal tissue MRLs cannot be applied. All of the active ingredients in Table 1 are used in the treatment of mite infestations. They represent the total evaluations for honey. Note that currently there are no antimicrobial substances listed and therefore approved. There is therefore currently *zero tolerance* for any antimicrobial in honey.

IV.2. Directive 96/23/EC

Group A
Antithyroid agents
Beta-agonists

Table I. EMEA-evaluated active ingredients for use in bee health (2377/90)

Active Ingredient	MRL (ppb)	Food Type	Annex
Amitraz	200	Honey	Annex I
Coumaphos	100	Honey	Annex I
Camphor	-	-	Annex II
Eucalyptol	-	-	Annex II
Flumethrin	-	-	Annex II
Formic Acid	-	-	Annex II
Oxalic acid			Annex II
Lactic Acid	-	-	Annex II
Menthol	-	-	Annex II
Tau-fluvalinate	-	-	Annex II
Thymol	-	-	Annex II
Cymiazole	1,000	Honey	Annex III

Resorcylic Acid Lactones (e.g., Zeronal)
Steroids
Stilbenes (plus derivatives, salts, esters)
Annex IV compounds

Group B
Antimicrobials
Other veterinary drugs (e.g., Carbamates, Pyrethroids)
Other substances & environmental contaminants:
OC's
OPs, General chemicals
Mycotoxins

The National Reference Laboratories also screen for the following range of antimicrobials:

Tetracyclines	Beta lactams
Cephalosporins	Sulphonamides
Chloramphenicol	Streptomycin
Aminoglycosides	Nitrofurans

(Piro and Mutinelli, 2003)

References

ACI (2005) Airports Council International Traffic statistics reports, Internet website http://www.aci.aero

AFSSA (2005) Agence Française de sécurité sanitaire des aliments, Internet website http://www.afssa.fr/

Ahl A. S., Acree J. A., Gipson P. S., McDowell R. M., Miller L., McElvaine M. D. (1993) Standardisation of nomenclature for animal health risk analysis, Rev. Sci. Tech. Off. .Int. Epizoot., 12, 1045-1053.

Allen M. (2003) Working with the Oxford Bee Company, Bee Biz 16, 23-27

Animal Health Australia (2005) Internet website http://www.animalhealthaustralia.com.au/

Apimondia (2005) Internet website http://www.beekeeping.com/apimondia/index_us.htm. Standing Commission of beekeeping for rural development

Apiservices (2005) Virtual beekeeping gallery, Internet website http://www.apiculture.com

APVMA (2005) Australian pesticides and veterinary medicines authority, Internet website http://www.apvma.gov.au/

AQIS (1998) The AQIS Import Risk Analysis Process Handbook.

AQIS (1999) National Risk Management and the SPS Agreement.

ARC-PPRI (2005) Beekeeping for Poverty relief, ARC Plant Protection Research Institute, Internet website http://www.arc.agric.za/institutes/ppri/main/divisions/beekeeping/poverty.htm

Baker R., Cannon R., Bartlett P., Barker I.(2005) Novel strategies for assessing and managing the risks posed by invasive alien species to global crop production and biodiversity, Ann. Appl. Biol.146, 177-192.

Barclay J.S., Moffett J.O. (1984) The pollination value of honey bees to wildlife. Am. Bee J. 124, 497-498.

Bees Abroad (2005) Internet website http://www.beesabroad.org.uk/

Bees for Development (2005) Internet website http://www.planbee.org.uk/

BFR (2005) Federal Institute for risk assessment, Internet website http://www.bfr.bund.de/

BIOBEST (2005) Commercial bumblebee production, Internet website http://www.biobest.be/

BOEING (2005) World demand for commercial airplanes: current market outlook, Internet website http://www.boeing.com/commercial/cargo/flash.html

BIO-BEE, (2005) Bio-bee biological systems, Internet website http://www.bio-bee.com/english/welcome.html

Biosecurity Australia (2005) Internet website http://www.affa.gov.au/biosecurityaustralia

Biosecurity New Zealand (2005) Varroa pest management strategy, Internet website http://www.biosecurity.govt.nz/pests-diseases/animals/varroa/index.htm

Borneck R., Merle B. (1989) Essai d'une évaluation de l'incidence économique de l'abeille domestique dans l'agriculture européenne, Apiacta 24, 33-38.

Bosch J., Kemp W. P. (2002) Developing and establishing bee species as crop pollinators: the example of *Osmia* spp. (Hymenoptera: Megachilidae) and fruit trees, Bull. Entomol. Res. 92, 3-16.

Bradbear N., Fisher E., Jackson H. (2002) Strengthening Livelihoods: Exploring the role of beekeeping in development, Bees for Development, Troy, UK.

