Climbing the walls

Behavioural manipulation of insects by baculoviruses

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This research was conducted under the auspices of the Graduate School of Production Ecology and Resource Conservation.

Climbing the walls

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Stineke van Houte

Thesis
submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. Dr M.J. Kropff,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Tuesday 21 January 2014
at 1.30 p.m. in the Aula.

Stineke (C.G.J.) van Houte
Climbing the walls
- Behavioural manipulation of insects by baculoviruses -
228 pages.

PhD thesis, Wageningen University, Wageningen, NL (2014)
With references, with summaries in English and Dutch

ISBN 978-94-6173-830-1

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Chapter

1

General introduction and thesis outline

A brief history of baculoviruses and Wipfelkrankheit

Even though the identity of baculoviruses was not revealed until several decades ago, their dramatic effect on insect hosts was recognized already centuries before. The earliest description of baculovirus infection in Western literature is attributed to the 16th-century Marcus Hieronymus Vida di Cremona, bishop of Alba in Northern Italy. His poem named 'De Bombycum' dates from 1527 and describes diseased silkworms, caterpillars of the silk moth *Bombyx mori*, as follows:

Protinus elucet languentibus aurea pellis
Deinde tumet, turpisque animis ignavia venit
Desidibus, tandem rumpuntur, et omnia tetro
Inficiunt tabo; sanies fluit undique membris

The poem was translated into modern English by N.A. Miller, as described in Miller (1997):

All at once, in the weak ones, the skin appears yellow. Then they swell up and a foul[1] inactivity[2] comes in the bodies of those who have fallen down. Finally they break open and everything is infected with repulsive putrid gore; diseased blood from all sides flows from the bodies.

[1] Connotation of smell; [2] Connotation of paralysis

The 'putrid gore' and 'diseased blood' that flow out of the caterpillars most likely represent the typical liquefaction or 'melting' of the hosts, which is characteristic for baculovirus infection (Fig. 1A).

The earliest known description of behavioural alterations in diseased insect hosts dates from as early as 1891 and likely represents the oldest written account of behavioural manipulation by parasites in history. In this work Hofmann illustrates how larvae of the nun moth *Lymantria monacha* climb up and die in tree canopies. These larvae had succumbed from an unknown agent which made them crawl out of their common hiding place to die on exposed parts of the trees. This phenomenon was named Wipfelkrankheit (Hofmann 1891), which is more commonly known in the Anglo-Saxon world as tree-top disease. It was to be discovered several decades later that these larvae had died due to an infection with a baculovirus, causing behavioural alterations in the infected caterpillars and liquefying them to efficiently disperse progeny viruses into the environment.

Baculoviruses – an introduction

The *Baculoviridae* (King et al. 2011) are a large family of invertebrate viruses. Members of this family infect over 700 insect species worldwide (reviewed in Miller (1997)). While baculoviruses can infect insects in the orders Diptera and Hymenoptera, the majority infects host species belonging to the order Lepidoptera, and this latter group of baculoviruses will be discussed in more detail here. In general, baculoviruses are highly pathogenic and host specific, a combination which has rendered them useful as biological control agents in, for example, the control of the beet army worm *Spodoptera exigua*, the codling moth *Cydia pomonella* and the cotton bollworm *Helicoverpa armigera* as alternative to chemical insecticides (reviewed in Moscardi (1999)). As a more recent development, baculoviruses are also used as expression vectors for the production of large amounts of heterologous proteins in insect cells or insects. As such, baculoviruses are important tools for applications such as vaccine production, health diagnostics and gene therapy (van Oers 2011).

Baculoviruses have circular double-stranded DNA (dsDNA) genomes ranging in size from 80 to 180 kilobasepairs (kbp) that may contain 90 to 180 open reading frames (ORFs) (reviewed in Blissard (1996), Miller (1997) and van Oers & Vlak (2007)). A unique feature of these viruses is the production of two different morphotypes during infection that are genetically identical. Budded viruses (BVs) mediate cell-to-cell spread, whereas occlusion-derived viruses (ODVs) are involved in horizontal transmission from insect to insect. BVs consist of a single, enveloped nucleocapsid and bud from the

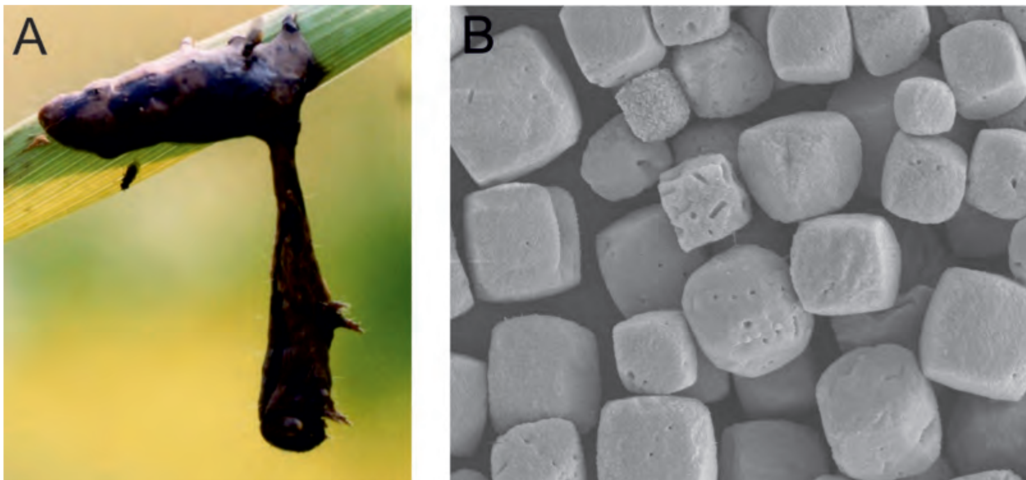


Figure 1. (A) Liquefied lepidopteran caterpillar that succumbed to a baculovirus infection (*photo credits: Michael Grove*); (B) Scanning electron microscopy picture of occlusion bodies (OBs) from *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) (*photo credits: Jan van Lent*).

membrane of infected cells to spread to neighbouring cells or via haemolymph to other tissues. In contrast, ODVs acquire their envelope in the nucleus by *de novo* assembly, and may contain single or multiple nucleocapsids within a single envelope, depending on the virus species. These ODVs are embedded in a paracrystalline proteinaceous occlusion body (OB) (Fig. 1B) varying in size from 0.5 to 3 µm that protects the virions against proteolytic attack and/or environmental decay. This paracrystalline matrix is predominantly made out of a single type of protein: polyhedrin for nucleopolyhedroviruses (NPVs) or granulin for granuloviruses (GVs) (see below). OBs are extremely stable and can remain infectious for years in the environment (Thomas et al. 1972), enabling the virus to persist in the absence of a host for longer periods of time.

Horizontal transmission occurs through ingestion of OBs by a foraging caterpillar (Fig. 2, step 1 and 2). The alkaline pH of the midgut dissolves the OB protein matrix, causing the release of ODVs (Fig. 2, steps 3 and 4). These ODVs then cross the peritrophic membrane, a chitin and protein layer lining the midgut (Hegedus et al. 2009), after which primary infection of midgut epithelial cells can take place (Miller 1997). After an initial round of virus replication in the nucleus of these cells the production of BVs starts (Fig. 2, step 5), although several studies suggest that BVs may also be produced by simply 'repackaging' ODV-derived nucleocapsids into BVs, thus circumventing the need for virus replication (Granados & Lawler 1981; Washburn et al. 2003a; Washburn et al. 2003b). BVs are transported to other tissues via haemolymph (circulatory fluid) and the trachea (respiratory system), causing the infection to spread through the whole insect. In the last phase of infection OB production occurs in the nuclei of these secondary infected cells. After the insect has succumbed to the infection, breakdown of internal structures by viral enzymes such as chitinase and cathepsin causes total rupture of the carcass (Hawtin et al. 1997), ensuring optimal release of newly formed OBs into the environment. Depending on the host species and developmental stage of the larvae, virus yields of up to 10^{10} OBs per larva have been reported (Entwistle & Evans 1985).

Baculoviruses share a very long co-evolutionary history with their insect hosts (Herniou et al. 2004). Ancestral dsDNA viruses first evolved with the first insects approximately 310 million years ago (Thézé et al. 2011), which makes the family *Baculoviridae* older than current age estimates for any other virus family. Subsequent diversification of holometabolous insects (insects that undergo complete metamorphosis), to which also insects in the orders Lepidoptera, Diptera and Hymenoptera belong, appears to have occurred in the same time frame as the diversification of baculoviruses (Herniou et al. 2004; Thézé et al. 2011). However, on a smaller scale, the phylogeny of baculoviruses does not exactly mirror the phylogeny of their hosts, suggesting that these viruses may have colonized new insect hosts many times during their evolution (Thézé et al. 2011).

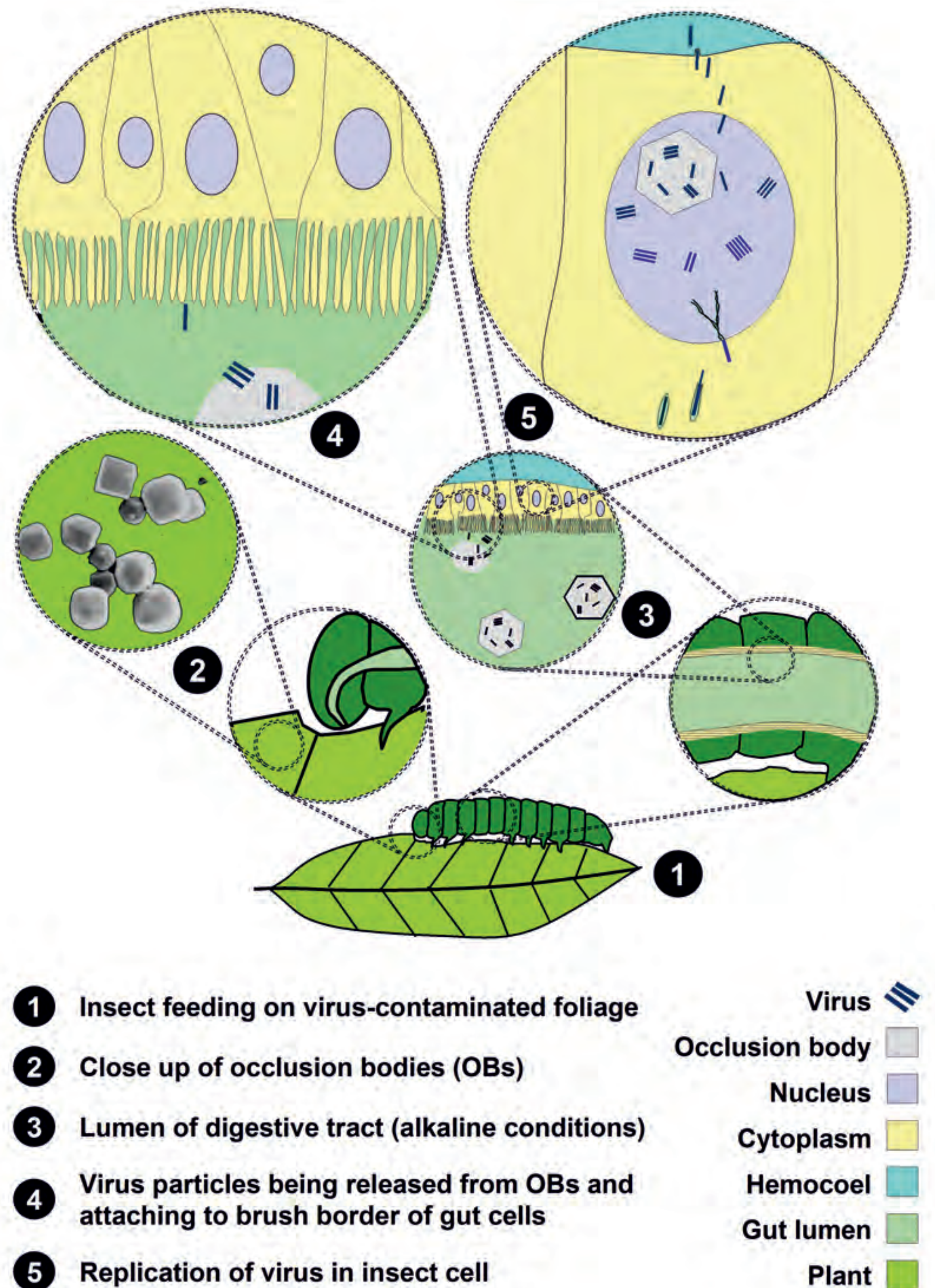


Figure 2. The infection cycle of a nucleopolyhedrovirus (NPV) in a lepidopteran host (*figure used with permission from Dwight Lynn*).

Based on their phylogenetic relationship four genera of baculoviruses are distinguished: (i) the genus *Alphabaculovirus* consisting of lepidopteran-specific NPVs, (ii) the genus *Betabaculovirus* consisting of lepidopteran-specific GVs, (iii) the genus *Gammabaculovirus* comprising hymenopteran-specific NPVs, and (iv) the genus *Deltabaculovirus* comprising dipteran-specific NPVs (Jehle et al. 2006). Members of the genus *Alphabaculovirus* are subdivided into group I and group II NPVs (Zanotto et al. 1993). Both of these groups comprise single NPVs (SNPVs), containing a single nucleocapsid within an ODV, and multiple NPVs (MNPVs) containing multiple nucleocapsids within an ODV (reviewed in Blissard (1996)). These two phenotypes (SNPVs and MNPVs) have no taxonomic meaning and the genetic basis for these phenotypes is unknown.

The insect hosts – an introduction

In the research presented in this thesis two lepidopteran host species were used, which are introduced below.

Spodoptera exigua

Spodoptera exigua (Fig. 3) is also known as the beet armyworm, a name derived from the caterpillars' 'army'-like behaviour in the field. A group of larvae eats every single plant in a patch until supplies are exhausted, after which all larvae 'march' to a new field patch. *Spodoptera exigua* is a polyphagous pest insect that causes enormous damage to several

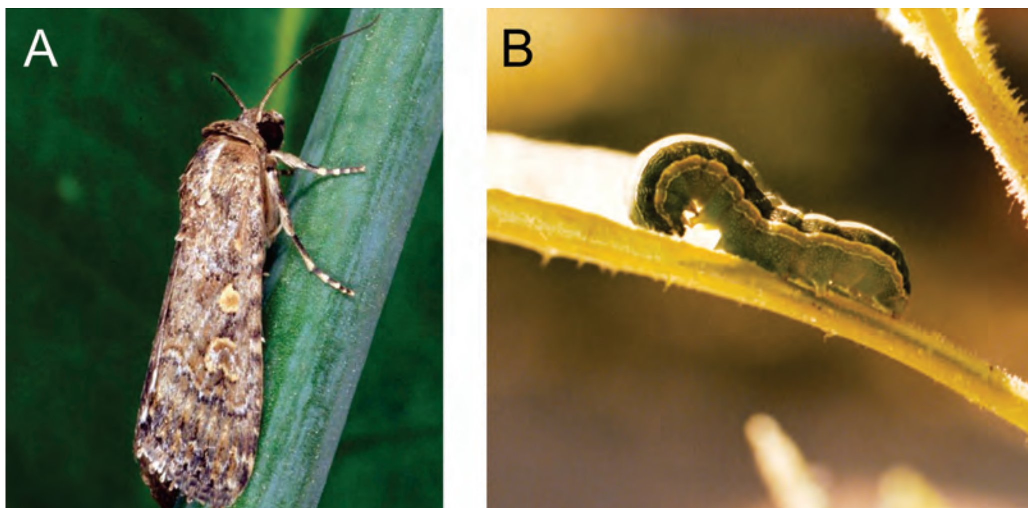


Figure 3. (A) Adult *Spodoptera exigua* moth (*photo credits: Merle Shepard, www.bugwood.org*); (B) *S. exigua* 5th instar larva (*picture from: Plos Biol (2004) 2(8): e250*).

cultivated crops and flowers, including beet, lettuce, tomato and chrysanthemum (Hill 1983; Smits et al. 1987). It occurs in many tropical and subtropical areas worldwide, and is nowadays also a serious pest in greenhouses (Smits et al. 1987). *Spodoptera exigua* belongs to the lepidopteran subfamily Noctuidae. At 27 °C, the generation time in the laboratory is approximately 20 days, in which the animals normally go through five larval stages (named L1 to L5, or 1st to 5th instar), a pupal and an adult stage. Adult female moths (Fig. 3A) commonly lay their eggs in clusters of 50 to 150 eggs on the underside of lower plant leaves within 10 cm from the soil surface (Fye & McAda 1972; Smits et al. 1987). At this temperature eggs hatch after 2-3 days. The development time for the L1 to L5 stages is 2, 1.5, 1.5, 2 and 3-4 days, respectively. While the younger larvae (L1 and L2) feed gregariously, a transition to solitary behaviour occurs in L3 larvae. L4 and L5 larvae (Fig. 3B) are entirely solitary feeders (Poe et al. 1973). Pupation takes place in the soil (Capinera 2001; Smits et al. 1987). The two baculovirus species used in this thesis, *Autographa californica* (Ac) MNPV and *S. exigua* (Se) MNPV, are both infectious to *S. exigua*. SeMNPV is a specialist virus that is highly pathogenic to its only host *S. exigua*. In contrast, AcMNPV is a generalist baculovirus that can infect over 32 different insect species from at least 15 families within the Lepidoptera (Cory & Myers 2003; Goulson 2003; Groener 1986). AcMNPV is less infectious to *S. exigua* larvae than SeMNPV.