Bradbear N. (2003) Beekeeping and sustainable livelihoods, FAO Diversification Booklet 1.

BRAVE (2005) Proceedings on the Bee Research and Virus in Europe Meeting BRAVE, Sophia-Antipolis, France 24-26 april, Aubert M., Ball B., Fries I., Milani N., Moritz R. (Eds.)

Brown M. (1999) Import Risk Analysis: Package bees from New Zealand Report produced by the CSL National Bee Unit on behalf of the Ministry of Agriculture, Fisheries and Food, London.

Brown M., Thompson H.M., Bew M.H. (2002) Risks to UK beekeeping from the parasitic mite *Tropilaelaps clareae* and the small hive beetle, *Aethina tumida*, Bee world 83 151-164.

Brown M. (2004) So you want to keep bees? Bee World 85, 65-66.

Buchmann S.L., Nabhan G.P. (1997) The forgotten pollinators, Island Press, Washington DC, USA.

Buchmann S.L., Ascher J.S. (2005) The plight of pollinating bees, Bee World 86, 71-74.

Büchler R. (2003) Information on the detection of Kashmir bee virus in Hesse, German Beekeeping Internet website http://www.biohonigbonn.de/Gesundheit/gesundheit.html#Viren

Büchler R., Pechhacker H. (2005) Apiculture in Europe (in German), Dtsch. Bien. J. 2, 18-19.

California Almond Board (2005) Internet web site http://www.almondboard.com/

Carreck N., Williams I. (1998) The economic value of bees in the UK, Bee World 79,115-123.

CBD (2005) Convention on biological diversity, Internet website alien species http://www.biodiv.org/programmes/cross-cutting/alien/

CDFA (2005) California Department of Food and Agriculture, Internet website http://www.cdfa.ca.gov/phpps/pdep/rifa/html/english/farm/beehive_information.html

CFIA (2005) Canadian Food Inspection Agency, Internet website http://www.inspection.gc.ca/english/anima/honmiel/honmiele.shtml

Chantawannakul P., Ward L., Boonham N., Brown M. (2006) A scientific note on the detection of honeybee viruses using real-time PCR (TaqMan) in varroa mites collected from a Thai honeybee (*Apis mellifera*) apiary, J. Invertebr. Path, in press.

Clavero M., Garcia-Berthou E. (2005) Invasive species area leading cause of animal extinctions, Trends Ecol. Evol. 20, 110.

CODEX (2005) Codex Alimentarius, Internet website http://www.codexalimentarius.net/web/index_en.jsp

COM (1999) Commission of the European Communities. White paper on food safety.

COM (2001) Commission report to the Council and European Parliament on the implementation of Council Regulation No. 1221/97.

COM (2004) Second report from the Commission to the Council and European Parliament on the implementation of Council Regulation No. 1221/97.

Corbet S. A., Williams I.H., Osborne J.L. (1991) Bees and the pollination of crops and wild flowers: changes in the European Community. EU report Bee World 72, 47-59.

Covello V.T., Merkoher M.W. (1993) Risk Assessment Methods. 'Approaches for assessing health and environmental risks', Plenum Press, New York.

Crane E. (1990) Bees and beekeeping: Science, Practice and World Resources, Comstock Publishing Associates, Ithaca/NY pp 475-491.

CSL (2005) Central Science Laboratory, Internet web site www.csl.gov.uk Analytical Services http://www.csl.gov.uk/prodserv/ana/

Davis M. (2003) Biotic globalization: Does competition form introduced species threaten biodiversity? Bioscience 53, 481-489.

Defra (2003) Review of non-native species legislation and policy, Report of the working group. http://www.defra.gov.uk/wildlife-countryside/resprog/findings/non-native/ecoscope.pdf

Defra (2005) Non native species, Internet website http://www.defra.gov.uk/wildlife-countryside/non-native/index.htm

Delaplane K.S., Mayer D.F. (2000) Crop Pollination by Bees, CABI Publishing, New York.

EFSA (2005) European food safety authority, Internet website http://www.efsa.eu.int/

Eischen F.A., Graham R.H., Cox R. (2005) Regional distribution of *Paenibacillus larvae* subspecies *larvae*, the causative organism of American foul brood, in honey bee colonies of the Western United States, J. Econ. Entomol. 98, 1087-1093.

Elton C.S. (1958) The ecology of invasions by animals and plants, Methuen, London.

EPPO (2002) Pest risk analysis on detection of a pest in an imported consignment, EPPO Bull. 32, 235-239.

EPPO (2005) European and Mediterranean Plant protection organisation (EPP0), Internet website http://www.eppo.org/index.htm

EMEA (2005) European Medicines Evaluation Agency, Internet website www.emea.eu.int/home.htm

EurBee (2004) Proceedings of the first European Conference of Apidology, Udine Italy, 19-23 sept, Bernardinelli I., Milani N. (Eds.), Arti Grafiche Friulane, Udine.

EUROPA (2005) European Union Food Safety, Internet websites. http://europa.eu.int/comm/food/index_en.htm, http://europa.eu.int/comm/dgs/health_consumer/index_en.htm. Rapid Alert System for Food and Feed http://europa.eu.int/comm/food/food/rapidalert/index_en.htm

EU (2004) European Commission on Agriculture. Experts meeting on Apiculture: Varroa control, 24th October 2003.

EURLEX (2005) Portal to European Law http://europa.eu.int/eur-lex/

Farrelly M.J. (2005) Varroa threatens USA agriculture, Bee World 86, 78.

FAO (1996) International Standards for Phytosanitary Measures: Guidelines for Pest Risk Analysis.

FAO (2003) FAO Yearbook on trade, Statistics Series No. 184, 188-190.

Fasham M., Trumper K. (2001) Review of non-native species legislation and guidance, Report for Defra European Wildlife Division.

Faucon J.P., Martel A.C., Antinelli J.F., Clément M.C., Zeggane S., Cordella C., Davico R., Rognone C., Aurières C. (2001) Sondage sur la qualité des miels de lavande-lavandin, Ann. Fals. Exp. Chim. 91, 251-261.

FDA (2005) US Food and Drug Administration, Internet website http://www.fda.gov/

Fléché C. (1997). Risques de dissémination des maladies apiaires par les mouvements internationaux des abeilles et de leurs produits. Rev. Sci. Tech. Off. Int. Epizoot. 16, 177-186.

Flanders R.V. (1999) Pest risk assessment: Importation of adult queens, package bees and germplasm of honey bees, *Apis mellifera* L. from New Zealand (revised March 2002) pas d'autres info ?

Free J.B. (1993) Insect pollination of crops (second edition), Academic press, New York.

FSIS (2005) USDA Food Safety and Inspection Service http://www.fsis.usda.gov/

FSA (2004) Food Law Guide http://www.food.gov.uk/multimedia/pdfs/foodlaw.pdf

FSA (2005) Food Standards Agency, Internet website http://www.food.gov.uk/news/newsarchive/2005/oct/nchoneyrecall

FSANZ (2005) Food Standards Australia New Zealand http://www.foodstandards.gov.au/aboutus/

FVO (2005) Food and veterinary Office http://europa.eu.int/comm/food/fvo/index_en.htm

Gibb R.A. (1991) The values of honeybee pollination to society, Acta Horticulturae 288, 63-68.

Gibbs M.H., Muirhead I.F. (1998) The economic value and environmental impact of the Australian beekeeping industry. A report commissioned by the Australian honey bee industry council (AHBIC) for the Australian beekeeping industry, [Online] http://www.honeybee.org.au/economics.htm

GISP (2005) Global Invasive Species Programme [Online] http://www.biodiv.org/programmes/cross-cutting/alien/gisp.asp

Goodwin M. (2004) Introduction and spread of Varroa in New Zealand, Bee World 85, 26-28.

Gordon J., Davis L. (2003) Valuing honeybee pollination, Rural Industries Research and Development Corporation Australia RIRDC Report 03/077.

GTZ (2005) Internet website http://www.gtz.de/en/

Harrison B. (2005a) US beekeepers make history with first Australian bee imports. Part I, Am. Bee J. 145, 287-290.

Harrison B. (2005b) US beekeepers make history with first Australian bee imports. Part II. Am. Bee J. 145, 377-380.

Harmon A. (1999) Illegal imports into New Zealand, Australian Honey bee industry council Newsletter July-August, [On line] http://www.honeybee.org.au/nljuaug.htm

Hini C. (2004) International Animal Trade, Surveillance 31, 4-7.

Hood W. (2004) The small hive beetle, *Aethina tumida*: a review, Bee world 85, 51-59.

Hulme P.E. (2003) Biological invasions: winning the science battles but losing the conservation war? Oryx 37, 178-193.

Ishibashi T., Wilson D. (2005) Trade issues and the WTO/OIE, in: BRAVE Proceedings on the Bee Research and Virus in Europe Meeting BRAVE, Sophia-Antipolis, France 24-26 april, Aubert M., Ball B., Fries I., Milani N., Moritz R. (Eds.), 289-295.

ISSG (2005) Global Invasive Species Database, Internet website www.issg.org

Jamieson, P. (2003) Contaminants of the bee colony, Bee Biz 16, 35-38.