Trichoplusia ni

Trichoplusia ni (Fig. 4) is commonly known as the cabbage looper, a name that derives from the way the caterpillar 'loops' its body during movement (Fig. 4B). Like *S. exigua*,

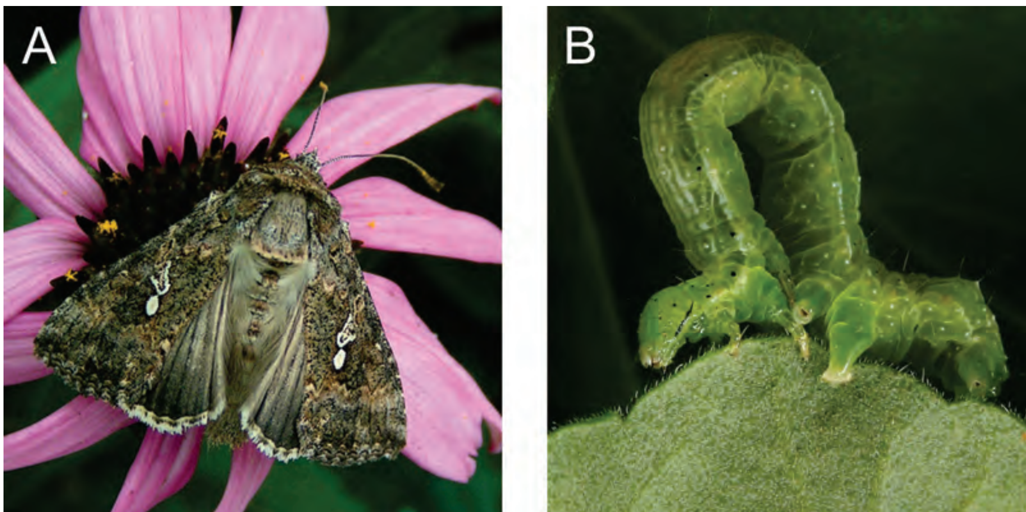


Figure 4. (A) Adult *Trichoplusia ni* moth (*photo credits: J. Clucier, www.fotopedia.com*); (B) *T. ni* 5th instar larva (*photo credits: David Cappaert, www.bugwood.org*).

it is a polyphagous insect that belongs to the Noctuidae. It is reported to feed on plants of 28 different families, including crops like cabbage, tomato and cucumber (Capinera 2001; Stamp & Casey 1993). At 22 °C, the generation time in the laboratory is approximately 33 days, in which the animals commonly have five larval stages, a pupal and an adult stage. In contrast to *S. exigua*, *T. ni* moths (Fig. 4A) lay their eggs singly on the upper side and underside of leaves (Jackson et al. 1969). At this temperature, eggs hatch after 4 days. The development time for the L1 to L5 stages is 2, 2, 2, 3 and 4 days respectively. At pupation a white web structure is produced on the underside of leaves in which the pupa is woven (Capinera 2001). *Trichoplusia ni* larvae are not susceptible to the abovementioned specialist SeMNPV, but are highly susceptible to the generalist AcMNPV.

Baculoviruses and parasitic manipulation – where do we stand?

Manipulation of host behaviour by a parasite, commonly referred to as parasitic manipulation, appears to be a widespread strategy of parasites to enhance their transmission and survival. One of the key questions concerns the mechanism that underlies these behavioural changes: Are there specific parasite genes that induce these behaviours, and if so, do they divert existing host behaviours to their own benefit? A detailed review on the concept of parasitic manipulation and on the current knowledge of these mechanisms will be given in Chapter 2.

Among the many types of parasites known to manipulate host behaviour, baculoviruses are an elegant model system for studying the mechanisms leading to parasitic manipulation. Unlike most manipulative parasites, well-developed genetic tools are available for baculoviruses, allowing the study of single viral genes for their potential role in behavioural manipulation. Furthermore, many baculovirus host insect species can be reared under laboratory conditions and are readily available. Lastly, compared to other parasites known to manipulate host behaviour, the cellular and molecular aspects of baculovirus infection in lepidopteran hosts are known in relatively great detail. This is largely attribu table to the long-time scientific interest in baculoviruses for their use as biological control agents and as expression vectors for heterologous protein production. It is partially due to these developments that some information is available on how baculoviruses exert their role as behavioural manipulators, as outlined below. This knowledge provides an excellent starting point to further explore the mechanism(s) underlying baculovirus-induced manipulation of host behaviour at the genetic and molecular level.

After the aforementioned description of behavioural alterations in baculovirus-infected

caterpillars by Hofmann (Hofmann 1891), several authors have reported higher dispersal rates of infected caterpillars as compared to healthy ones and aberrant climbing behaviour of infected caterpillars leading to death at elevated positions (Evans & Allaway 1983; Smirnoff 1965). The first quantitative studies on baculovirus-induced changes of host behaviour were performed using *Mamestra brassicae* larvae infected with the baculovirus *M. brassicae* (Mb) MNPV (Goulson 1997; Vasconcelos et al. 1996). Infected larvae were found to move three to five times further than uninfected ones, and the majority of larvae died on the apical, more exposed part of plant leaves. Strikingly, at that time the scientific interest in the concept of behavioural manipulation by parasites – the idea of an animal's brain controlled by a parasite – was minimal. Most studies concerning baculoviruses and their ability to alter host behaviour were focusing on their potential contribution to the development of insect pest biocontrol programs. For a long time, nothing was known about the proximate mechanisms that might govern these behavioural changes. In 2005, Kamita et al. identified the first 'behavioural' gene in a baculovirus by demonstrating the involvement of the protein tyrosine phosphatase (*ptp*) gene from *B. mori* (Bm) NPV in hyperactive behaviour in larvae of the silkworm *B. mori* (Kamita et al. 2005). Furthermore, a study on *Lymantria dispar* (Ld) MNPV showed that the baculovirus ecdysteroid UDP-glucosyl transferase (*egt*) gene is required for death at elevated positions (tree-top disease) of gypsy moth (*L. dispar*) larvae (Hoover et al. 2011).

Although these studies certainly paved the road towards a deeper understanding of the mechanisms governing baculovirus-mediated behavioural manipulation, many questions remain to be answered and many intriguing new questions arise from these findings. For example, the intermediate steps occurring between expression of the viral 'behavioural' gene and manifestation of behavioural manipulation are unknown. What are the molecular factors, be it host and/or viral, affected by the behavioural gene product? Do these factors exert a direct effect in the brain of an infected individual or is the virus-induced signal somehow transmitted to the brain, for example through secretion of a (neuro-)hormone? Thinking at a more evolutionary level, it is unclear how well-conserved these manipulative strategies are among members of the *Baculoviridae f*amily. Is behavioural manipulation only seen in virus-host associations that share a long and intimate co-evolutionary history or is it a general response? Does a single baculovirus species induce distinct behavioural changes dependent on the host species and ecology of that host? And what is the adaptive value of inducing hyperactivity and tree-top disease, if any?

Outline of the thesis

This thesis investigates possible mechanisms that baculoviruses use to induce behavioural changes in caterpillar hosts. While it touches upon several of the questions asked above, the thesis concentrates on the following main topics: i) the molecular mechanism that underlies hyperactive behaviour induced by the viral protein tyrosine phosphatase (*ptp*) gene; ii) understanding whether hyperactivity and tree-top disease are independent strategies for which distinct virus genes are responsible; and iii) the mechanisms that underlie tree-top disease in different lepidopteran hosts.

In **Chapter 2** the concept of parasitic manipulation is introduced and the currently known examples of behavioural manipulation by parasites in invertebrates are reviewed. Furthermore, several host genes are discussed that are known behavioural determinants and therefore could be suitable targets for parasites to enable behavioural changes.

In **Chapter 3** the hypothesis is tested that baculovirus protein tyrosine phosphatase (*ptp*)- induced hyperactivity is a conserved strategy of group I NPVs to manipulate insect host behaviour. The *ptp* gene in BmNPV was previously shown to cause enhanced larval dispersal during infection of *B. mori* (Kamita et al. 2005). As a homolog of this gene is present in AcMNPV, the involvement of *ptp* in hyperactive behaviour of *S. exigua* larvae is experimentally investigated.

The finding that the PTP phosphatase activity is needed for the induction of hyperactivity by AcMNPV, led to the question which (host) proteins are targeted by PTP to achieve this behavioural change. **Chapter 4** describes the identification of host and viral proteins that interact with the AcMNPV PTP protein. This is achieved by purification of PTP from infected insect cells and subsequent identification of co-purified proteins using proteomic analysis.

Within the baculoviruses two different genes are found that encode protein tyrosine phosphatases, *ptp* and *ptp2*. While the *ptp* gene induces hyperactivity (described in Chapter 3), no function has yet been assigned to the *ptp2* gene. To obtain more insight in its putative function during baculovirus infection, a molecular characterization of the baculovirus *ptp2* gene was performed, which is described in **Chapter 5**. This characterization was done using a variety of methods, including phylogenetic inference, substrate analysis and transient expression assays in insect cells.

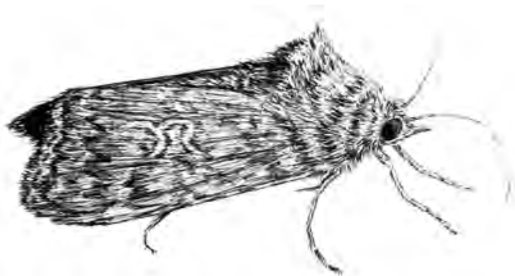
In addition to the induction of hyperactive behaviour in caterpillars during baculovirus

infection, the induction of tree-top disease was also investigated. **Chapter 6** deals with the question whether hyperactivity and tree-top disease are induced by the same mechanism, or whether these two behaviours have a distinct genetic basis. This question is addressed by investigating whether AcMNPV *ptp*, a known inducer of hyperactivity, is involved in inducing tree-top disease in the host *S. exigua*.

Chapter 7 aims to answer the question whether tree-top disease induced by the generalist baculovirus AcMNPV is dependent on the host species, *S. exigua* and *T. ni*. Furthermore, the question is addressed whether the baculovirus *egt* gene, known to be necessary for death at elevated positions in another baculovirus-host system (Hoover et al. 2011), plays a role in the observed tree-top disease induced by AcMNPV.

Besides the involvement of the baculovirus AcMNPV in behavioural manipulation, the effect of the specialist baculovirus *S. exigua* (Se) MNPV on tree-top disease in the host *S. exigua* was studied (**Chapter 8**). In this chapter the hypothesis was tested that SeMNPV-induced tree-top disease is a light-dependent response.

Finally, in **Chapter 9** the results of the different research chapters described in this thesis are discussed and the findings are placed in a broader perspective on parasitic manipulation. Furthermore, an outlook and some directions for future research are given.



Chapter

2

Walking with insects: Molecular mechanisms behind parasitic manipulation of host behaviour

This chapter has been published as:

Stineke van Houte*, Vera I.D. Ros* and Monique M. van Oers (2013) Walking with insects: molecular mechanisms behind parasitic manipulation of host behaviour. *Molecular Ecology* 22, 3458-3475.

* These authors contributed equally to this paper

Abstract

Parasitic infections are often followed by changes in host behaviour. Numerous and exquisite examples of such behavioural alterations are known, covering a broad spectrum of parasites and hosts. Most descriptions of such parasite-induced changes in host behaviour are observational reports, while experimentally confirmed examples of parasite genes inducing these changes are limited. In this paper we review changes in invertebrate host behaviour observed upon infection by parasites, and discuss such changes in an evolutionary context. We then explore possible mechanisms involved in parasite-induced changes in host behaviour. Genes and pathways known to play a role in invertebrate behaviour are reviewed, and we hypothesize how parasites (may) affect these pathways. This review provides the state of the art in this exciting, interdisciplinary field by exploring possible pathways triggered in hosts, and suggesting methodologies to unravel the molecular mechanisms that lead to changes in host behaviour.

Introduction

Parasites often have profound effects on the animal hosts they invade by affecting development, physiology, morphology, evolution and ecology (Beckage 1997; Lefèvre et al. 2009a; Lefèvre et al. 2009b; Price 1980; Ros et al. 2008; Thomas et al. 2010). In many cases, host behaviour is also altered upon parasitic infection (Beckage 1997; Lefèvre & Thomas 2008; Libersat et al. 2009; Moore 2002). These changes range from slight alterations of already existing behavioural traits to the exhibition of completely new activities. Fascinating examples include *Toxoplasma*-infected rodents that lose their innate aversion to cats (Berdoy et al. 2000), Gordian worm-infected crickets committing suicide (Thomas et al. 2002), and lancet liver fluke-infected ants climbing into grass blades (Hohorst & Graefe 1961; Libersat et al. 2009; Moore 1995). These observed changes in host behaviour are often thought to be beneficial to the parasite as they may increase the rate of transmission and survival. Parasites that change host behaviour are diverse and comprise viruses, bacteria and a wide range of eukaryotes, including fungi, protozoa, parasitoids (including parasitic wasps) and parasitic worms. The latter encompass nematodes (roundworms), trematodes (flukes), cestodes (tapeworms), nematomorphs (Gordian worms or horsehair worms), and acanthocephalans (thorny-headed or spiny-headed worms). Besides the ability of parasites to manipulate host behaviour, an increasing body of evidence suggests that non-parasitic microbes (e.g. so-called 'beneficial microbes' like gut microbes) can also alter host behaviour (see Ezenwa et al. (2012) for a review).

Most descriptions of parasite-induced changes in host behaviour are observational reports, while experimentally confirmed examples of parasite genes inducing these changes are limited (Hoover et al. 2011; Kamita et al. 2005; Lefèvre et al. 2009a; Lefèvre et al. 2009b; Libersat et al. 2009; van Houte et al. 2012). As a consequence, the underlying mechanisms behind parasite-induced behavioural alterations remain enigmatic. In addition, little is known on host pathways translating the parasite-induced signal to a particular behaviour. Insights into such mechanisms should provide important knowledge on the evolution of parasitic manipulative strategies and on animal behaviour in general. However, unravelling these mechanisms is a challenging task as both the processes by which the behavioural changes are induced and the behaviour itself are often complex and multidimensional (Thomas et al. 2010). For example, a single host may be infected by multiple parasites, and a single parasite can induce multiple behavioural alterations in a host.

This review describes different aspects of changes in invertebrate host behaviour observed

upon infection by parasites, illustrated with several appealing examples. The adaptiveness of changes in host behaviour is addressed, and the concepts of multidimensionality and convergence of behavioural alterations are discussed. Possible mechanisms involved in parasite-induced changes in host behaviour are explored, and genes and pathways known to play a role in invertebrate behaviour (in non-parasitised conditions) are discussed. As the components of such pathways provide excellent entries for parasites to change host behaviour, we hypothesize how parasites (may) affect these pathways. We did not aim to provide an exhaustive research of all available examples of behavioural alterations upon parasitic infection (see Moore (2002) for an excellent review). Since the majority of the published data on this topic concerns invertebrates, vertebrate behaviour will not be addressed. For the latter we refer to reviews by Klein (2003) and Tomonaga (2004).

On the extended phenotype and (non-)adaptiveness

In an evolutionary context, changes in host behaviour upon parasite infection are examples of an extended phenotype, a concept introduced by Dawkins (1982). He stated that the observed host phenotype is a consequence of a parasite's gene being expressed. As such, parasitic manipulation can be defined as the alteration by the parasite of a host phenotypic trait in a way that enhances the parasite's probability of transmission and survival (Hughes 2013; Lefèvre & Thomas 2008; Thomas et al. 2012). This can be achieved for example by directly enhancing host-to-host transmission, by increasing the chance of finding a mate, or by dissemination of the parasite in a suitable location. In this view, changes in host behaviour are adaptive to the parasite.

However, observed changes in host behaviour are not necessarily beneficial to the parasite. Such changes may as well be adaptive to the host, aimed at reducing the fitness costs of infection, or may be pathological side-effects (Moore 2013; Poulin 2010; Thomas et al. 2012). Host adaptive changes are for example seen in carpenter ants (*Camponotus aethiops*) that upon infection by the pathogenic fungus *Metarhizium brunneum* become unsociable to reduce the risk of dissemination of infection in the entire colony (Bos et al. 2012). Other host adaptive strategies include self-medication (e.g. feeding on compounds toxic to parasites) or behavioural fever (seeking temperature conditions unfavourable for parasite growth) to reduce parasitic load (see de Roode & Lefèvre (2012) for a review).

Other theories elaborate on the existence of alternative adaptive strategies. For example, infected hosts might apply a 'mafia-like' strategy where they cooperate with manipulative parasites instead of resisting them, thereby reducing fitness costs associated with manipulation (when cooperation is less costly than resistance) (Lefèvre et al. 2009a; Ponton et

al. 2006). Likewise, parasites might exploit host compensatory responses to their own benefit (Lefèvre et al. 2009a; Lefèvre & Thomas 2008). Although well described theoretical frameworks exist for such strategies, experimental evidence for their existence is lacking and hard to obtain.

In many cases behavioural alterations upon infection are intuitively interpreted as being adaptive to the parasite. Ideally, such assumptions require experimental evidence to be critically evaluated. However, this may be a challenging task, since measuring fitness of host (e.g. survival rates or fecundity) and parasite (e.g. transmission rates) is difficult in many host-parasite systems (Moore 2013; Poulin 2010). Throughout this paper we will use the term 'parasitic manipulation' in cases where this seems applicable, even in cases where evidence that changes in host behaviour are actually adaptive to the parasite might be lacking.

Even if host behavioural changes are a result of manipulation by parasites, the observed changes should be regarded as a shared phenotype resulting from the expression of host and parasite genes. This shared phenotype is the result of an evolutionary arms race, and is determined by changes induced by the parasite and counteracted by the host (Lefèvre et al. 2008; Lefèvre & Thomas 2008; Poulin et al. 1994). The actual outcome and magnitude of changes might vary over time and space, and is dependent on many factors, including host and parasite genetics and non-genetic factors like age, developmental stage, parasitic load, time after infection and environmental conditions (Moore 2013; Poulin et al. 1994; Thomas et al. 2011).

Multidimensionality: multiple alterations and multiple parasites

Instead of regarding parasitic manipulation as a 'simple' alteration of a single host beha vioural trait, it should be seen as a multidimensional phenomenon (Cézilly et al. 2013; Thomas et al. 2010; Thomas et al. 2012). Single parasites may cause multiple behavioural alterations within a host (simultaneously or sequentially), and, likewise, a single host can be infected by more than one parasite at the same time (Cézilly et al. 2013; Cézilly et al. 2000; Lefèvre & Thomas 2008; Perrot-Minnot 2004). For example, the cockroach *Periplaneta americana* can be parasitised by the parasitoid *Moniliformis moniliformis*, leading to increased locomotion activity (Moore 1983; Wilson & Edwards 1986), while the same cockroach species can be parasitised by the tropical wasp *Ampulex compressa*, inducing a zombie-like state and preventing it from any spontaneous locomotion (Gal & Libersat 2008). These parasites may have conflicting interests, and in case of co-parasiti-

sation this might be expressed in different manipulation patterns than for singly infected hosts. Parasites could for instance sabotage the manipulation induced by other parasites (Cézilly et al. 2013; Haine et al. 2005; Thomas et al. 2011). On the other hand, effects may become additive, leading to enhanced (behavioural) changes, or some parasites may profit from changes induced by other parasites. Also, the presence of one parasite might prevent infection or manipulation by another parasite. *Wolbachia*, for example, has been reported to reduce (Hedges et al. 2008; Martinez et al. 2012) or increase (Graham et al. 2012) the susceptibility of its host to virus infections. Therefore, when studying host manipulation, it should be taken into account that hosts might harbour more parasites that can influence the outcome of the studied manipulation.

Convergence: similar manipulations in different systems

Different parasites may encounter similar selective pressures when infecting a host, and consequently may develop similar strategies to manipulate host behaviour (Ponton et al. 2006; Poulin 1998). Such convergent manipulative behaviour can be a consequence of similar proximate mechanisms, but can also be achieved by different mechanisms. Gordian worms (phylum Nematomorpha) induce suicidal behaviour in some arthropods, forcing these hosts to drown themselves by jumping into water, where the worms are released to mate (Thomas et al. 2002). A similar behaviour is observed for ants infected by mermithids (phylum Nematoda, unrelated to nematomorphs) (Maeyama et al. 1994). Another example concerns two other unrelated parasites, the trematode *Microphallus papillorobustus* and the acanthocephalan *Polymorphus minutus* that both manipulate the behaviour of gammarid species (small freshwater crustaceans) by inducing them to move towards the water surface, where they are easily visible to aquatic birds that serve as the final hosts. In both parasite-gammarid associations, similar proteins are involved, suggesting molecular convergence in the proximate mechanisms of these manipulations (Ponton et al. 2006). Climbing behaviour upon infection is also observed in different systems, including caterpillars infected by baculoviruses (Hoover et al. 2011), ants infected by *Ophiocordyceps* fungi (Hughes et al. 2011) or by lancet liver flukes (*Dicrocoelium dendriticum*) (Hohorst & Graefe 1961; Libersat et al. 2009). Whether these alterations are caused by similar mechanisms is not yet clear.

In contrast to the above, related parasites may rely on different mechanisms to alter host behaviour. Baculoviruses induce enhanced locomotion activity and climbing behaviour in caterpillars (Fig. 1A). For two of these viruses (*Bombyx mori* nucleopolyhedrovirus (BmNPV) and *Autographa californica* nucleopolyhedrovirus (AcMNPV)) the viral protein tyrosine phosphatase (*ptp*) gene was found to be responsible for inducing

enhanced locomotion activity in insect hosts (*Bombyx mori* and *Spodoptera exigua* larvae, respectively) (Kamita et al. 2005; van Houte et al. 2012). However, the underlying mechanism appears to be different; while in AcMNPV the phosphatase function of the encoded PTP protein is crucial for behavioural manipulation (van Houte et al. 2012), this is not the case for BmNPV. In the latter case absence of the PTP protein affected viral gene expression levels, possibly leading to the observed behavioural change through an unknown mechanism (Katsuma et al. 2012). Although the involvement of PTP in behavioural changes might have a common origin, the exact mechanism might have diverged over time.

Phylogenetic analyses can give valuable insights into the evolutionary history of behavioural manipulations, and may help to understand whether certain manipulations have a common origin or evolved independently. In addition, it can be useful to assess the adaptive significance of such manipulations (Moore & Gotelli 1996; Poulin 1998). If similar changes in behaviour are observed in two distantly related host species, induced by two distantly related parasite species, this strongly directs at an adaptive significance (to either host or parasite) of the behavioural change.

Changes in host behaviour

The bodyguard: using the host to avoid enemies

Some parasites turn their host into a bodyguard to ensure protection of the parasite from enemies like predators or hyperparasitoids (Grosman et al. 2008; Harvey et al. 2008; Maure et al. 2011). For example, *Thyrinteina leucocerae* caterpillars protect pupae of the braconid parasitoid *Glyptapanteles sp*. (Grosman et al. 2008). Once the parasitoid larvae leave the host to pupate, the host defends the pupae by knocking off predators with violent head-swings, resulting in reduced mortality rates of the parasitoid pupae. A similar phenomenon is observed with *Pieris brassicae* caterpillars parasitised by the braconid parasitoid *Cotesia glomerata*. Upon parasitoid egression from the host, the caterpillar spins a silk web over the parasitoid pupae, and responds aggressively when disturbed, thus protecting the pupae from hyperparasitoids and predators (Brodeur & Vet 1994; Harvey et al. 2008). Bracoviruses and ichnoviruses (*Polydnaviridae*) appear to play beneficial roles in the development of some braconid and ichneumonid parasitoids (Burke & Strand 2012). The genomes of these viruses contain many *ptp* genes (although they seem to be evolutionarily unrelated to the baculovirus *ptp* gene inducing hyperactive behaviour), of which at least some play a role in immunomodulation of the caterpillar host (Falabella et al. 2006; Ibrahim & Kim 2008; Suderman et al. 2008). It is unknown

whether these polydnaviral *ptp* genes play a role in the observed behavioural alterations in caterpillars infected by parasitoids.

A change in behaviour presumably aimed to reduce hyperparasitism and predation is also seen in parasitised *Macrosiphum euphorbiae* aphids, with a different behavioural change depending on the physiological state of the parasitoid *Aphidius nigripes*. Aphids containing non-diapausing parasitoids move to the upper surface of leaves to mummify, while those containing diapausing parasitoids leave the host plant and mummify in concealed sites. In both cases the parasitoids appear to seek the optimal microhabitat for survival (Brodeur & McNeil 1989, 1992).

It should be noted, however, that host predation is not always detrimental to the parasite, and in some cases could even be advantageous. Host predation can increase parasite transmission, as is seen in baculovirus-infected caterpillars that climb to the top of plants, where they are more visible to birds that predate on caterpillars. Baculoviruses survive a passage through the bird gut (where the pH is lower than in the larval midgut), and consequently the viruses disseminate more widely in the environment (Vasconcelos et al. 1996).

Where to go? Changes in locomotion behaviour and phototactic or geotactic behaviour

Many parasites alter their host's locomotion behaviour, by changing the speed or the direction of locomotion (including climbing behaviour, described below). This might increase the area over which the parasite (e.g. fungal spores or virus particles) is spread and increase the probability of finding a new host or a suitable place for survival. A change in host behaviour can also be adaptive to the host, for example to prevent contamination of conspecifics. Changes in the direction of locomotion might be a response to e.g. gravity (geotaxis), light stimuli (phototaxis), or odours. A clear example of manipulation of host locomotion is observed in caterpillars infected with baculoviruses (see above).

Another fascinating and well-described case is the previously mentioned water-seeking behaviour of arthropods infected with a Gordian worm (Thomas et al. 2002) (Fig. 1B). A recent study demonstrated that the water-seeking behaviour is the consequence of an altered response to light (positive phototaxis), combined with an increase in locomotion activity (Ponton et al. 2011). The behaviour is time-regulated and is only observed at night (not during the day or after 2-3 am). By using a parasitoproteomics approach, Biron et al. (2005) found that during a Gordian worm (*Spinochordodes tellinii*) infection of a grasshopper (*Meconema thalassinum*) a protein (CG31732-PD (isoform D)) was

differentially expressed in the host's central nervous system (CNS). This protein is known to be involved in control of geotactic behaviour, suggesting that it may play a role in the observed behaviour (Biron & Loxdale 2013).

Several examples of other parasitic worms manipulating host locomotion behaviour have been reported (reviewed in Adamo (2002)). The family Gammaridae comprise small freshwater crustaceans, which are intermediate hosts for parasitic worms, including the acanthocephalans *P. minutus* and *Pomphorynchus laevis*, and the trematode *M. papillorobustus* (Bethel & Holmes 1977; Cézilly et al. 2000; Helluy 1984). Uninfected gammarids dive downwards when disturbed and cover themselves in the mud. On the other hand, gammarids infected by parasitic worms (Fig. 1C) glide along the water surface to seek a

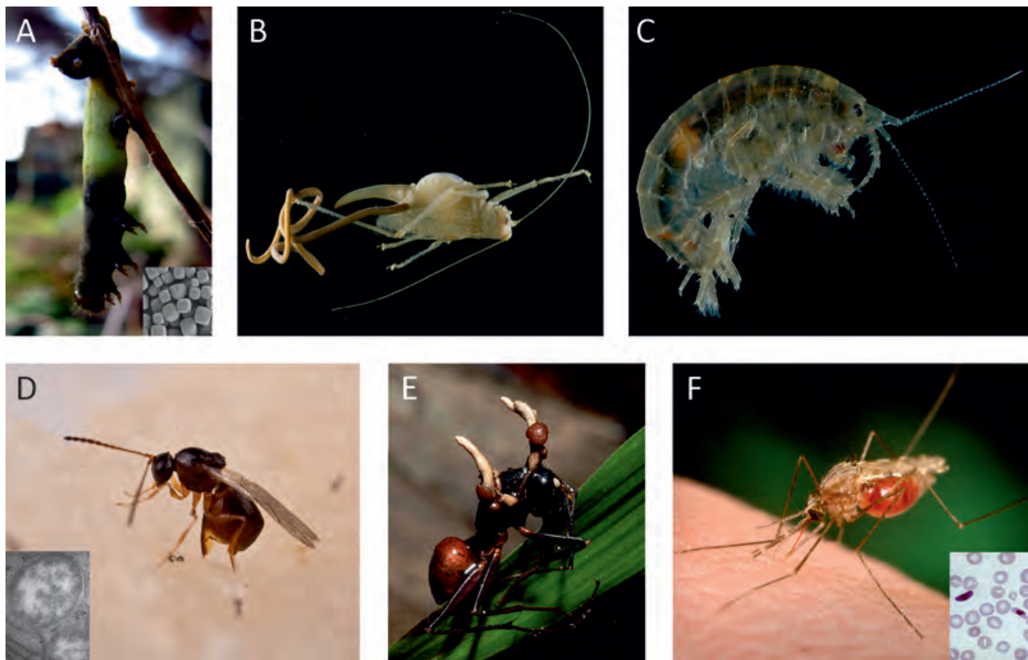


Figure 1. Examples of parasitic manipulations of invertebrate behaviour. A) Baculoviruses (inset picture) manipulate locomotion and climbing behaviour of their lepidopteran hosts; B) The Gordian worm *Spinochordodes tellinii* manipulates water-seeking behaviour of its *Meconema* grasshopper host; C) The parasitic worm *Pomphorhynchus laevis* induces phototactic behaviour in its host *Gammarus pulex*; D) *Wolbachia* bacteria (inset picture) decrease locomotion activity of *Leptopilina heterotoma* wasps; E) The fungus *Ophiocordyceps* induces climbing behaviour of *Camponotus* ants; F) The protozoa *Plasmodium falciparum* (inset picture) alters blood-seeking and feeding behaviour of the insect vector *Anopheles gambiae*. [*Photo credits: A) W.I. Tyne (caterpillar); Jan van Lent, Wageningen Electron Microscopy Centre (baculovirus); B) Andreas Schmidt-Rhaesa; C) F. Cézilly, UMR CNRS/Université de Bourgogne 5561 Biogéosciences; D) Hans Smid, www.bugsinthepicture.com (Leptopilina); Elliot Kitajima (Wolbachia); E) http://hortlog.blogspot.com; F) James D. Gathany/CDC (Anopheles); Mae Melvin/CDC (Plasmodium)*]

solid support. This typical clinging behaviour exposes the gammarid to predators such as frogs, fish and birds, which are the parasites' next host. In gammarids infected with *M. papillorobustus* this clinging behaviour is characterized by positive phototaxis, negative geotaxis, and aberrant evasive behaviour (Ponton et al. 2006). *Polymorphus minutus* does not induce positive phototaxis, but negative geotaxis and aberrant evasive behaviour were clearly observed in parasitised *Gammarus pulex* (Cézilly et al. 2000). A comparative proteomics study revealed an increased expression of a protein (aromatic L-amino acid decarboxylase) involved in serotonin synthesis in *Gammarus insensibilis* infected with *M. papillorobustus*, but not in *G. pulex* infected with *P. minutus*. This suggests that serotonin may function in positive phototaxis (Ponton et al. 2006). Another gammarid species, *Echinogammarus stammeri*, carrying *P. laevis* worms showed increased locomotion activity compared to conspecifics without worms (Maynard et al. 1998). This behavioural change is likely to be adaptive to the parasite, since there is increased consumption of infected compared to uninfected *E. stammeri* by the bullhead host, *Cottus gobio* (Lagrue et al. 2007).

An increase in locomotion activity has also been observed in hosts parasitised by parasitoids or infected with bacteria. The cockroach *P. americana* parasitised by the parasitoid *M. moniliformis* shows increased locomotion activity and becomes positively phototactic (Moore 1983; Wilson & Edwards 1986). In the parasitoid *Leptopilina heterotoma* (Fig. 1D), the endosymbiotic bacterium *Wolbachia* decreases locomotion activity (Fleury et al. 2000), while in flies of the genus *Drosophila* it either decreases or increases activity, depending on the *Drosophila* species and the bacterial strain (Peng et al. 2008). *Wolbachia* infection of the mosquito *Aedes aegypti* leads to increased mosquito locomotion, accompanied by an increase in metabolic rate. Since *Wolbachia* infects brain tissue (Dobson et al. 1999) it was speculated that the increase in activity may be due to physiological changes in the CNS (Evans et al. 2009).

Towards the top: enhancing parasite dispersal

Remarkable are some examples of hosts showing climbing behaviour upon infection. This behaviour has been observed for different host-parasite associations, where the induced climbing is thought to contribute to enhanced transmission of the parasite. Caterpillars infected with a baculovirus climb to the top of plants, where they eventually die and liquefy, and in the process release progeny virus (Fig. 1A) (Goulson 1997; Kamita et al. 2005; Smirnoff 1965; Vasconcelos et al. 1996). The virus has a higher chance to disseminate to lower parts of the plant when it is released from the top rather than from the lower branches (Goulson 1997; Vasconcelos et al. 1996). This behaviour has been described as 'Wipfelkrankheit' or 'tree-top disease' (Evans 1986; Smirnoff 1965). Hoover

et al. (2011) recently showed that a viral gene, ecdysteroid UDP-glucosyl transferase (*egt*) is involved in inducing tree-top disease of *Lymantria dispar* caterpillars infected with *Lymantria dispar* multiple nucleopolyhedrovirus (LdMNPV).

Another remarkable example is the fungus *Ophiocordyceps* that turns ants into zombies (Hughes et al. 2011). Infected ants drop down from canopy nests, and climb up again in understory vegetation to bite into leaf veins before dying (Fig. 1E). This death grip is timed around solar noon, and occurs at ca. 25 cm above the soil surface. This position is optimal for fungal sporulation, where the fungal fruiting body grows from the ant's brain and releases its spores (Andersen et al. 2009; Roy et al. 2006). Alterations in climbing behaviour occur in several other fungus-infected arthropods as well (see Roy et al. (2006) for a review). An example is the arctiid caterpillar *Chionarctia nivea* infected by the entomopathogenic fungus *Entomophaga aulicae* (Yamazaki et al. 2004). Sick caterpillars crawled up dead grass and herb stems to die at the highest parts, while healthy larvae wandered on the ground.

Lancet liver flukes use ants, including *Formica fusca*, as intermediate hosts between snails and cattle. The flukes encyst in the ant's haemocoel, except for one individual fluke, which enters the subesophageal ganglion (SEG) of the ant. Similar to the fungus *Ophiocordyceps*, lancet liver flukes induce the ants to climb and to anchor onto grass blades with their mandibles (Romig 1980; Schneider & Hohorst 1971). This behavioural change appears to be regulated in a clock-wise manner: ants move to the top of grass blades in the evening, and, if not consumed by cattle, descend again the next morning (Hohorst & Graefe 1961; Libersat et al. 2009).

Climbing behaviour might be related to geotactic or phototactic behaviour. In a dense canopy, light intensity is often higher at the top than at the lower parts of the canopy. Moving toward the light might be involved in climbing behaviour, although this would only be relevant during daytime. Alternatively, negative geotaxis might be involved.

Under the spell: paralysing the host

Paralysing the host is a widespread strategy among parasitoid wasps (Libersat et al. 2009). Some of these cases involve the injection of (neuro-)toxins or venoms directly into the host's nervous system (Adamo 2002; Gal et al., 2005; Libersat et al. 2009). The tropical wasp *A. compressa* induces a zombie-like state in the cockroach *P. americana*, preventing it from any spontaneous locomotion. This enables the wasp to lead the cockroach to the wasp's nest, where it lays an egg on one of the cockroach's legs and subsequently buries the cockroach in the nest. The cockroach finally serves as a food supply for the emerging

wasp larva (Gal & Libersat 2008). The authors hypothesized that this hypokinetic state is induced by neurotoxins present in the wasp's venom affecting the SEG (Gal & Libersat 2010).

The parasitoid *Cotesia congregata* feeds on larvae of the moth *Manduca sexta*. On the day prior to the emergence of the wasps' larvae, feeding and locomotion activity of parasitised *M. sexta* larvae decreases, and ceases completely until its death (Adamo 1998; Adamo et al. 1997). Removal of the host SEG restores locomotion activity, indicating that the parasite inhibits locomotion activity via a neural pathway (Adamo 2002).