Jiwa F. (2003) Honey Care Africa's Tripartite Model: An innovative approach to sustainable beekeeping in Kenya, in: XXXVIIIth Apimondia International Congress, Ljubljana, Slovenia.

Jones R. (2004) European beekeeping in the 21st century: strengths, weaknesses, opportunities, and threats, Bee World 85, 77-80.

Jukes D. (2005) Food Laws, Internet Website University of Reading http://www.foodlaw.rdg.ac.uk/

Juliano S. A., Lounibus L. P. (2005) Ecology of invasive mosquitoes: effects on resident species and on human health, Ecology Letters 8, 558-574.

Kemp W.P. (2000) The future of crop pollination, Am. Bee J. 140, 851-853.

Koppert (2005) Internet website http://www.koppert.nl/e005.shtml

Logan Bee Lab USDA (2005) Internet website http://www.loganbeelab.usu.edu/

Lodesani M., Costa C. (2003) Bee breeding and genetics in Europe, Bee World 84, 69-85.

Lumpkin D, (2005) Overcoming obstacles during 2005 almond pollination, Am. Bee J. 145, 290-295.

Mack R. N., Lonsdale W. M. (2001) Humans as global plant dispensers: getting more than we bargained for, Bioscience 51, 95-102.

Manchester S. J., Bullock J. M. (2000) The impacts of non-native species on UK biodiversity and the effectiveness of control, J. Appl. Ecol. 37, 845-864.

MAF (2000) Varroa in New Zealand: economic impact assessment, Ministry of Agriculture and Forestry report.

MAF (2002a) Review of the Varroa economic impact assessment, Ministry of Agriculture and Forestry report.

MAF (2002b) Standard for surveillance of diseases of honeybees, MAF Biosecurity Authority, Wellington, New Zealand

MAF (2005) New Zealand biosecurity Authority, Internet website http://www.biosecurity.govt.nz/

Malka Queens (2005) Internet website http://www.malkaqueens.com/espanol/home.htm

MacLeod A., Evans H.F., Baker R.H.A. (2002) An analysis of pest risk from an Asian longhorn beetle (*Anoplophora glabripennis*) to hardwood trees in the European community, Crop protection 21, 635-645.

Marris E. (2005) Shoot to Kill, Nature 438, 272-273.

Martel A. C., Zeggane, S. (2002) Determination of acaricides in honey by high-performance liquid chromatography with photodiode array detection, J. Chromatogr. A 954, 173-180.

Martel A. C., Zeggane S. (2003) HPLC determination of sulfathiazole in French honeys, J. Liq. Chromatogr. Relat. Technol. 26, 953-961.

Martin P. (2004) The honey regulations 2003, Bee Craft June, 13-15.

Martin P. (2004) Honey International Packers Association (HIPA), Working group report, enhanced government activity including enforcement, Celle.

Matheson A. (1993) World bee health update 1993, Bee World 74, 176-212.

Matheson A. (1995a) All change for global trade and the bee world, Bee World 76, 165-168.

Matheson A. (1995b) World bee health update 1995, Bee World 76, 31-39.

Matheson A. (1996) World bee health update 1996, Bee World 77, 45-51.

Matheson A. (1997) International trade agreements and honey bee diseases, in : Morse, R., Flottom, K. (Eds), Honey bee pests, predators and diseases, third edition, A I Root, Medina, pp 578-602 Appendices 10, 11.

Matheson A. (2000) Managing risks in world trade in bees and bee products, Apiacta 35, 1-12.

MAVIS (2005) Medicines Act Veterinary Information Service http://www.vmd.gov.uk/

McKinney M. L., Lockwood J. L. (1999) Biotic homogenization: a few winners replacing many losers in the next mass extinction, Trends Ecol. Evol. 14, 450-453.

McGrath S. (2005) Alien invaders, National Geographic March, 92-117.

McMillan D. (2001) Apicultural exotic disease surveillance report – 2000, Surveillance 28, 15-16.

McMillan D. (2003) Apicultural exotic disease surveillance report, Surveillance 30, 8-9.

Michener C. D. (2000) The bees of the world, Johns Hopkins University Press, Baltimore, USA and London, UK.

Miksha R. (2004) Mites, in: Bad Beekeeping, Trafford Publishing, Victoria B.C., pp 217-230.

Mooney (2005) Alien invasive species: a new synthesis, Scientific committee on problems of the environment (SCOPE), Island press, Washington DC.

Morse R.A., Calderone N.W. (2000) The value of honey bee pollination the United States, Bee Culture 128,3 15 page supplement.

Murilhas A. M., (2004) *Aethina tumida* arrives in Portugal. Will it be eradicated? Eurbee newsletter No. 2, 7-9.