Sex and the parasite: changes in reproductive behaviour

Manipulation of reproductive behaviour has been reported for several parasites. *Leptopilina boulardi* filamentous virus (LbFV) infects the parasitoid *L. boulardi* and increases the tendency of *L. boulardi* females to superparasitise *Drosophila* larvae (i.e. larvae that have already been parasitised by another female wasp) (Patot et al. 2009; Varaldi et al. 2003; Varaldi et al. 2009). Superparasitism is supposedly beneficial for the virus (Varaldi et al. 2009; Varaldi et al. 2006), since it permits horizontal transmission between *L. boulardi* embryos from different mothers. *Leptopilina boulardi* wasps use their ovipositors to detect chemical cues reminiscent of previous infestations and it was hypothesized that LbFV affects chemoreceptor neurons in the ovipositors (Varaldi et al. 2009). In addition, virus infection reduces locomotion activity of *L. boulardi* females (Varaldi et al. 2005; Varaldi et al. 2006).

The endosymbiotic bacterium *Cardinium* manipulates the oviposition choice of the parasitoid wasp *Encarsia pergandiella* (Kenyon & Hunter 2007). These parasitoid wasps are autoparasitoids; in sexual (haplodiploid) forms of this species, female (diploid) eggs are laid in nymphal whiteflies (the primary hosts), while male (haploid) eggs are laid in conspecific or heterospecific pupal parasitoid wasps developing within the whitefly cuticle (secondary hosts). *Cardinium* induces thelytokous parthenogenesis in *E. pergandiella*, by which females develop from unfertilized, haploid, eggs. Uninfected wasps lay unfertilized eggs in secondary hosts (developing into males), but for *Cardinium*-infected hosts, a shift is seen towards primary hosts, where successful development into females is possible. This change in oviposition behaviour seems to be induced by *Cardinium* (Kenyon & Hunter 2007). Likewise, the endosymbiotic bacterium *Wolbachia* has been shown to influence mate choice in spider mites (Vala et al. 2004) and fruit flies (Miller et al. 2010).

Helicoverpa zea nudivirus 2 (HzNV-2; *Nudiviridae*) infects the corn earworm moth *Heli-*

coverpa zea and has been found to alter the moth's mating behaviour (Burand et al. 2012; Burand & Tan 2006; Burand et al. 2005). HzNV-2 (a.k.a. gonad-specific virus (GSV), Hz-2V) is sexually transmitted when males mate with an infected female and transfer the virus to uninfected females in subsequent mating attempts. While uninfected females stop calling after mating, infected females continue calling (Burand et al. 2005). Similarly, healthy females mating with healthy males cease mate calling, while females mating with infected males continue calling (Burand & Tan 2006; Burand et al. 2005). Most likely, the transfer of anti-calling factors, part of the seminal fluid, is blocked by a 'virus-plug' (Burand & Tan 2006). In addition, healthy males are more attracted to infected females, which exhibit a five- to seven-fold increased pheromone production than uninfected females (Burand et al. 2005). Infected males show no preference for infected over uninfected females (Burand & Tan 2006). The observed alterations in physiology and behaviour likely enhance virus transmission (Burand & Tan 2006; Burand et al. 2005).

Some parasites are known to completely feminise their male hosts. *Wolbachia* bacteria, for example, are transmitted by females only and manipulate host reproductive behaviour to increase the number of females in a population. One way is by inducing feminisation: hosts that would develop as males develop into functional females (Engelstädter & Hurst 2009), changing them morphologically and behaviourally. The exact mechanism is unknown, but somehow *Wolbachia* interferes with the sex-determination pathway, probably by suppressing an androgenic gland (Werren et al. 2008). Feminisation is also observed for the mayfly *Baetis bicaudatus* infected with the nematode G*asteromermis sp*., where male hosts are feminised resulting in the formation of intersexes and complete sex reversals (Vance & Peckarsky 1996).

Modifying the vehicle: vector manipulation

Arthropods not only suffer from parasite infections themselves, but often serve as vectors for transmitting human and veterinary parasites from one host to another. Evidence is accumulating that such vector-borne parasites not simply use their vector as a vehicle, but also alter vector traits to increase transmission rates, including alterations in feeding and probing, locomotion, host seeking, and reproductive behaviour (Bennett et al. 2008; Hurd 2003; Lefèvre et al. 2006; Lefèvre & Thomas 2008). A better understanding of such alterations of vector behaviour and associated changes in parasite transmission may benefit the development of disease control strategies.

Arthropod-borne viruses (arboviruses) increase probing time and/or frequency of blood feeding by mosquitoes (Bennett et al. 2008; Grimstad et al. 1980; Lima-Camara et al. 2011; Platt et al. 1997). *Aedes triseriatus* infected with La Crosse virus (*Bunyaviridae*)

probes more, while engorging less blood per feeding (Grimstad et al. 1980). Dengue virus (*Flaviviridae*) causes a longer probing and feeding time in *A. aegypti* females (Platt et al. 1997) and recently, Lima-Camara et al. (2011) reported a 50% increase in locomotion activity of *A. aegypti* mosquitoes carrying this virus.

Several studies show that the protozoan *Plasmodium* (Fig. 1F), a causative agent of malaria, increases feeding frequency and feeding persistence of its mosquito vector (Anderson et al. 2000; Anderson et al. 1999; Koella & Packer 1996; Koella et al. 1998; Moore 1983). Likewise, infection with *Leishmania* parasites increases feeding persistence and enhances feeding on multiple human individuals (Rogers & Bates 2007). *Trypanosoma* parasites infecting tsetse flies (Diptera: Glossinidae) promote their transmission by manipulating tsetse feeding behaviour (van den Abbeele et al. 2010). The parasites achieve this by modifying saliva composition, resulting in a reduced anti-haemostatic potential of the saliva. This hampers feeding performance and, consequently, prolongs feeding. Such behavioural alterations are thought to enhance parasite transmission by an increased contact rate between vector and host.

Examples showing a reduction in feeding or locomotion behaviour of insect vectors are also known. Vesicular stomatitis virus (*Rhabdoviridae*) reduces blood feeding of *Culicoides sonorensis* midges significantly at the peak of the virus titer (Bennett et al. 2008). Decreased flight activity was observed for *Culex tarsalis* mosquitoes infected with Western equine encephalomyelitis virus (*Togaviridae*) (Lee et al. 2000). Flight ability is an important epidemiological factor in arbovirus transmission. Whether the last two examples are the consequence of parasitic manipulation or rather reflect pathological side-effects of infection or vector adaptations is not known.

Behavioural changes have not only been found for vectors transmitting human or veterinary parasites, but also for insect vectors transmitting plant viruses. Recently it was reported that male *Frankliniella occidentalis* thrips infected with Tomato spotted wilt virus (*Bunyaviridae*) show an increase in feeding frequency, thereby enhancing the chance of virus transmission from vector to plant (Stafford et al. 2011). Although many examples of parasites affecting vector behaviour have been described (see Lefèvre & Thomas (2008) for an overview), direct evidence that the observed changes indeed increase parasite transmission is still scarce.

Exploring molecular mechanisms behind invertebrate behaviour

Approaches to unravel underlying mechanisms

Despite the wide range of examples of manipulation of host behaviour, little is known concerning mechanisms behind these manipulations. The question arises how parasites manipulate the behaviour of their host, or more specifically, which parasite and host genes and/or proteins are involved in this change in behaviour? To date, only two parasite genes (the baculovirus genes *ptp* and *egt*) inducing host behavioural changes have been identified, alluded to above (Hoover et al. 2011; Kamita et al. 2005; van Houte et al. 2012). Also, wasp venoms are known to induce host behavioural changes (Gal & Libersat 2010), although responsible components within the venom still have to be identified. To date no host genes involved in behavioural manipulation have been identified. To comprehend mechanisms of behavioural manipulation by parasites, a thorough understanding of the genetic pathways underlying invertebrate behaviour as such – i.e. not in the context of parasitic manipulation – is required. Studying the interplay between genes and behaviour is a relatively new discipline that is rapidly expanding. A wealth of genetic information has become available with the whole-genome sequencing of invertebrate model organisms, including *Drosophila melanogaster*, *Caenorhabditis elegans* and *A. aegypti*. This knowledge, combined with insights from neurobiology, provide a firm base for research on behavioural genetics. In recent years, several genes have been found to play a role in the modulation of specific behaviours (reviewed in Sokolowski (2001)) and many pathways of behavioural adaptation appear to be conserved across species and, in some cases, even across phyla (Beck 2006). This high degree of conservation has led to the implementation of the candidate gene approach in the field of behavioural genetics (Fitzpatrick et al. 2005; Hoedjes et al. 2011). This is an experimental approach based on the assumption that the involvement of a specific gene in a behavioural phenotype is conserved among organisms. The foraging (*for*) gene, encoding protein kinase G (PKG), was the first example of a gene implicated in feeding-dependent locomotion across species (Fitzpatrick & Sokolowski 2004) (Fig. 2 and see below).

The fact that many pathways underlying particular behavioural traits are highly conserved renders these pathways suitable targets for manipulation by parasites. We therefore propose to extend the candidate gene approach to the level of parasitic manipulation. Not only the role of a specific gene in determining a behavioural phenotype is assumed to be conserved among different species, but the pathways that are targets for behavioural manipulation by parasites may also be conserved. Although exact mechanisms of interference with these pathways may vary among parasites, this approach aids in identifying

potential manipulation targets exploited by parasites.

The main drawback of this approach is that it provides a relatively narrow view and only those pathways already known to be implicated in behaviour can be considered. To reduce the risk of overlooking (unknown) pathways, additional genome- and proteome-wide approaches therefore need to be exploited. Highly sensitive and quantitative transcriptomic and proteomic analyses are relatively new disciplines that have become very important in studying parasite-host interactions at a molecular level. Techniques used for gene expression profiling include serial analysis of gene expression (SAGE) or deep sequencing of transcriptomes that may be combined with microarrays for more precise quantification (Knox & Skuce 2005; Ramsay 1998; Yoshino et al. 2001). Such approaches provide differential expression profiles between parasitised/infected and uninfected individuals, revealing genes affected by parasitism, although not necessarily with a relation to host behaviour. If the parasite gene(s) that induces a behavioural trait is known, a more directed search is possible by comparing individuals infected with a 'wild type' parasite with individuals infected with a 'mutant' parasite in which the gene of interest is knocked out.

Proteomic quantification methods have the advantage not only to show whether a gene is expressed, but also provide information on protein levels and/or modifications. Such data are highly relevant since many signalling pathways use alterations in the phosphorylation status of proteins as a signal. 'Parasitoproteomics' aiming to identify peptides expressed by the host and the parasite, and their possible cross-talk, has already been applied in several parasite-host systems (Biron et al. 2005; Kariithi et al. 2011; Lefèvre et al. 2009a; Ponton et al. 2006). An example relevant to behavioural manipulation is the proteomic analysis of insects infected and manipulated by Gordian worms (see above), revealing the presence of parasite proteins that mimicked host proteins belonging to the Wnt family, parasite proteins involved in neurotransmitter release, and a host protein involved in geotactic behaviour (Biron et al. 2005; Biron et al. 2006).

Both genome- and proteome-wide approaches often render complex data sets, from which the genes/proteins/pathways that are related to changes in behaviour, need to be extracted. Whatever strategy is chosen, the candidate gene approach may assist in selecting particular pathways exploited by parasites for the purpose of manipulating host behaviour. Below an overview is given of possible points of interference and a schematic on how the various pathways could be interconnected (Fig. 3).

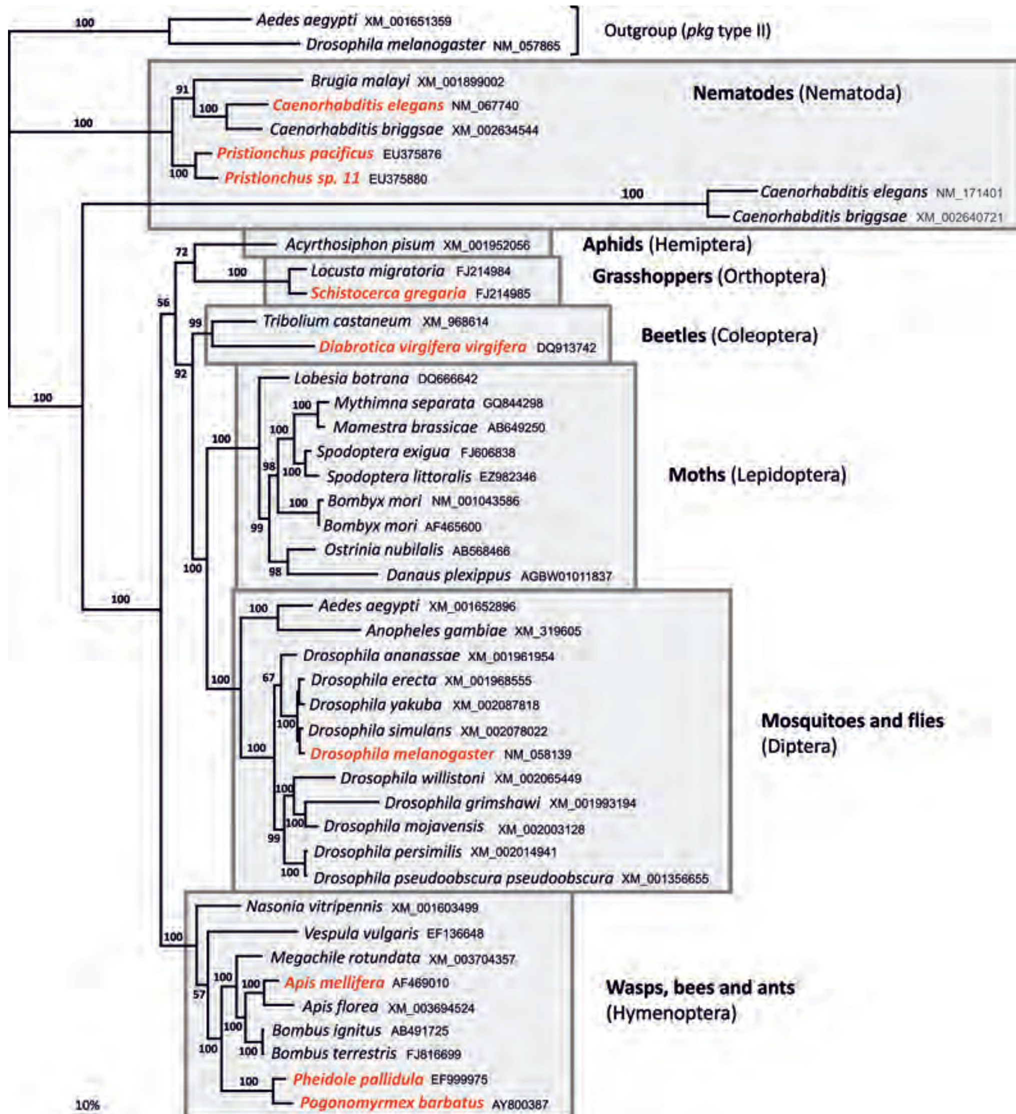


Figure 2. Bayesian phylogeny of nematode and insect *pkg* nucleotide sequences. For species depicted in red, a role for PKG in (feeding-related) locomotion behaviour was found (species in black were not investigated so far). The pattern suggests a conserved role for PKG in locomotion. Numbers depict Bayesian posterior probabilities. The bar at the bottom indicates a branch length of 10% distance. [Methods: GenBank was explored to obtain *pkg* nucleotide sequences (only *pkg* sequences of type I were included, as these are known to be involved in locomotion (Fitzpatrick & Sokolowski 2004); two homologous *pkg* type II sequences were used as an outgroup). The *pkg* cDNA sequence from *S. exigua* (Genbank accession FJ606838) was obtained using a RACE approach (Schaefer 1995). BLAST (National Center for Biotechnology Information) was used for nucleotide and predicted amino acid sequence homology searches. Nematode and insect *pkg* genes were included in the analysis. Phylogenetic Bayesian inference was performed as described in van Houte et al. (2012)].

Genes involved in (feeding-related) locomotion behaviour

Research on invertebrate behavioural genetics led to the discovery of a number of genes involved in the induction of locomotion behaviour. For some of these genes their role as behavioural determinant is conserved across several species, and in one case, even across phyla (NPY, see below). One of the most extensively studied examples is the *for* gene, encoding a cGMP-dependent protein kinase (PKG). This gene was first discovered in *D. melanogaster*, where allelic variation in *for* was associated with a natural polymorphism in foraging behaviour. 'Rover' larvae show a higher locomotion activity than 'sitter' larvae and move over a larger distance when placed on a nutritive source. This increase in activity is accompanied by higher *for* mRNA and PKG protein levels in rovers compared to sitters (Osborne et al. 1997). In *Apis mellifera*, a similar link exists between the *for* homolog *Amfor* and a behavioural transition from nursing to foraging bees (Ben-Shahar et al. 2003), and subsequently a link between PKG and variation in locomotion was also identified in ants (Ingram et al. 2011; Ingram et al. 2005; Lucas & Sokolowski 2009), locusts (Newland & Yates 2006), beetles (Garabagi et al. 2008), and even beyond the order Insecta, in several nematode species (Fujiwara et al. 2002; Hong et al. 2008). These data plus phylogenetic analyses suggest that modulation of PKG levels is a highly conserved mechanism for the regulation of (feeding-related) locomotion behaviour, since a link between PKG and behaviour has so far been found in four different insect genera and in nematodes (Fig. 2 and Fitzpatrick & Sokolowski (2004)).

Another gene involved in the regulation of locomotion behaviour is the *npy* gene, encoding neuropeptide Y (NPY, a.k.a. NPF) (Brown et al. 1999). Its role seems to be conserved across animal phyla: *npy* regulates foraging and social behaviour in worms and flies (de Bono & Bargmann 1998; Wu et al. 2003) while its mammalian homolog is responsible for the regulation of feeding behaviour in vertebrates (Beck 2006; Stanley & Leibowitz 1985). A mutation in a *C. elegans* NPY receptor-like gene is associated with a behavioural variation between social and solitary foragers (de Bono & Bargmann 1998), while in *D. melanogaster* NPY is implicated in the transition from feeding to wandering behaviour during larval development (Wu et al. 2003).

The metabolic neuropeptide adipokinetic hormone (AKH) plays a role in starvation-induced locomotion as was first described in *D. melanogaster* (Isabel et al. 2005; Lee & Park 2004). Upon starvation, wild type flies display increased locomotion behaviour prior to death, while AKH-deficient flies lack this increase in locomotion and are more resistant to starvation-induced death (Isabel et al. 2005; Lee & Park 2004). AKH also mediates transport of lipids from the insect fat body to the haemolymph (Beenakkers et al. 1985) and increases haemolymph sugar levels (Gade et al. 1997). The double function of AKH

in regulating locomotion activity and raising haemolymph lipid and sugar levels may be considered complementary, since AKH-induced locomotion probably results from AKH-regulated lipid and sugar metabolism, thereby maximizing survival chances for the fly (Isabel et al. 2005; Lee & Park 2004). In moths, cockroaches and firebugs injection of AKH into the CNS induced locomotion (Kodrik et al. 2000; Milde et al. 1995; Wicher et al. 2006). Variation in *akh* mRNA levels also coincides with the natural occurrence of rover and sitter *D. melanogaster* larvae that display an allelic variation in the *for* gene (Kaun et al. 2008). It is hypothesized that for directly regulates *akh* transcription, or alternatively, that the abovementioned *npy* acts as a link between *for* and *akh* expression.

Tachykinins form a large family of vertebrate neuropeptides, implicated in numerous processes in the nervous, gastrointestinal and vascular systems (Otsuka & Yoshioka 1993). In gerbils, activity of a brain-specific tachykinin receptor is involved in locomotion behaviour (Nordquist et al. 2008). In invertebrates, an ancestrally related family of tachykinin-related peptides (TKRPs) has been identified (Nässel 1999), which function in modulation of muscular activity, regulation of diuresis in the Malpighian tubules, and as release factors for AKH (Nässel 2002). A role for TKRPs in locomotion was shown in *D. melanogaster*, where TKRP-deficient flies displayed aberrant olfactory perception and enhanced locomotion activity (Winther et al. 2006), and aberrant spatial orientation behaviour (Kahsai et al. 2010). These studies suggest a function for TKRP in the modulation of locomotion activity in flies (Nässel & Winther 2010).

Given the apparent similarity between PKG, NPY, AKH and tachykinin in the modulation of feeding-related locomotion behaviour, a single pathway may exist in which (some of) these molecules act together (Sokolowski 2003) (Fig. 3). The highly conserved role that these genes play in modulating behaviour across species or even taxa renders them optimal targets for parasites to intervene with host behaviour. Although the evidence is scarce, several studies indicate that (some of) these pathways could indeed be altered by parasites, as discussed below.

Parasitic manipulation involving AKH has not been reported so far, however, it is well established that many parasites affect host metabolic processes, some of which could be AKH-mediated. Several studies have shown increased sugar levels in the haemolymph of parasitised insects (Dahlman 1975; Dahlman & Vinson 1975, 1976; Thompson 1982). For example *Heliothis virescens* larvae parasitised by the braconid wasp *Microplitis croceipes* showed elevated haemolymph sugar levels and higher fat body glycogen levels until five and six days after parasitic oviposition, respectively. In addition, haemolymph lipid levels were elevated (Nakamatsu & Tanaka 2004; Rivers & Denlinger 1995). AKH may also be involved in manipulation of the beetle *Tenebrio molitor* by the tapeworm *Hymenolepis*

diminuta, a parasite that affects oviposition behaviour (Hurd 2009). Parasitised hosts showed lower levels of vitellogenin, a protein thought to be regulated by AKH (Hurd 2009).

In the freshwater snail *Lymniae stagnalis* increased expression of the *npy* gene is observed in the CNS upon parasitisation by the trematode *Trichobilharzia ocellata*, a parasite which alters egg-laying behaviour of its host (Hoek et al. 1997). Direct injection of *Lymniae* NPY peptide in non-parasitised snails inhibits egg-laying, indicating that the parasite may alter host reproductive behaviour by modulation of host *npy* expression (de Jong-Brink et al. 1999).

A parasite protein (e.g. the abovementioned baculovirus PTP protein) could target one of these host proteins by interfering directly with its expression or synthesis, or by targeting proteins more upstream in a pathway. For example, PTP could target PKG, which could subsequently lead to changes in neurohormones such as NPY, AKH and tachykinin (Fig. 3).

Neurotransmitters and locomotion behaviour

Locomotion in invertebrates also involves the action of several monoamine molecules, including dopamine, serotonin and octopamine (Bicker 1999; Saraswati et al. 2004; van Swinderen & Andretic 2011) (Fig. 3) that may act as neurotransmitters or neurohormones. For example, dopamine and octopamine are known to enhance locomotion activity in *D. melanogaster*, while tyramine decreases locomotion (Akasaka et al. 2010; Draper et al. 2007; Saraswati et al. 2004), and in *A. mellifera* dopamine enhances locomotion activity (Fussnecker et al. 2006), but injection of octopamine and its precursor tyramine led to decreased locomotion activity (Fussnecker et al. 2006).

In several cases of behavioural manipulation, neurotransmitter levels were found to be altered (Adamo 2002). Lefèvre et al. (2009a) hypothesized that host phototactic behaviour can be altered by interference with the serotonergic system. For example, higher levels of brain serotonin seem to be functionally linked to the positive phototactic behaviour observed in *G. pulex* infected with *P. laevis* (see above) and *Pomphorhynchus tereticollis* (Tain et al. 2006), and in *G. insensibilis* infected with *M. papillorobustus* (Ponton et al. 2006) (see above). *Manduca sexta* larvae parasitised by the wasp *C. congregata* have increased octopamine levels (Adamo et al. 1997), which may coincide with the observed decrease in movement (see above). In the snail *Biomphalaria glabrata*, levels of dopamine, its precursor L-dopa, and serotonin decreased upon parasitisation by the human blood fluke *Schistosoma mansoni* (Manger et al. 1996). Serotonin levels also decreased

due to the uptake of host serotonin by these larval parasites (Yoshino et al. 2001). In *P. americana* cockroaches stung by the parasitoid *A. compressa*, dopamine was detected in the wasp venom injected into the cockroach's central nervous system (Gal et al. 2005). A proteomic analysis of tsetse flies (*Glossina*) infected with *Trypanosoma* shows the presence of two pyridoxal-dependent decarboxylases, enzymes that catalyse the final step in the synthesis of dopamine and serotonin, suggesting a modification of dopamine and/or serotonin in the brain of the tsetse flies (Lefèvre et al. 2007). Parasites that manipulate host behaviour via neurotransmitters (e.g., parasitic worms infecting gammarids, see above) most likely do this by interfering with the synthesis or release of these agents in the CNS (Lefèvre et al. 2009a) (Fig. 3).

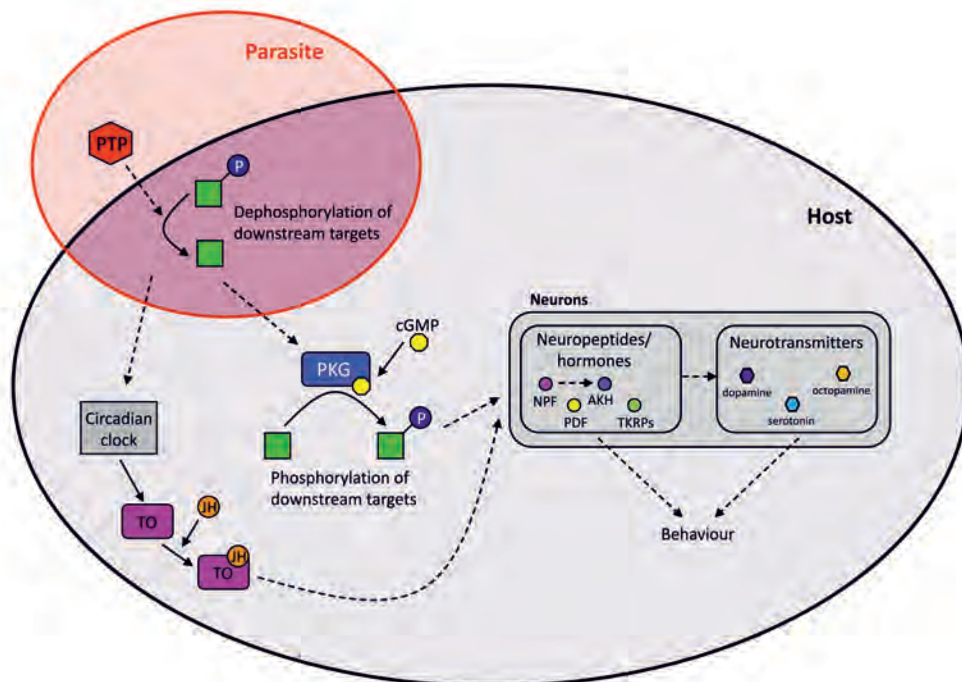


Figure 3. Schematic overview of signalling pathways potentially involved in parasite induced behavioural manipulation. A parasitic protein (e.g., protein tyrosine phosphatase (PTP) in the case of baculoviruses manipulating caterpillar behaviour (Kamita et al. 2005; van Houte et al. 2012) may directly or indirectly affect the host PKG pathway and/or the circadian clock pathway, eventually leading to behavioural changes. Takeout (TO) is thought to link circadian rhythms and feeding behaviour and may act as a juvenile hormone (JH) carrier protein. Neuropeptides and hormones (e.g. neuropeptide Y (NPY), tachykinin-related peptides (TKRPs), pigment-dispersing factor (PDF), adipokinetic hormone (AKH) and neurotransmitters (e.g. octopamine, dopamine, serotonin)) are possibly affected downstream of PKG and/or TO, leading to changes in host behaviour (Johnson & Leroux 2010; Kaun et al. 2008; Meunier et al. 2007; Morton & Hudson 2002; Sokolowski 2003).

Circadian-clock regulated genes involved in feeding-related locomotion and geotaxis

Insect behaviour is controlled in a 24-hour cycle by the circadian system, allowing the organism to respond to rhythmic environmental changes related to e.g. light, temperature and food availability (reviewed in Allada & Chung (2010)). The circadian system consists of three components; 1) the pacemaker, representing the core circadian clock; 2) the input pathway, synchronizing the clock to the environment; and 3) the output pathway, comprising genes that are involved in circadian-clock regulated behaviour, thereby representing a link between circadian rhythm and behaviour (Allada & Chung 2010; Jackson et al. 2001). Parasitic alterations of host behaviour may also be regulated in a clock-wise manner. For example, water-seeking behaviour in arthropods infected with Gordian worms is observed at night only (Ponton et al. 2011) and ants parasitised by lancet liver flukes climb to the top of grass blades in the evening (Libersat et al. 2009). Clock-genes are possibly involved in such behavioural manipulations.

Takeout (*to*) (Fig. 3) is a circadian-clock regulated gene that regulates feeding behaviour in *D. melanogaster* (Sarov-Blat et al. 2000). Flies kept under starvation conditions displayed increased locomotion activity, which was accompanied by an increase of *to* mRNA and protein levels. This increase in activity under starvation was not observed in mutant flies carrying a deletion in the 3'UTR of the *to* gene. In addition, *to* expression was blocked in all arrhythmic central clock mutants, indicating that *to* acts as a molecular link between the circadian clock and the regulation of feeding behaviour under starvation conditions (Meunier et al. 2007; Sarov-Blat et al. 2000). *Takeout* also acts as a modulator of juvenile hormone (JH) levels (Meunier et al. 2007), which is a hormone also involved in locomotion (Belgacem & Martin 2002) (Fig. 3). Although no reports on parasitic manipulation of the *takeout* pathway are currently known, parasites may influence circadian rhythms in their hosts. Recently, Biernat et al. (2012) showed that a DNA repair protein (PHR2) encoded by the baculovirus *Chrysodeixis chalcites* nucleopolyhedrovirus (ChchNPV) (van Oers et al. 2005) can mimic the function of mammalian cryptochromes, essential regulators of the circadian clock. Whether this affects insect circadian rhythms remains to be elucidated.

The gene encoding the neuropeptide pigment-dispersing factor (*pdf*) (Fig. 3) is highly expressed in a subset of pacemaker neurons and is needed for proper circadian locomotion activity (Renn et al. 1999). PDF is involved in geotactic behaviour in *D. melanogaster* as was demonstrated with two fly lines displaying opposing geotactic behaviour, in which *pdf* was identified as one of the differentially expressed genes in a cDNA microarray analysis (Toma et al. 2002). In addition, *pdf*-null mutants showed strong negative geotaxis

while transgenic insertion of an extra copy of the *pdf* gene resulted in a modest increase in positive geotaxis (Toma et al. 2002). This is in line with the finding that a neuronal PDF receptor is involved in both circadian rhythmicity and geotaxis in *Drosophila* (Mertens et al. 2005).

Besides inducing hypermobile behaviour (see above), baculoviruses also induce larval hosts to move to elevated positions of plants or trees (Evans 1986; Goulson 1997; Smirnoff 1965). Some baculovirus infections, however, give opposite effects: infected *Operophtera brumata* larvae tend to migrate downwards (Raymond et al. 2005). If the climbing behaviour of baculovirus-infected larvae is an example of modulation of geotaxis (alternatively it could be a modulation of phototaxis), then the *pdf* gene provides an excellent candidate to play a role in the induced climbing behaviour. This can also be the case for ants infected with the lancet liver fluke *D. dendriticum* (Romig 1980; Schneider & Hohorst 1971) and for *Ophiocordyceps*-infected ants (Hughes et al. 2011).

Conclusions and outlook

Research on behavioural manipulation by parasites is at a turning point; a shift from mere observations to unravelling molecular mechanisms by which parasites alter host behaviour is dawning. In this review we discussed different examples of parasitic manipulation of invertebrates, and we explored possible mechanisms behind such manipulations, confirming that experimental data on underlying mechanisms are still scarce. Relatively novel techniques such as transcriptomics and proteomics made important initial contributions to this field, and will be invaluable in the future. Combining the information obtained in such genome-/proteome-wide analyses with what is known from the genetic basis of behaviour in unparasitised animals is a relevant next step in unraveling mechanisms of parasitic manipulation, and several studies indeed support the hypothesis that genes known to have a conserved role in insect behaviour could be targets for manipulation by parasites. Therefore, the 'parasite-extended' candidate gene approach, based on the assumption that parasites hijack existing signalling pathways involved in behavioural traits, will be an important new tool to deepen our understanding of the intricate strategies by which parasites alter host behaviour. Vast amounts of genomic information have become available for many organisms and will aid in this research, providing novel insights into the molecular basis of behavioural manipulation by parasites.

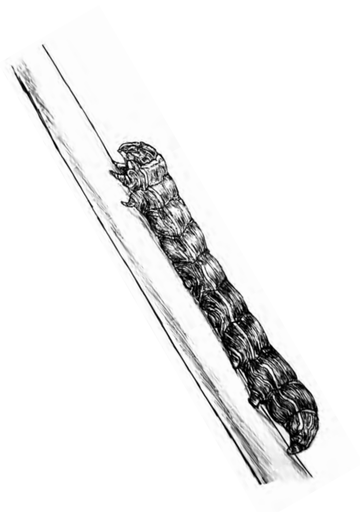
Acknowledgements

The authors thank Katja Hoedjes, Hans Smid, Just Vlak and Louise Vet for useful discussions and reading previous versions of this manuscript and four anonymous reviewers for constructive and helpful comments. Basil Arif is sincerely thanked for critically reading the manuscript. SVH and VIDR are both supported by the Program Strategic Alliances of the Royal Dutch Academy of Sciences (project 08-PSA-BD-01) and VIDR is supported by a VENI grant of the Netherlands Organisation for Scientific Research (project 863.11.017).

Data accessibility

DNA sequence: GenBank accession FJ606838.

Phylogenetic data: TREEBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S13731).



Chapter

3

Protein tyrosine phosphatase-induced hyperactivity is a conserved strategy of a subset of baculoviruses to manipulate lepidopteran host behaviour

This chapter has been published as:

Stineke van Houte, Vera I.D. Ros, Tom G. Mastenbroek, Nadia J. Vendrig, Kelli Hoover, Jeroen Spitzen, Monique M. van Oers (2012) Protein tyrosine phosphatase-induced hyperactivity is a conserved strategy of a subset of baculoviruses to manipulate lepidopteran host behavior. *PLoS ONE* 7(10), e46933.

Abstract

Many parasites manipulate host behaviour to increase the probability of transmission. To date, direct evidence for parasitic genes underlying such behavioural manipulations is scarce. Here we show that the baculovirus *Autographa californica* nucleopolyhedrovirus (AcMNPV) induces hyperactive behaviour in *Spodoptera exigua* larvae at three days after infection. Furthermore, we identify the viral protein tyrosine phosphatase (*ptp*) gene as a key player in the induction of hyperactivity in larvae, and show that mutating the catalytic site of the encoded phosphatase enzyme prevents this induced behaviour. Phylogenetic inference points at a lepidopteran origin of the *ptp* gene and shows that this gene is well-conserved in a group of related baculoviruses. Our study suggests that *ptp*-induced behavioural manipulation is an evolutionarily conserved strategy of this group of baculoviruses to enhance virus transmission, and represents an example of the extended phenotype concept. Overall, these data provide a firm base for a deeper understanding of the mechanisms behind baculovirus-induced insect behaviour.

Introduction

Modification of host behaviour upon parasitic infection is an intriguing phenomenon that has been observed in a wide range of organisms (Beckage 1997; Lefèvre et al. 2009a; Moore 2002). Parasites can affect a large variety of behavioural traits, including feeding behaviour, mating, odour response, and locomotion activity (Moore 2002). Many of these behavioural changes are thought to represent manipulative strategies of the parasite to increase parasite transmission rates. As such, the altered behaviour of the host is regulated by the expression of parasitic genes, thus representing the parasite's extended phenotype (Dawkins 1982).