Murray N. (2002) Import Risk Analysis Animals and Animal products, MAF Biosecurity, New Zealand.

NISC (2005) National Invasive Species Council, Internet website. http://www.invasivespecies.gov/

Neumann P., Elzen P. (2004) The biology of the small hive beetle (*Aethina tumida*, Coleoptera: Nitidulidae): gaps in our knowledge of an invasive species, Apidologie 35, 229-247.

Ogaba M. (2003) Household poverty reduction through beekeeping amongst Uganda rural women, XXXVIIIth Apimondia International Congress, Ljubljana, Slovenia.

Ogden S., Reid M. (1996) Trade liberalization: opportunities and bee health implications, Surveillance 23, 12-13.

OIE (2005a) Office International des Épizooties, Internet website. www.oie.int

OIE (2005b) Terrestrial Animal Health Code (12th edition) [online] http://www.oie.int/eng/normes/mcode/en_sommaire.htm

Oxford Bee Company (2005) Oxford bee company, commercial solitary bee production, Internet website. http://www.oxbeeco.com/

Pacific Queens (2005) Pacific queens, Internet website http://www.apicultura.cl/pacificqueens/03pacific/pacific.html

Pharo H. (2004) Import risk analysis: Honey bee products, Biosecurity New Zealand, Ministry of Agriculture and Forestry.

Pimentel, D., Lach L., Zuniga R., Morrison D. (2000) Environmental and economic costs of non indigenous species in the United States, Biosci. 50, 53-65.

Pimentel D., McNair; S., Janecka J., Wightman J., Simmonds C., O'Connell C., Wong E., Russel L., Zern J., Aquino T., Tsomondo T. (2001) Economic and environmental threats of alien plant, animal and microbe invasions, Agric. Ecosyst. Environ. 84,1-20.

Piro R., Mutinelli F. (2003) The EU legislation for honey residue control, Apiacta 38,15-20.

Protect New Zealand (2005) Protect New Zealand Biosecurity, Internet web site. http://www.protectnz.org.nz/

Reid M. (2002) American foul brood, Surveillance 29, 21-22.

Rixon. (2005) Invasion risks posed by the aquarium trade and live fish markets on the Laurentian Great Lakes, Biodivers. Conserv. 14, 1365-1381.

Robinson W.S., Nowogrodski R., Morse R.A. (1989a, 1989b). The value of honey bees as pollinators of U.S. crops, Am. Bee J. 129, 411-423 and 477-487.

Saha J. (2003) Beekeeping for rural development, its potentiality and beekeeping against poverty – Bangladesh perspective, XXXVIIIth Apimondia International Congress, Ljubljana, Slovenia.

Sanford M. T. (2000) Managing honey bees and profitability in the new millennium, Bee Biz 12, 15-20.

Sansford C. (2001) Import Risk Analysis package bees from Australia: potential for transmission of plant pathogens in consignments, Central Science Laboratory (CSL) PRA report for Defra Plant Health Division, York.

Shimanuki H. (1997) Bacteria, in: Morse, R., Flottom, K. (Eds), Honey bee pests, predators and diseases, third edition, A.I. Root, Medina, pp 35-54.

Siede R., Derakhshifar I., Otten C., Berenyi O., Bakonyi T., Koglberger H., Büchler R. (2005) Prevalence of Kashmir bee virus in Central Europe, J. Apic. Res. 44, 129.

Simberloff D. (2004) A rising tide of species and literature: a review of some recent books on biological invasions, Biosci. 54, 247-254.

Simpson B. (2002) Summary of assessments of the economic impact of varroa in the South island of New Zealand, Ministry of Agriculture and Forestry report.

Smith G. C., Henderson I. S., Roberson P.A. (2005) A model of ruddy duck *Oxyura jamaicensis* eradication for the UK, J. Appl. Ecol. 42, 546-555.

SPS (2005) SPS agreement http://www.wto.org/english/docs_e/legal_e/15-sps.pdf

SNFA (2005) Swedish National Food Administration, Internet website http://www.slv.se/

Stanghellini M.S, Ambrose J.T., Hopkins, D.I. (2000) Bumble bee colonies as potential alternative hosts for the small hive beetle (*Aethina tumida* Murray), Am. Bee J. 130, 71-75.

Tentcheva D., Gauthier L., Zappulla N., Dainat B., Cousserans F., Colin M.E., Bergoin M. (2004) Prevalence and seasonal variations of six bee viruses in Apis mellifera L. and Varroa destructor mite populations in France, Appl. Environ. Microbiol. 70, 7185-7191.