Aquatic isopods (*Asellus intermedius*), for example, display hyperactive behaviour when they are infected with the parasitic worm *Acanthocephalus dirus* (Moore 1995). This behavioural change is thought to make the isopods more susceptible to predation by fish, which serve as a final host for the acanthocephalan worm (Combes 1991). Also arthropod-borne parasites may alter the behaviour of their hosts to increase their chances of transmission (Bennett et al. 2008; Lefèvre & Thomas 2008; Lima-Camara et al. 2011). *Aedes aegypti* mosquitoes carrying Dengue virus (*Flaviviridae*) display a 50% higher locomotion activity than uninfected mosquitoes (Lima-Camara et al. 2011), and show a longer probing and feeding time (Platt et al. 1997), thereby potentially increasing the transmission rates of the virus. Although numerous examples of such manipulations are known, direct evidence for parasitic genes underlying these phenomena is scarce and the molecular mechanisms by which such genes alter behaviour are still largely enigmatic (Lefèvre et al. 2009a; Lefèvre et al. 2009b; Libersat et al. 2009).

Baculoviruses are known to induce behavioural changes in their caterpillar hosts, consisting of abnormal climbing behaviour, named 'Wipfelkrankheit' or 'tree-top disease', and hyperactivity (Goulson 1997; Hofmann 1891). Both behavioural changes are thought to enhance the spread of progeny virions released from dead and liquefied caterpillars over a large surface of plant foliage, thus increasing transmission rates to subsequent generations of caterpillar hosts. Although the molecular mechanisms underlying baculovirus-induced behaviour are still largely unknown, recent work provided some first clues about two baculoviral genes involved in either one of these extended phenotypes. Firstly, the ecdysteroid uridine 5′-diphosphate (UDP)-glucosyl transferase (*egt*) gene of *Lymantria dispar* multiple nucleopolyhedrovirus (LdMNPV) was found to induce climbing behaviour in the European gypsy moth *L. dispar* (Hoover et al. 2011), resulting in tree-top disease. On the other hand, Kamita et al. (2005) and Katsuma et al. (2012) showed the involvement of the *Bombyx mori* nucleopolyhedrovirus (BmNPV) *ptp* gene, encoding a

protein tyrosine phosphatase, in the induction of enhanced locomotion activity in larvae of the silkmoth *B. mori* (Kamita et al. 2005; Katsuma et al. 2012). However, at present it is unclear how well-conserved the *ptp* gene is among the members of the family *Baculoviridae*, and whether this *ptp*-induced behaviour reflects an evolutionary conserved strategy of baculoviruses to manipulate their insect host. Moreover, notwithstanding the considerable advances that the study by Kamita et al. (2005) has made in understanding the molecular basis of this parasite-induced hyperactive extended phenotype, it is unknown to what extent this particular pathogen-host system reflects ecologically meaningful interactions between baculoviruses and lepidopteran hosts. This is a relevant question, as since the foundation of sericulture, approximately 5000 years ago, *B. mori* became a fully domesticated insect that has undergone extensive inbreeding and artificial selection, mainly for silk production optimization purposes (Xia et al. 2009). During this selection process *B. mori* lost several typical behavioural traits, including flight and predator and disease avoidance, whereas it gained other characteristics, e.g. increased tolerance to larval crowding (Xia et al. 2009). Gain or loss of such traits can be expected to strongly affect phenomena such as parasitic manipulation of behaviour. This is illustrated by the fact that the *B. mori* strain used in the locomotion activity assays showed exceptionally low endogenous activity levels in the absence of virus infection (Kamita et al. 2005), while the wild silkmoth *B. mandarina*, which is considered the ancestral species from which *B. mori* originated (Goldsmith et al. 2005), is known to display relatively high activity levels in the field (Shimada 1999). Therefore, it is important to understand whether a possible virus-induced behavioural manipulation is conserved in insect species that did not undergo such extensive artificial selection, and presumably have evolved strategies to counteract parasitic manipulation. Behavioural alterations observed in such insects are more likely to reflect a natural situation in which the manipulation is the result of a long coevolutionary history between parasite and host.

A homolog of the BmNPV *ptp* gene is present in the baculovirus type species *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) (Ayres et al. 1994). Previous work on this AcMNPV *ptp* gene has shown that it is not essential for virus replication (Li & Miller 1995b). The enzyme encoded by this gene contains the HC signature motif that is characteristic for all members of the PTP protein family (reviewed in Tonks (2006)), and dephosphorylates both RNA (Gross & Shuman 1998; Takagi et al. 1998) and protein substrates (Sheng & Charbonneau 1993) *in vitro*. Mutating the catalytic Cys-119 residue in the HC motif to either alanine (C119A) or serine (C119S) was shown to abolish enzymatic activity of the AcMNPV PTP protein (Gross & Shuman 1998; Sheng & Charbonneau 1993; Takagi et al. 1998).

To examine whether baculoviral *ptp*-induced behaviour reflects an evolutionarily

conserved strategy, we used AcMNPV and its lepidopteran host *Spodoptera exigua* (Hübner) (Lepidoptera, Noctuidae) as a model system for studying behavioural manipulation and its underlying mechanisms. AcMNPV represents the best characterized baculovirus to date, for which well-developed genetic tools exist (Luckow et al. 1993). *Spodoptera exigua* or the beet armyworm is a polyphagous insect pest species that occurs worldwide in tropical and subtropical areas. Although AcMNPV has a broad host range, with over 32 insect species known to be susceptible to the virus (Groener 1986), *S. exigua* was chosen as a host model, as the virus is highly infectious to this natural host. This combination of virus and host is relatively commonly used as *S. exigua* can be easily reared under laboratory conditions.

We show that AcMNPV induces hyperactivity in *S. exigua* larvae at three days after infection. Subsequently, the possible involvement of the AcMNPV *ptp* gene in this behavioural change was studied, and we analysed whether the phosphatase activity of its encoded enzyme is required for this behavioural change. Phylogenetic analyses were performed to gain insight into the origin of the baculovirus *ptp* gene and to determine its degree of conservation within the family *Baculoviridae*. Our results strongly suggest that *ptp*-mediated manipulation of behaviour represents an evolutionarily conserved strategy among a subset of baculoviruses. Overall, these findings contribute to a better understanding of the mechanisms governing parasite-induced behavioural changes.

Materials and methods

Insect cells and larvae

Spodoptera frugiperda 9 (Sf9) cells (Invitrogen) were cultured as monolayers in Sf900II serum-free medium (Invitrogen) supplemented with 5% fetal bovine serum (Invitrogen) and 0.1% gentamycin (Invitrogen). *Spodoptera exigua* larvae were reared on artificial diet at 27 ºC with 50% relative humidity as described before (Smits et al. 1986), and a 14:10 h light:dark photoperiod.

Generation of recombinant bacmids

The AcMNPV E2 bacmid was used as wild type (WT) virus in this study (Smith & Summers 1979). An AcMNPV bacmid with a deletion of the *ptp* gene (Δ*ptp*), derived from this WT bacmid, was kindly provided by Linda Guarino of Texas A&M University, USA (Li & Guarino 2008). The Δ*ptp* virus was originally created by replacing nucleotide (nt) positions 509 to 1080, a region spanning the complete *ptp* ORF, with a Zeocin resistance marker gene (Li & Guarino 2008). To enable oral infection of larvae, the open

reading frame (ORF) of the AcMNPV polyhedrin (*polh*) gene was placed back into the WT and Δ*ptp* genomes. For this purpose, Bac-to-Bac transposition (Luckow et al. 1993) was performed with a modified pFastBacDual vector (pFBDpolhΔp10), in which the AcMNPV *polh* ORF was cloned downstream of the *polh* promoter, and from which the *p10* promoter was removed, both as described in Peng et al. (2010) (Fig. 1). To ensure that a possible phenotype of the Δ*ptp* recombinant virus was not due to any other genome mutations, a repair bacmid was created for which the Δ*ptp* bacmid was used as a backbone. On the AcMNPV genome (Ayres et al. 1994) the *homologous repeat* (*hr*) 1 region is mapped at nt positions 133883 to 133894 and 1 to 445, ending 48 nt upstream of the *ptp* ORF (which is AcORF1). As baculovirus *hr*s are known to be involved in enhancing the expression of downstream genes (Blissard 1996), the repair bacmid was generated in such a way that the *ptp* gene was placed back together with the upstream *hr1* sequence. The coding sequence of the *ptp* ORF and the upstream 532 base pairs (bp), containing the *hr1* repeat region and the putative *ptp* promoter sequence (Kim & Weaver 1993), were PCR amplified with the proofreading polymerase Phusion (Finnzymes), using primer 1 combined with primer 2 (Table S1 in van Houte et al. (2012)). To allow subsequent cloning, *Nco*I and *Nsi*I restriction sites (underlined in the primer sequence, Table S1 in van Houte et al. (2012)) were introduced with these primers. The sequenced

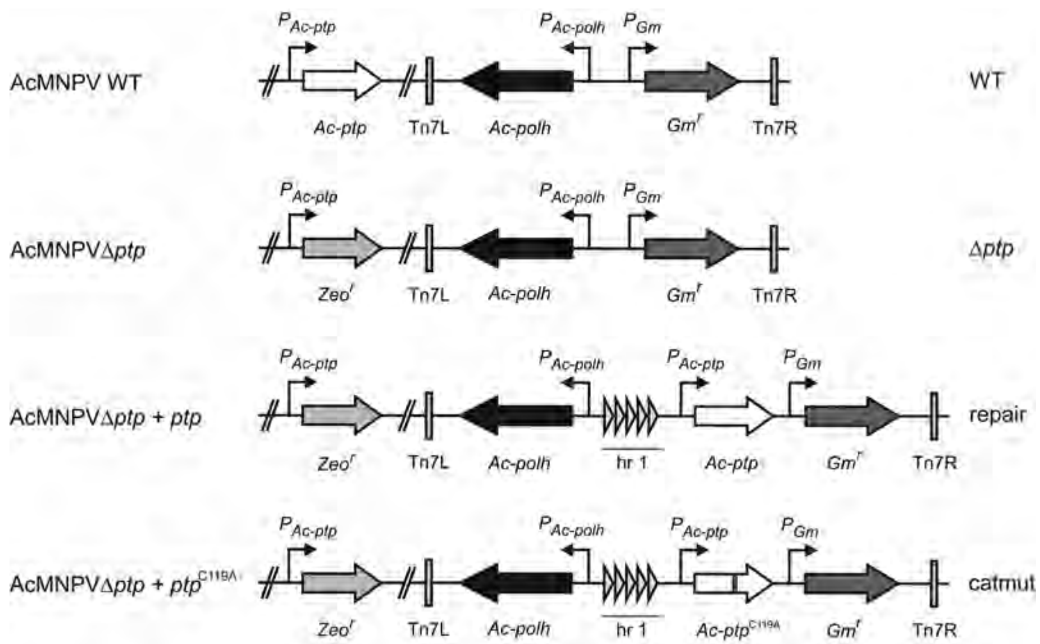


Figure 1. Overview of the recombinant bacmids used in this study. On the left the names of the recombinant bacmids are indicated, while on the right the corresponding abbreviated names as they are used in this paper are shown.

PCR product was cloned as *Nco*I/*Nsi*I fragment into pFBDpolhΔp10. Subsequently, the transposon carrying the *ptp* and *polh* sequences was introduced into the Δ*ptp* bacmid as described above (Fig. 1).

For construction of a recombinant bacmid encoding a catalytically inactive PTP protein (catmut), a mutation was introduced in the HC signature motif. The Cys-119 residue was replaced with an alanine (C119A) by overlap extension PCR with Phusion polymerase, using primer 3, which introduced two point mutations at nt 355 and 356 relative to the ATG start codon, combined with primer 2 (Table S1 in van Houte et al. (2012)). The resulting 241 bp product was then used as reverse primer combined with primer 1, thus generating the ptp^{C119A} ORF with the upstream *hr1* sequence and putative promoter sequence. This sequenced fragment was cloned as *Nco*I/*Nsi*I fragment into the pFBDpolhΔp10 vector, and inserted into the Δ*ptp* bacmid as described above (Fig. 1).

Amplification and purification of virus

To produce recombinant viruses, Sf9 cells were transfected with the above described recombinant bacmids (WT, Δ*ptp*, repair and catmut) using CellFectin II transfection reagent (Invitrogen), and the resulting budded virus (BV) stock was amplified once in cells. The viral occlusion bodies (OBs) generated in these infected cells were amplified in *S. exigua* 3rd (L3) and 4th (L4) larval instars. Purification of OBs from infected larvae was done by grinding deceased larvae in water and filtering through a double layer of cheese cloth. The suspension was first centrifuged at 500x*g*, after which the supernatant was centrifuged at 4000x*g* to pellet OBs. Finally, OBs were resuspended in water and stored at 4 °C.

Infectivity assays

Infectivity assays were performed to determine the 50% lethal virus concentration (LC_{50}) for each virus. *Spodoptera exigua* larvae were grown until they were late 2nd instar. Larvae were starved overnight for 16 h and allowed to moult during that time. Newly moulted 3rd instar larvae were selected and infected using droplet feeding. Dilutions of viral OB stocks were prepared in a 10% sucrose solution containing 0.4% (w/v) Patent Blue V food colouring dye (Sigma-Aldrich). For each virus (WT, Δ*ptp*, repair, and catmut) the following dilutions were prepared: 10^4, 10^5, 10^6, 10^7, 10^8 and 10^9 OBs/ml. For each dilution, 24-36 larvae were allowed to drink from the virus suspension for 15 minutes. Mock-infected larvae were used as controls and were droplet fed using a virus-free 10% sucrose solution containing 0.4% (w/v) Patent Blue V food colouring dye. Only larvae with a completely blue-coloured gut were selected, as these were assumed to have ingested

an equal volume of virus suspension. These larvae were reared individually on artificial diet in 12-well plates. Once a day, larvae were scored for mortality until all larvae were either dead or had pupated. To determine the 50% lethal time (LT_{50}) values for each of the viruses, larvae infected with an $LC_{90\text{-}95}$ dose (10^8 OBs/ml) were checked twice a day for mortality until all larvae were dead or had pupated (mock). Median LC_{50} values were determined by Probit analysis, and median LT_{50} values were determined using Kaplan-Meier survival analysis. Both analyses were performed using SPSS 19.0.

Movement assays

Late 2nd instar larvae were starved for 16 h and allowed to moult as described above. Newly moulted 3rd instar larvae were infected with an $LC_{90\text{-}95}$ dose (10^8 OBs/ml) of virus using droplet feeding, and subsequently fed on artificial diet. To measure larval activity, individual larvae were placed in an arena consisting of a plain surface with dimensions of 120 x 90 cm, which was equipped with a digital video camera (Sony) positioned at 200 cm above the arena surface. The camera was flanked by two photography studio lights of 40 W each to ensure an equal light distribution throughout the arena surface. The temperature at which the measurements were done was 24.5 ºC ± 0.7. While being recorded, each larva was allowed to move freely for 10 minutes in the arena. To calculate the distance (i.e. locomotion activity) travelled by individual larvae, digital recordings were analysed using EthoVision tracking software (Noldus Information Technology, The Netherlands). This is a commonly used software analysis method for different types of behavioural assays (for a review see Martin (2003)).

In the first experiment, the distance moved by mock- and WT-infected larvae was measured at one, two and three days post-infection (dpi). Two replicates were performed, and data were analysed using a linear mixed model (Proc Mixed procedure, SAS Inst. Inc., 2004) with treatment, days post-infection, experiment number, and their two-way interactions as fixed effects, days post-infection as within-subject repeated measures, and an unstructured covariance structure. The full-model was reduced using backward stepwise elimination of fixed effects based on Kenward-Rogers approximate F-test ($\alpha>0.05$), and least square means differences for treatment, days post-infection and their interaction were then tested for significance using Bonferroni-adjusted P-values.

In the second experiment, distance moved by mock-, WT-, Δ*ptp*-, repair-, and catmut-infected larvae was measured at three dpi. For statistical analysis of the data, distances travelled were normalized using a Box-Cox transformation (Box & Cox 1964). In a single movement assay two or three treatments were measured, one always being the WT treatment, so that it could be used as an internal standard for the different assays performed.

Two or three replicates were performed per treatment. The relation between distance travelled at three days post-infection and treatment (mock, WT, Δ*ptp*, repair and catmut) was analysed using general linear models (Proc GLM procedure, SAS Inst. Inc., 2004) with treatment and experiment number, and their interaction, as fixed effects. The full-model was reduced using backward stepwise elimination of fixed effects based on the F-test ($\alpha>0.05$) with type III sum of squares, and least square means differences of treatments were then tested for significance using Bonferroni-adjusted P-values.

RNA isolation and RT-PCR

Newly moulted 3rd instar larvae were infected by droplet feeding with an LC_{90-95} dose of virus as described above. At three dpi total RNA from single larvae was extracted by homogenizing in 250 µl Trizol reagent (Invitrogen) and total RNA was isolated following the manufacturer's instructions. The RNA pellet was dissolved in 50 µl water and heated for 10 min at 55 °C. Any contaminating DNA was removed with the DNA*free* kit (Applied Biosystems) according to the company's protocol. Production of cDNA was performed using SuperScript III Reverse Transcriptase (Invitrogen) according to the company's protocol. RT-PCR was performed using primers to amplify i) 426 bp within the AcMNPV *ptp* ORF (primers 5 and 6, Table S1 in van Houte et al. (2012)), ii) 512 bp within the AcMNPV *ie1* ORF (primers 7 and 8), and iii) 486 bp of the *S. exigua* host *Se-eIF5A* ORF (primers 9 and 10). For each RT sample, a control sample was run in which the RT step was omitted (non-RT) to check for DNA contamination. In addition, a negative control without template was processed for each primer pair.