Temple M.L., Emmett B.J., Scott P.E., Crabb R.J. (2001) An economic evaluation of Defra's bee health programme. A report prepared for Economics and resource Use Division, Defra, London. [on line] http://statistics.defra.gov.uk/esg/evaluation/beehealth/default.asp

Thompson H., Waite R.J., Wilkins S., Brown, M.A., Bigwood T., Shaw M., Ridgway C., Sharman M. (2005) Effects of European foulbrood treatment regime on oxytetracycline levels in honey extracted from treated honeybee (*Apis mellifera*) colonies and toxicity to brood, Food Addit. Contam. 22, 573-578

Thompson H., Waite R.J., Wilkins S., Brown M.A., Bigwood T., Shaw M., Ridgway C., Sharman M. (2006) Effects of shook swarm and supplementary feeding on oxytetracycline levels in honey extracted from treated colonies, Apidologie, in press.

TRACE (2005) TRACE: delivering integrated traceability systems to enhance consumer confidence in the authenticity of food, [online] http://www.trace.eu.org/

Traynor J. (2005) Almond Pollination, 2006 and beyond, Bee Culture, Nov., 29-40.

UKCIP (2005) UK climate impacts programme, Internet website. http://www.ukcip.org.uk/

US Environmental Protection Agency (2007) Integrated Pest Managment (IPM) and Food Production, Internet website. http://www.epa.gov/pesticides/factsheets/ipm.htm

Van Driesche J., Van Driesche R. (2000) Nature out of place: biological invasions in the global age, Island Press, Washington DC.

VMD (2005) Veterinary Medicines Directorate Internet website http://www.vmd.gov.uk/

Villenueva R, Roubik D.W., Colu-Ucan,W. (2005) Extinction of *Melipona beecheii* and traditional beekeeping in the Yucatan peninsula, Bee World 86, 35-41.

Vitousek P., D'antonio C.M., Loope L.L., Rejmanek M., Westbrooks R. (1997) Introduced species: a significant component of human caused global change, New Zealand J. Ecol. 21, 1-16.

Waite R.J., Brown M.A., Thompson H.M. (2003) Controlling European foulbrood with the shook swarm method and oxytetracycline in the UK, Apidologie 34, 569-575.

Wehling W.F. (2000) Pest Risk Assessment: Importation of adult queens, package bees and germplasm of honey bees, *Apis mellifera* L., from Australia, Plant Protection and Quarantine, Animal and Plant Health Inspection Service, APHIS, (revised March 2002).

Wooldridge M. (1996) Risk analysis, risk assessment, animal health and the decision making process, State Vet. J. 6, 4-6.

Wooldridge M. (2000) Risk analysis in aquatic animal health, in Rodgers C.J. (Ed), Proceedings of an OIE International Conference, Paris, February 2000, pp 11-18.

WHO (2005) World health organisation, Internet website. http://www.who.int/en/

WTO (2005) World Trade Organisation, Internet website http://www.wto.org/

Annex

RECOMMENDATIONS OF THE WORKING GROUPS GATHERED DURING THE BRAVE MEETING HELD IN SOPHIA-ANTIPOLIS (24-26 APRIL 2005).

Table of Contents

Working group
Characterisation of viruses of honey bees and related species and pathogenesis

Rapporteur : Mike CARTER

A. Characterisation of viruses of honey bees and related species

1. State of the art.

Major advances have taken place in insect virology over recent years. Insect virology offers the most fruitful zone for the discovery of new viruses and replication strategies which in turn offer the potential for furthering our understanding of cell biology.

Study of bee viruses is advancing with this movement but more remains to be done. These opportunities for academic progress mean that there is an increasing and world-wide interest in the subject.

2. Challenges and problems :

- Many bee viruses remain poorly characterised.

- The study of bee viruses is impeded by lack of certified virus-free bees and *in-vitro* cultivation systems.

- There is a lack of characterised standard reagents and reference sera.

- Disease burden is underestimated; persistent infections are common but dismissed. Only gross disruption is detected, less obvious effects (decreased lifespan) must impact on productivity. Objective measures are required.

- Varroa has changed the rules, opening a new route for infection and possibly altering the behaviour of viruses by selecting mite-replicating variants. This has increased the significance of persistent infections

3. Actions reagents and techniques required:

- Sequencing of bee viruses should be completed (for those whose sequencing has only being partly done), or performed and new agents sought,

- PCR procedures should be developed for newly sequenced agents,

- Recombinant antigens are needed to produce standardised antisera and antigens,

- An active (interventionist) approach is required to develop bee cell lines, whereas considering the numerous attempts with no avail made by numerous workers, this approach is now rarely encouraged.

B. Pathology and Pathogenesis :

High mutation rate and the organisation of viruses into quasi-species underlies potential for rapid RNA virus adaptive mutation have implications for host- and tissue tropism. However, these phenomenon are is poorly characterised.

Among the most urgent needs in this area, the group identified :

- the need to locate virus-infected tissues following naturally acquired, acquired following parasitism by Varroa, and artificially acquired (following injection or other artificial infection procedure),

- the need to understand more of the dynamics of virus interaction with Varroa in *Apis mellifera* and particularly in *Apis cerana*, its original host.

Working group
Diagnostics techniques for virus diseases in honey bees

Rapporteur : Mark STEVENS

1. To develop an international database of appropriate diagnostic protocols, methods and tools such as antibodies and PCR primers.

Further sequencing of bee viruses will allow the opportunity to develop new PCR primer sets that can be exploited for diagnostics. It will also be necessary to develop standardized protocols for the collection, storage and initial processing of bee samples prior to any diagnostic tests, especially if samples being sent long distances.

2. To undertake a survey of the distribution and impact of the key viruses of bees within individual countries in order to determine their presence (for any quarantine lists that may be instigated) and to understand the epidemiology of these viruses.

3. To determine, via the diagnostic methods, the thresholds of detection for specified viruses and the thresholds at which these viruses cause 'damage' to bees.

Such methods could be exploited to standardize the terms used within bee virology, for example, quantification of terms such as 'unapparent' and 'latent'.

4. To undertake a series of ring tests among international laboratories to standardize methods for key viruses and to establish standard operating procedures for each system.

5. To establish a specific working group for KBP/ABPV.

Working group
Genetics, physiology, behaviour

Rapporteur : Robin MORITZ

Researches to be developed must be carried out at the individual level or at the colony level.

A. Research need at the individual (bee) level.

Among the most urgent needs the group recommended :

1. screening for genetic variance of resistance to viral diseases in European honeybees,

2. to develop techniques for infecting drones with virus to take advantage of the haplo/diploid genetic structure of the bee species,

3. to identify disease resistance genes and their regulation (including the use of candidate genes from other model systems).

4. to develop molecular tools to assist in selection for virus disease resistance,

5. to study the effects of virus infection on the physiology of host organs (including gene expression in the host, structure, cell death, endocrine regulation).

B. Research need at the colony level.

1. Resistance to virus must be studied not only on groups of bee isolated from their hive but also at the colony level. Whereas simpler, the study at the level of groups of bee isolated from their hive, cannot be considered as a good model for entire bee colonies for two reasons : (i) isolated bees die rapidly (either they are infected with virus or not) and (ii) colonies may develop strategies to overcome virus infections.

2. Any behavioural changes in honeybees induced by virus infections that may affect colonial traits should be identified : e.g defence, hygienic behaviour, flight behaviour, intracolonial behaviour of infected and non infected workers.

Working group
Insect immunity and virus latency

Rapporteurs : Brenda BALL, Peter ROSENKRANZ and Jean-Luc IMLER

The insect response to infection is based on cellular and humoral defences but differs significantly from vertebrate systems in the degree to which recognition and memory contribute to immunity. The haemocytes of the honey bee have been characterised but in many instances their role and function are incompletely described or understood. Similarly, a range of antibacterial proteins produced by bees in response to various challenges have been recognised, but it is not known whether either of these defences systems are activated or effective against virus pathogens. Additionally to cellular and humoral defences, social insects can adapt behavioural responses to pathogen challenge at both the individual and colony level. A detailed investigation and understanding of all kinds of the innate immune and adaptive behavioural responses of populations, and the different life stages within them, would make a significant contribution to epidemiological studies.

Many of the viruses of bees persist within individuals at levels that are not readily detectable and that apparently cause no gross pathology. However, it has been demonstrated experimentally that these sequestered viruses can be activated and induced to multiply to lethal levels by a number of diverse triggers. Progress in the elucidation of some of the physiological and immunological pathways involved in these events has been made in other insects, facilitated by knowledge of the insect genome and functional approaches (genetics, transgenesis, RNAi). It is to be hoped that the honey bee genome project will ultimately make an important contribution in this area.

Insect immunity and virus latency are important fundamental areas that could provide valuable insights into infection processes and disease epidemiology, but of which at present we have only limited knowledge and understanding. Because of the close interdependence of these two areas research, progress would be greatly enhanced by bringing together specialists in each discipline for the design and undertaking of integrative studies. Some key questions and priority areas for further investigation were identified.

1. Investigation and characterisation of the response of honey bees to

pathogen or other challenges could initially be based on currently known immune systems and pathways in other insects. Looking for homologies and differences to model systems would provide a simple first approach : in particular as infection markers are known for other insects, they should be looked for in infected honey bees.