Phylogenetic analysis

GenBank and Butterflybase/InsectaCentral (Papanicolaou et al. 2008) were explored to obtain *ptp* nucleotide sequences (Table S2 in van Houte et al. (2012)). A homologous *ptp* sequence from a *S. exigua* EST bank was provided by Salva Herrero, Universitat de València, Spain (Pascual et al. 2012). BLAST (National Center for Biotechnology Information) was used for nucleotide and predicted amino acid sequence homology searches. Multiple searches were performed, using different baculovirus and lepidopteran *ptp* sequences as a query. All known invertebrate (20), baculovirus (15), and poxvirus (2) *ptp* genes were included in the analysis. Sequences were translated in frame to proteins and aligned using MAFFT version 6 with default settings (Katoh et al. 2005). Protein alignment was converted back into the corresponding codon alignment using PAL2NAL (Suyama et al. 2006). Gblocks (Castresana 2000) was used for trimming sequences to select conserved domains. PAUP* version 4.0b10 (Swofford 2002) was used to select the optimal evolution model, as described in Ros et al. (2009). ML analysis (heuristic

search, 100 bootstrap replicates) was performed in PAUP, using a submodel of the General Time Reversible Model with invariable sites and a gamma distribution of rate heterogeneity (GTR + I + G) with rate class 'abcdec'. Bayesian inference was conducted using MRBAYES 3.1.2 (Ronquist & Huelsenbeck 2003), using the GTR + I + G model (default settings, six million generations, burn-in of 25%).

A *lef-8* phylogeny was also constructed as described above (model: GTR + I + G, rate class: 'abccde') and includes the *lef-8* sequences of all baculoviruses that have a *ptp* and/or *ptp2* gene (except for *Iragoides fasciata* NPV for which the *lef-8* sequence is not available in GenBank). In addition, we included all Alpha- and Betabaculoviruses for which the genome sequence is available in GenBank and for which the absence of both *ptp* and *ptp2* genes was confirmed (Table S3 in van Houte et al. (2012)).

Results

Larval infectivity is not affected by deletion of the AcMNPV *ptp* gene

To investigate whether AcMNPV induces altered behaviour in the permissive host *S. exigua*, and to determine whether the viral *ptp* gene plays a role in this, we performed behavioural studies using four different viruses: WT, Δ*ptp*, repair and catmut (Fig. 1). First, the lethal concentration (LC_{50}) and lethal time (LT_{50}) of these virus stocks were determined in *S. exigua* 3rd instar larvae. The LC_{50} values for WT, Δ*ptp*, repair, and catmut were $10^{6.4}$, $10^{6.5}$, $10^{6.6}$, and $10^{6.7}$ OBs/ml, respectively (Table 1), and overlapping 95% fiducial limits indicated that these values were not significantly different. Likewise, upon infection with an $LC_{90\text{-}95}$ viral dose, no significant differences in the time to death were found among the viruses, with LT_{50} values of 112, 120, 100, and 112 h, respectively (Table 1) (χ^2=1.291, df=3, P=0.731), although the 95% fiducial limits interval of the LT_{50} value of AcMNPV Δ*ptp* was quite large (38 h). It is possible that differences in LT_{50} may exist upon infection with lower viral doses, although this would not be relevant for this study as only $LC_{90\text{-}95}$ viral doses were used in the movement

Table 1. Dose-mortality response (log LC_{50}) and time-mortality response (LT_{50}) of 3rd instar *S. exigua* larvae infected with WT, Δ*ptp*, repair and catmut virus. LT_{50} determined for a virus concentration of 10^8 OBs/ml (~90-95% mortality).

Virus	Log LC_{50} (OBs/ml)	Log 95% fiducial limits (OBs/ml)		LT_{50} (h)	95% fiducial limits (h)	
		upper	*lower*		*upper*	*lower*
WT	6.4	6.1	6.8	112	97	127
Δ*ptp*	6.5	6.2	6.8	120	101	139
repair	6.6	6.3	6.9	100	92	108
catmut	6.7	6.4	7.0	112	104	120

Table 2 . Variation between different replicates of the movement assays.

(A). Variation between different replicates in movement assays of mock- and WT-infected larvae at one, two and three dpi. Minimum, maximum and mean (± SE) distances moved of all replicates are displayed.

Days post-infection (dpi)	Treatment	Distance moved (mm)		
		Minimum	*Maximum*	*Mean ± SE*
1 dpi	Mock	501.1	532.5	516.8 ± 51.45
	WT	494.8	540.7	520.1 ± 60.07
2 dpi	Mock	392.0	548.3	474.1 ± 45.99
	WT	525.9	561.3	541.8 ± 50.29
3 dpi	Mock	481.0	503.6	492.6 ± 42.68
	WT	803.3	830.0	815.3 ± 45.72

(B). Variation between different replicates in movement assays of mock-, WT-, Δ*ptp*-, repair- and catmut-infected larvae at three dpi. Minimum, maximum and mean (± SE) distances moved of all replicates are displayed.

Treatment	Distance moved (mm)		
	Minimum	*Maximum*	*Mean ± SE*
Mock	403.7	630.3	455.5 ± 54.90
WT	575.1	937.6	687.2 ± 44.55
Δ*ptp*	376.8	573.6	481.9 ± 54.68
repair	663.2	686.4	674.3 ± 107.07
catmut	265.6	267.1	266.3 ± 39.05

assays. Overall, this indicates that viral infectivity and speed of kill are not affected by the absence of the *ptp* gene (Δ*ptp*) or the presence of a mutant *ptp* gene (catmut) during infection of *S. exigua* larvae. Li and Miller (1995) reported that upon amplification in Sf21 insect cells, the AcMNPV Δ*ptp* virus infectivity was reduced 50% compared to a WT strain (Li & Miller 1995a). However, they also showed that this potency difference disappeared upon oral infection in *S. frugiperda* larvae, and that the lethal concentration and lethal time were similar between WT and Δ*ptp* virus, which is in correspondence with our findings for *S. exigua*.

AcMNPV induces hyperactive behaviour in its host *S. exigua*

To investigate whether AcMNPV induces hyperactive behaviour in the permissive host *S. exigua*, movement assays were performed in an arena. Mock- and WT-infected larvae were tracked in the arena at one, two and three days post-infection (dpi) to check for differences in locomotion activity. A summary of the variation in the original data between different replicates, indicating the minimum and maximum distances measured for a certain treatment, is given in Table 2A. Results showed that treatment (mock/WT) altered host mobility ($F_{(1,67.4)}$ = 5.27; P=0.0248), depending on the dpi ($F_{(2,65.7)}$ = 9.25; P=0.0003), and a highly significant interaction was found between treatment and dpi (treatment*day: $F_{(2,65.7)}$ = 11.00; P<0.0001). On one and two dpi, distances moved

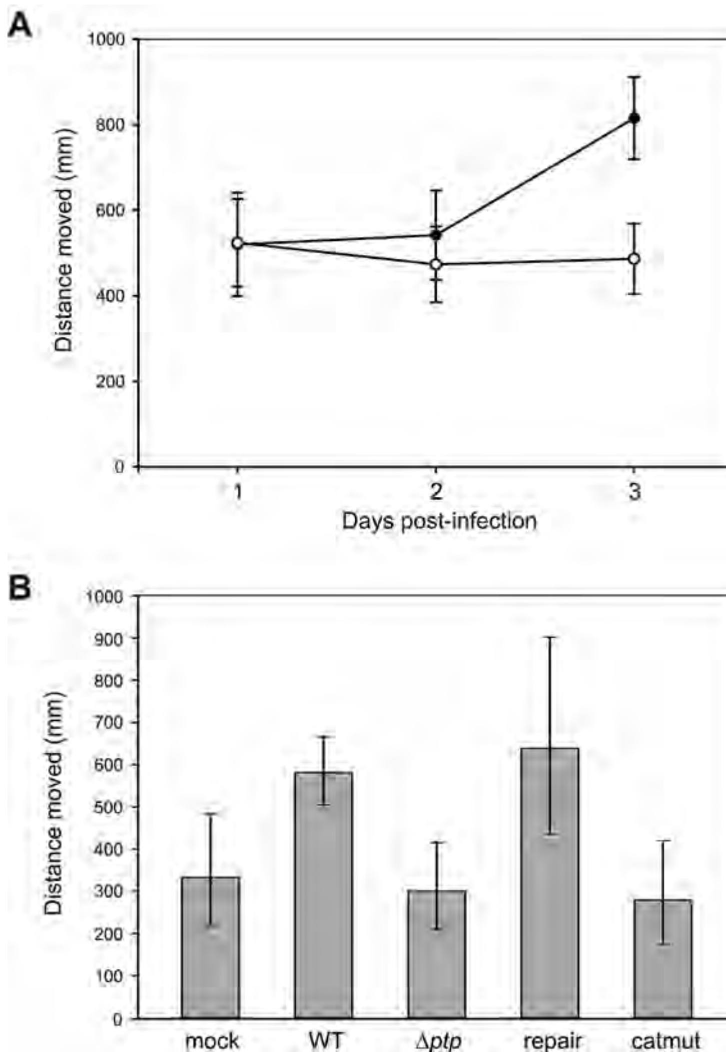


Figure 2. AcMNPV induces hyperactive behaviour, and requires the phosphatase activity of the *ptp* encoded enzyme. (A) Estimated marginal means of distances moved (mm) by mock-infected (open circles) and WT-infected (closed circles) larvae in a 10-min interval at one, two and three dpi. Mock: n=41, WT: n=29. At three dpi, WT-infected larvae show higher activity than mock-infected ones (P<0.0001). Error bars represent 95% confidence intervals. (B) Estimated marginal means of distances moved (mm) by mock-, WT-, Δ*ptp*-, repair- and catmut-infected larvae in a 10-min interval at 3 dpi. Mock: n=35, WT: n=113, Δ*ptp*: n=39, repair: n=23, catmut: n=24. WT-infected larvae show significantly higher activity than mock-, Δ*ptp*- and catmut-infected larvae (P=0.0295, P=0.001 and P=0.0013, respectively). Error bars represent 95% confidence intervals.

were not significantly different between the two treatments (mock day 1: 524 mm, WT day 1: 520 mm, P=1.000; mock day 2: 473 mm, WT day 2: 542 mm, P=1.000) (Fig. 2A). However, at three dpi WT-infected larvae showed a significantly higher locomotion activity than mock-infected larvae (mock: 486 mm, WT: 815 mm, P<0.0001). Movement data later than three dpi were not included, as virus-infected larvae started to become moribund at four dpi.

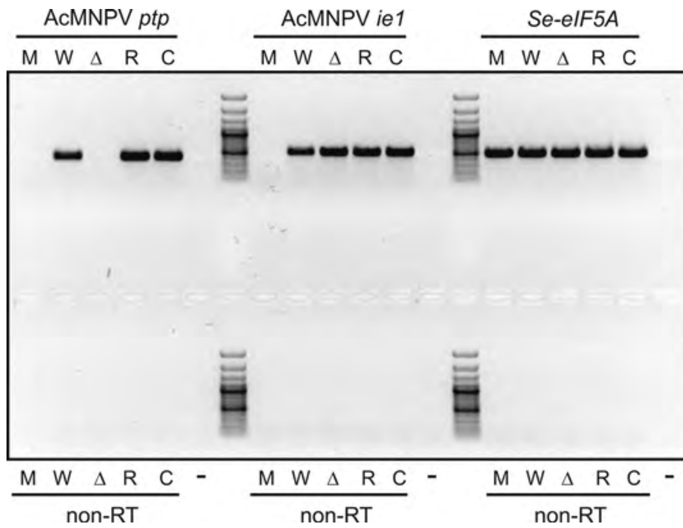


Figure 3. The AcMNPV *ptp* gene is expressed in the WT-, repair- and catmut-infected larvae. RT-PCR analysis on mock- (M), WT- (W), Δ*ptp*- (Δ), repair- (R) and catmut-infected (C) larvae. Expression of the AcMNPV *ptp* gene, the AcMNPV *ie1* gene and the host *Se-eIF5A* gene was analysed. For each RT sample, a PCR without RT step (non-RT) was performed in parallel. For each primer pair, a no-template control was processed (-). The GeneRuler 100 bp ladder (Fermentas) was included in the agarose gel to estimate PCR fragment sizes.

Hyperactive behaviour is induced by the AcMNPV *ptp* gene, but not by a mutant *ptp* gene encoding a catalytically inactive enzyme

Next, we examined a possible role for the *ptp* gene in the induction of the observed hyperactivity, by analyzing locomotion activity of mock-, WT-, Δ*ptp*, repair- and catmut-infected larvae at three dpi. Table 2B summarizes the variation in the original data between different replicates, indicating the minimum and maximum distances measured for all different treatments. As no interaction was detected between experiment and treatment ($F_{(5,228)}$ = 0.17; P=0.9724), this term was excluded from the model. After correction of the transformed data for between-experiment variation ($F_{(6,223)}$ = 2.31; P=0.0350), results showed a clear relation between virus treatment and larval activity ($F_{(4,223)}$ = 7.73; P<0.0001). WT-infected larvae again showed a higher activity than uninfected control larvae (WT: 581 mm, mock: 333 mm, P=0.0295), while Δ*ptp*-infected individuals moved a distance of 301 mm, which was significantly lower than WT-infected individuals (P=0.001) (Fig. 2B). Infection with a repair virus, in which the *ptp* gene was placed back in the viral genome, restored the hyperactive phenotype observed in WT-infected larvae (639 mm, P=1.000). Larvae infected with a catmut recombinant virus, carrying a catalytically mutated *ptp* gene, showed significantly lower activity than WT-infected larvae (279 mm, P=0.0013).

To exclude the possibility that the observed behavioural phenotype of the catmut-infected larvae was due to inappropriate *ptp* expression, RT-PCR was performed on total RNA isolated from mock- or virus-infected single whole larvae at three dpi. The AcMNPV

ptp gene was expressed in WT-, repair- and catmut-infected larvae, but expression was, as expected, absent in the mock- and Δ*ptp*-infected individuals (Fig. 3). The AcMNPV *ie1* gene, included as a control for virus infection, was expressed in all the virus-infected larvae, but not in the mock-infected ones. The *S. exigua eIF5A* gene, encoding eukaryotic translation initiation factor 5A (van Oers et al. 1999), was included as a host control and showed expression in both mock- and virus-infected larvae.

The AcMNPV *ptp* gene is likely acquired from an ancestral lepidopteran host

We investigated how *ptp* genes from baculoviruses and lepidopteran hosts were related using a phylogenetic approach, including all available invertebrate and baculovirus *ptp* sequences (Fig. 4). Two related poxvirus *ptp* sequences were included to see whether viral *ptp* homologs have a common origin. BLAST searches also revealed homologous vertebrate *ptp* sequences, but these were too distantly related to include in the phylogenetic analysis.

To better understand the origin of the baculovirus *ptp* gene, a baculovirus phylogeny was constructed using the baculovirus core gene *lef-8*, which encodes a subunit of the baculovirus RNA polymerase (Guarino et al. 1998). This highly conserved gene is very suitable for constructing a baculovirus phylogeny, distinguishing Alphabaculovirus group I NPVs, Alphabaculovirus group II NPVs and Betabaculoviruses (granuloviruses (GVs)) (Herniou & Jehle 2007).

All the *ptp* sequences derived from baculovirus genomes form a monophyletic group, and form a well-supported clade with lepidopteran *ptp* sequences from *S. exigua*, *Bicyclus anynana*, *Danaus plexippus* and one of the two *ptp* sequences from *B. mori*, and with the *ptp* sequence from *Amsacta moorei* entomopoxvirus. As this clade localizes within the insect *ptp* sequences (using nematode *ptp* sequences as outgroup), a transfer of a *ptp* gene from a lepidopteran host to an ancestral baculovirus appears to be a likely evolutionary scenario (see below). Since the baculovirus and lepidopteran *ptp* sequences are not mixed in this phylogeny, this transfer presumably happened once, after which the gene was spread within the family *Baculoviridae*. The *ptp* from *A. moorei* entomopoxvirus (AMEV, *Poxviridae*) also localizes within this clade, although its exact position is unresolved. It could have been acquired from an (lepidopteran) insect host by transfer to an ancestral virus that gave rise to both the pox- and the baculoviruses, however, this scenario is unlikely as the *ptp* from the canarypoxvirus (CNPV, *Poxviridae*) is located elsewhere in the phylogeny. This does not support a common origin of *ptp* in viruses. Possibly, a transfer of *ptp* between an (ancestral) baculovirus and an (ancestral) entomopoxvirus occurred.