2. The genetically regulated susceptibility to infection is well documented in *Drosophila* as proven by the lethal infection of mutant immunodeficient individuals infected by opportunistic bacteria or fungi. The new information on the bee genome should be used in similar approaches.

3. Are the latent/occult honey bee viruses transcriptionally active and in what form do they persist; as intact particles or as RNA? What are the main tissues or cells in which these viruses persist and does this differ with different virus types and different life stages?

Working group
Evolutionary epidemiology

Rapporteurs : Brenda BALL, Mark JF BROWN and Ingemar FRIES

1. Research shall be oriented to determine the basic biology of bee virus infections :

- transmission routes and persistence of viruses,
- transmission rates (vertically and horizontally between individuals, colonies, apiaries),
- impact, especially with respect to environmental stress, eg. nutrition, environmental pollution by pesticides and other xenobiotics, etc.

Whereas some knowledge is available on transmission routes and persistence of some virus infections, virtually nothing is known about transmission rates. There is an urgent need for quantification of different transmission routes and for much better documentation of the persistence of virus infections both in the absence and presence of *Varroa destructor*.

2. Standardised epidemiological surveys should be conducted using appropriate direct sampling and detection techniques for measuring incidence, prevalence and distribution of bee virus infections across the EU.

3. Epidemiological and evolutionary models should be created, based on the knowledge acquired through the researches described in paragraphs 1 and 2, to understand bee/virus associations and predict their development.

4. Biogeographical studies of virus isolates should be developed to create a phylogeny of honey bee viruses.

Finally, the group proposed that studies initially may be concentrated on one particular honey bee virus to produce a detailed understanding of such a system.

Working group
Management of bee diseases: economic impact

Rapporteur : Keith S. DELAPLANE

1. Successful control of bee diseases and pests will depend on a sound understanding of the biology underlying the host/pest/parasite relationship.

2. Specific management programs should be built on the principles of Integrated Pest Management (IPM), stressing reduced reliance on the use of acutely toxic pesticides. Components of IPM include but are not limited to: development of economic (action) thresholds, genetic host tolerance, cultural practices that interfere with parasite life history, social immune systems, beneficial disease-antagonistic organisms, and semiochemicals that can be used to trap pests or disrupt their mating. IPM can be expected to delay onset of economic thresholds and reduce overall use of acutely toxic pesticides.

3. Once economic thresholds are achieved, acutely toxic pesticides should only be used at lawfully-prescribed rates and in rotation, such that active ingredients are not used successively for avoiding or at least delaying the onset of resistance of the pest to the active ingredients.

4. The expected outcomes of IPM-based disease and pest control include, but are not limited to: reduced residues of acutely toxic pesticides in bee hive products and the environment, reduced occupational exposure of beekeepers to toxins, prolonged years of useful life for a limited pool of pesticides, improved reproductive performance of queens and drones, and increased colony population sizes and productivity.

5. Appropriate response to unknown causes of bee mortality requires establishment of standardized diagnostic avenues, establishment of regionalized baseline record keeping to discern historic trends in disease incidence, and beekeeper education programs to improve accurate diagnoses at the local level.

Working group
Regulatory issues

Rapporteur : Mike BROWN

1. to conduct delimiting surveys and monitoring programmes within the EU and elsewhere using recognised standard diagnostic methods and protocols to assist with: improving information on distribution and status for recognised bee viruses, assessing and identifying emerging or potential risks. In this way it will be possible to get a "handle" on what we may be dealing with, what their distribution is and what the economic impact might be.

2. to establish methods to measure pathological effects, impact in general including economic impacts, and set up studies to fill in identified gaps in knowledge.

3. to collect more detailed data on disease agents; epidemiology of honey bee virus infections to provide better information both to policy personnel at Commission and Member State level, for researchers and beekeepers. These information should include definitions and descriptions of the diseases, tests used, causative agents. (In order to consider legislation it is necessary to know what dealing with, what the distribution is, what are the disease signs, what is the impact, how to diagnose and so forth).

4. to provide better training and education for beekeepers to identify disease problems, provide information on good husbandry, and information on international trade rules. This may help to reduce the risks of illegal or uncertified trade in honeybees and any introductions of exotic pests.

5. to rapidly investigate the causes of any new significant losses of honey bee colonies when they occur, and this could be through collaborative efforts across Europe.

Information obtained could be placed on a website, eg OIE but also EU wide website.

INDEX

Symbols

A

B

C

D

E

F

G

H

I

J

K

L

M

N

O

P

Q

R

S

T

U

V

W

Y

European Commission

EUR 21937 — Virology and the Honey Bee

Luxembourg: Office for Official Publications of the European Communities

2008 — 460 pp. — format, 17,5 x 25 cm

ISBN 92-79-00586-3