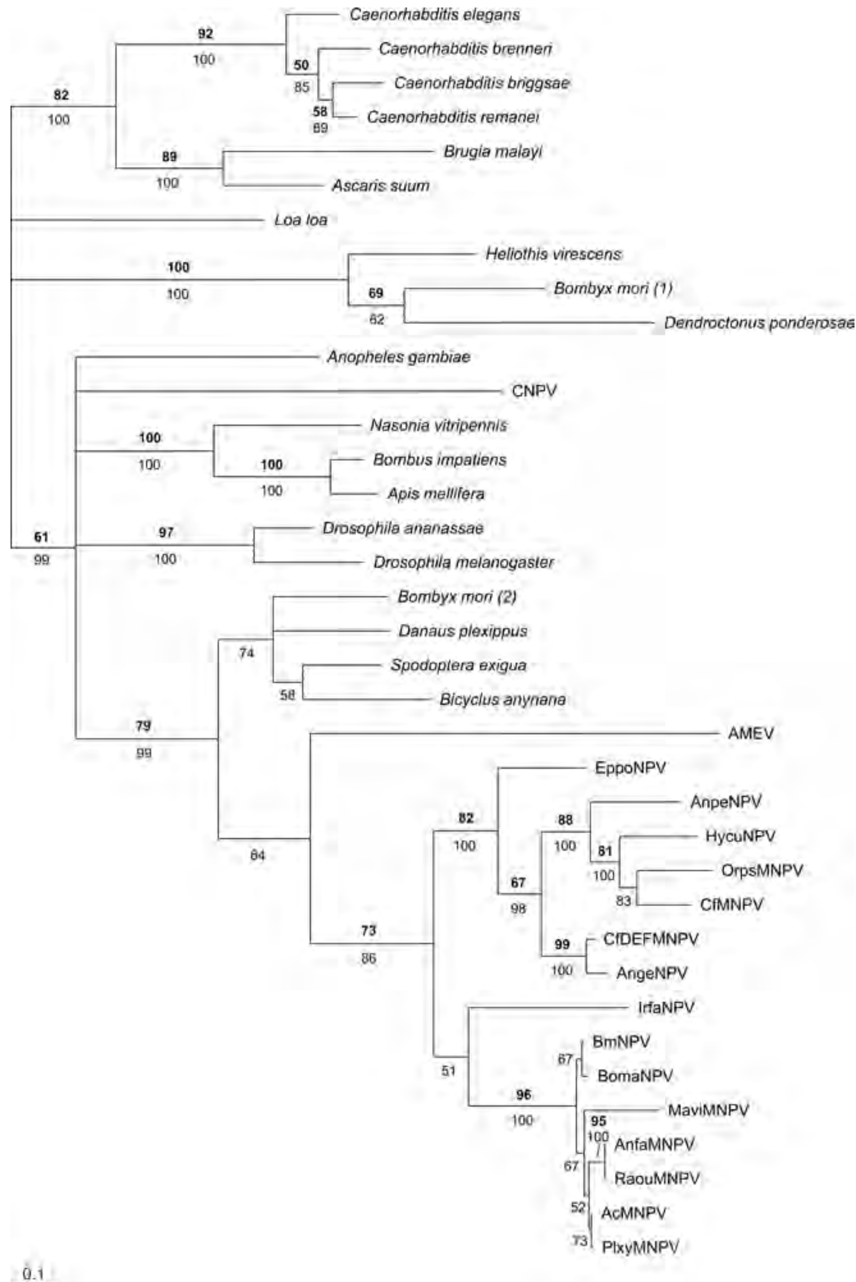


Figure 4. The baculovirus *ptp* gene was presumably acquired from a lepidopteran host by an ancestral NPV. Bayesian phylogeny of *ptp* nucleotide sequences from diverse invertebrate taxa. GenBank and Butterflybase/InsectaCentral accession numbers are given in Table S2 (van Houte et al. 2012). Numbers in bold indicate maximum likelihood bootstrap values based on 100 replicates, while plain numbers depict Bayesian posterior probabilities. Only values ≥ 50 are indicated for both analyses. The bar at the bottom indicates a branch length of 10% distance.

The baculovirus phylogeny (Fig. 5) shows that the *ptp* gene is present in all group I NPVs in the genus Alphabaculovirus, and absent in all Alphabaculovirus group II NPVs and Betabaculoviruses (GVs). The current hypothesis is that GVs and NPVs arose from a common ancestor, and that speciation of group I and II NPVs took place after divergence of GVs and NPVs (Herniou et al. 2004). The fact that only group I NPVs carry *ptp* indicates that the transfer of a lepidopteran host *ptp* to an ancestral baculovirus occurred after the group I and II NPVs diverged, or that the *ptp* gene was acquired before this divergence, and subsequently lost in (an ancestor of) group II NPVs and GVs. A transfer to an ancestral group I NPV baculovirus is the most parsimonious scenario.

In addition to the *B. mori ptp* sequence that is part of the lepidopteran clade showing high similarity to baculovirus *ptp*, *B. mori* also carries another *ptp* gene that is positioned in a different clade, together with *ptp* from *Heliothis virescens* (Lepidoptera) and *Dendroctonus ponderosae* (Coleoptera) (Fig. 4). The presence of this different *ptp* sequence within the *B. mori* genome could indicate an ancient gene duplication event, after which the different *ptp* copies diverged in sequence and, possibly, function. Alternatively, these two genes were acquired during two separate gene transfer events. Not all lepidopteran species carry two *ptp* genes: for *B. anynana* and *D. plexippus* only one copy was found. Whether *S. exigua* and *H. virescens* carry two copies is unknown since no whole genome information is available for these organisms.

Besides the presence of *ptp* in group I NPVs, another gene encoding a putative PTP protein is present in several baculovirus genomes. This gene, named *ptp2*, is present in a number of group II NPVs and in two GVs (Fig. 5).

Discussion

Baculoviruses have long been known to alter behaviour of their insect hosts (Goulson 1997; Hofmann 1891; Vasconcelos et al. 1996), but the mechanisms underlying these behavioural changes are still poorly understood. The involvement of the viral *ptp* gene in hyperactivity has been shown for *B. mori* larvae infected with BmNPV in a study that has provided valuable first insights into baculovirus-induced behavioural changes (Kamita et al. 2005). However, it remained uncertain whether *B. mori*, which underwent massive artificial selection throughout its domestication, reflects a truly ecologically relevant model system to study such manipulative strategies. We used the baculovirus AcMNPV and its natural host *S. exigua* as a model system to study the degree of conservation of *ptp*-induced behavioural manipulation in lepidopteran hosts. We show that AcMNPV induces hyperactive behaviour and that this behavioural change is absent when the cata-

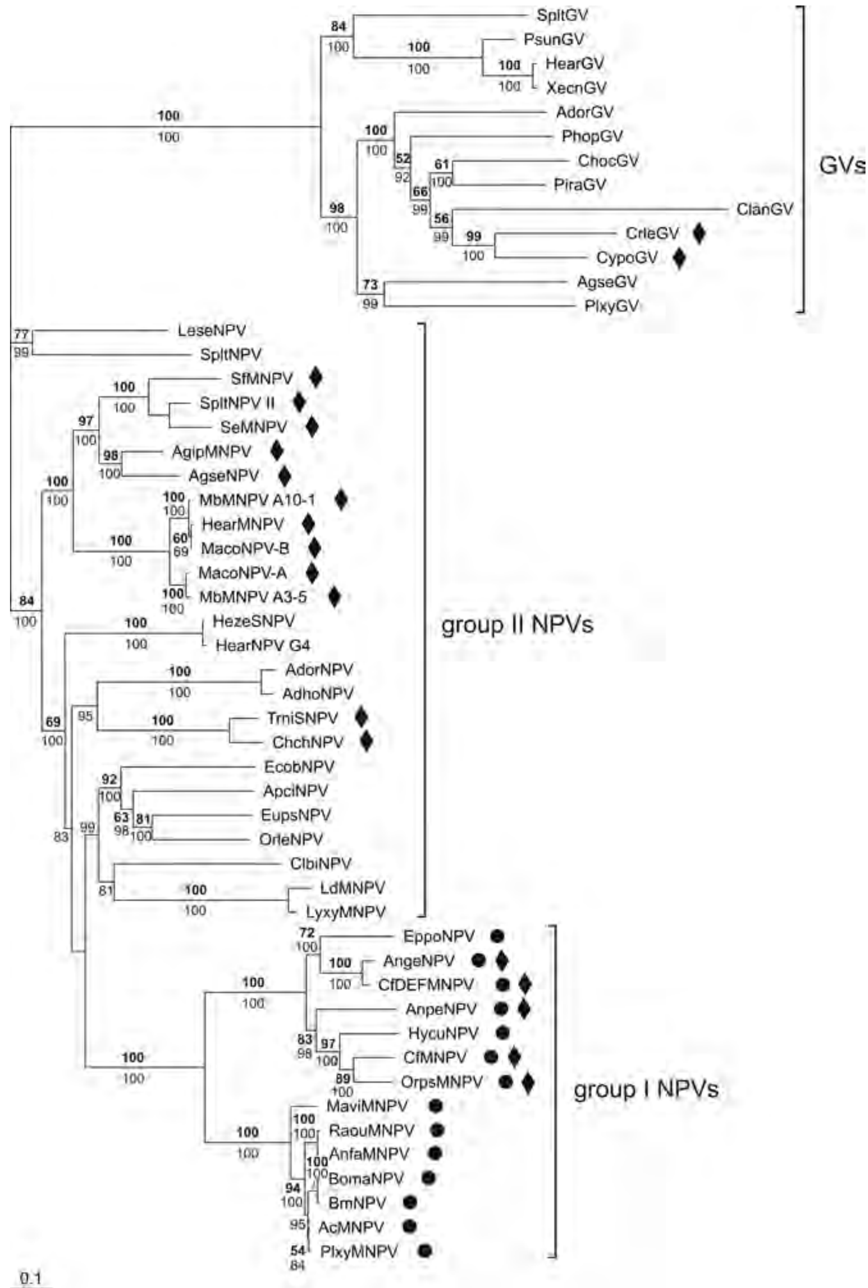


Figure 5. The *ptp* gene is present in all Alphabaculovirus group I NPVs. Bayesian phylogeny of baculoviruses based on the l*ef-8* gene. GenBank accession numbers are given in Table S3 (van Houte et al. 2012). Numbers in bold indicate maximum likelihood bootstrap values based on 100 replicates, while plain numbers depict Bayesian posterior probabilities. Only values ≥ 50 are indicated for both analyses. The bar at the bottom indicates a branch length of 10% distance. Baculoviruses possessing a *ptp* gene are marked by a black dot, while baculoviruses possessing a *ptp2* gene are marked by a black diamond.

lytic site of the encoded phosphatase enzyme is mutated. These data, together with the finding that the *ptp* gene is well-conserved in a group of related baculoviruses (group I NPVs), provide solid evidence that *ptp*-induced hyperactivity represents an evolutionary conserved strategy to manipulate host behaviour.

In the same study by Kamita et al. (2005) it was also shown that insertion of a *B. mori* host-derived *ptp* homolog in a BmNPVΔ*ptp* genome partially restored the hyperactive phenotype (Kamita et al. 2005). Our phylogenetic analysis suggests that the baculovirus *ptp* gene has a lepidopteran origin, indicating that an ancestral virus may have acquired this gene from its host in order to manipulate endogenous host pathways involved in behaviour. Our analysis also demonstrates the presence of *ptp* in all group I NPV genomes sequenced so far. Besides its presence in baculoviruses, arthropods and nematodes, a *ptp* homolog was also found in the poxviruses AMEV and CNPV. To our knowledge no behavioural changes have been described for poxvirus-infected hosts so far.

An important issue to address is the ecological relevance of the observed increased host activity, i.e. how could it contribute to virus transmission? Firstly, the increased movement of infected larvae ensures that progeny virions will be released over a larger surface, contributing to a higher chance of encountering a new host. Secondly, it is also thought that infected larvae start to 'leak' virus at a certain time after infection. Indeed, during our experiments, we occasionally observed larvae leaving a liquid trail, possibly containing virus particles, at three dpi (data not shown). Similar observations were reported during other studies (Kamita et al. 2005). Thirdly, increased activity in the field may also increase visibility to predator animals such as birds, which could serve as dispersal agent for the virus (Vasconcelos et al. 1996). An alternative explanation might be that increased movement of infected larvae reflects an adaptive strategy of the host to prevent virus transmission to conspecifics, as it could result in removal of viral inoculum from the insect population (Goulson 1997).

Regarding the magnitude of the observed behavioural changes, it is interesting to note that in the studies performed with BmNPV-infected *B. mori* larvae, activity differences between WT- and mock-infected larvae were large (approximately 80-fold), while in our work an approximately 1.5-fold difference in activity between mock- and WT-infected individuals was observed. However, as discussed in the introduction, the *B. mori* insect strain used for the hyperactivity study by Kamita et al. (2005) displayed an exceptionally low level of endogenous activity (Kamita et al. 2005). In contrast, the *S. exigua* larvae used in our experiments were not selected for any particular behavioural trait (although selection pressures affecting behavioural traits in cultured insect populations cannot be excluded). Therefore, the differences in activity found in the current work may better

reflect the natural situation. In our studies, mock-infected control larvae show a high endogenous activity, moving an average distance of 400-450 mm in 10 min. A 1.5-fold increase in distance moved of the observed population would over a 24 h time span lead to an approximately 40 meter difference in distance travelled between WT- and mock-infected larvae (excluding feeding and resting periods). For an efficient spread of virus particles over a large area such a difference could be a considerable factor.

Our findings seem to be in contrast with a recent study by Katsuma et al. (2012), reporting that in BmNPV-infected *B. mori* larvae PTP phosphatase activity was not required for the induction of hyperactivity. BmNPVΔ*ptp*-infected larvae showed reduced OB and BV production, and lower viral expression in many larval tissues, including the brain (Katsuma et al. 2012). The authors hypothesize that this reduction of viral replication in the brain causes the behavioural phenotype of BmNPVΔ*ptp*-infected larvae. Although this is an interesting observation that could explain the fact that in cell culture the deletion of the AcMNPV *ptp* gene results in lower viral infectivity (Li & Miller 1995b), it does not explain the mechanism by which this lower level of BmNPV viral expression in the host brain would contribute to altered behaviour in *B. mori*. The difference in behavioural phenotype between the *ptp* catalytic mutant in the BmNPV/*B. mori* system and the AcMNPV/*S. exigua* system may imply that distinct mechanisms underlie hyperactivity in these two systems. Several studies have shown that AcMNPV PTP is associated with virions, similar to BmNPV PTP (Li & Miller 1995a; Wang et al. 2010). Possibly, AcMNPV PTP functions both as a structural protein and a phosphatase. Minor variations in amino acid sequence (the proteins have 97% similarity) may play a role in this difference. Distinct mechanisms may also contribute to the difference in magnitude of the observed hyperactive phenotype.

In addition, there are several important differences in experimental setup between the studies that need to be considered. Firstly, *B. mori* 5th instar larvae were used in the study by Katsuma et al. (2012), while in the current study *S. exigua* 3rd instar larvae were used. It is known from other studies that behaviour, including locomotion activity, is influenced by developmental stage (Goulson 1997; Vasconcelos et al. 1996). Furthermore, movement assays with *B. mori* were performed with groups of larvae, while in this study movement assays were performed with individual larvae. This difference in experimental setup could have important behavioural consequences. For solitary host species, such as the *S. exigua* 3rd instar larvae used in this study (the 1st and 2nd instar are gregarious, after which the larvae become solitary (Smits et al. 1987)), the motivation to move away from conspecifics could be high, while gregarious species may tend to remain together. Future research should provide more insight in whether these factors are of importance in the observed behavioural differences between the two studies.

The finding that in AcMNPV a *ptp* gene encoding a catalytically active PTP enzyme is needed for the induction of hyperactivity has important implications for understanding the molecular mechanisms underlying baculovirus-induced behaviour. It suggests that AcMNPV PTP targets one or more phosphorylated substrate(s), either virus- or host-derived, which subsequently leads to the observed behavioural alteration. This opens up exciting new possibilities for further research to understand in more detail the mechanism of PTP-induced behavioural manipulation. For example, a differential transcriptome and (phospho-)proteome analysis could be employed in which WT- and Δ*ptp*-infected larvae are compared. Such experiments will shed light on possible host proteins and pathways affected by PTP expression. In addition, a host candidate-gene approach (Fitzpatrick et al. 2005) could be followed in which host genes known to be involved in a behavioural phenotype in different insect species are investigated for their involvement in parasitic manipulation of behaviour and their possible link with PTP.

Although the role of *ptp* in behavioural manipulation, despite possible variation in mechanism, appears to be conserved within group I NPVs, none of the group II NPVs or GVs carries a *ptp* gene (Fig. 5). Nevertheless, several studies indicate that group II NPVs may also alter host behaviour. For example, Goulson (1997) reported that *Mamestra brassicae* larvae infected with *M. brassicae* nucleopolyhedrovirus (MbMNPV) showed higher locomotion activity in both laboratory and field experiments compared to uninfected individuals (Goulson 1997). As the MbMNPV genome sequence has not been fully sequenced, we cannot exclude the possibility that MbMNPV carries a *ptp* gene, which may explain these behavioural changes. However, its position within the group II NPVs (Fig. 5), in which no baculovirus known so far contains a *ptp* gene, suggests that *ptp* is absent from its genome. This would imply that baculoviruses have developed different strategies to manipulate host behaviour. Interestingly, MbMNPV, together with a subset of the group II NPVs and two GVs (Fig. 5), carries a *ptp2* gene encoding a protein that belongs to the same PTP protein family as AcMNPV PTP, although the similarity between the two proteins is very low (13% amino acid similarity). Whether this gene may have a similar function as *ptp* in manipulating host behaviour is unknown.

Besides hyperactivity, baculoviruses are also known to alter climbing behaviour of their insect hosts (tree-top disease) (Goulson 1997; Vasconcelos et al. 1996). A recent study showed involvement of the ecdysteroid UDP-glucosyl transferase (*egt*) gene from *L. dispar* multiple nucleopolyhedrovirus (LdMNPV) in the induction of tree-top disease in *L. dispar* larvae (Hoover et al. 2011). LdMNPV is a group II NPV that does not carry a *ptp* gene. Hyperactivity was not consistently observed in LdMNPV-infected *L. dispar* larvae (K. Hoover, unpublished results), possibly indicating that tree-top disease and hyperactivity are two distinct behaviours induced by baculoviruses, for which different

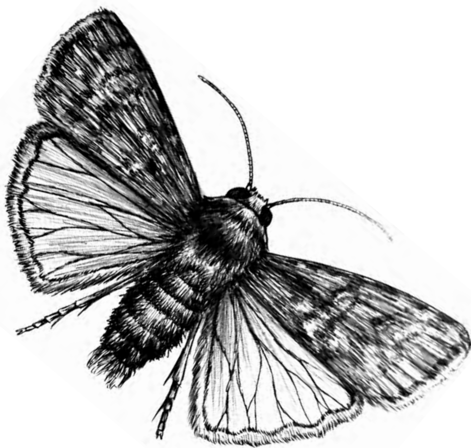
viral genes are responsible.

In addition to *ptp* and *egt*, other viral genes might affect larval behaviour. Recently, Biernat et al. (2012) showed that a DNA repair protein (PHR2) encoded by the baculovirus *Chrysodeixis chalcites* nucleopolyhedrovirus (ChchNPV) can mimic the function of mammalian cryptochromes, essential regulators of the circadian clock (Biernat et al. 2012). It remains to be elucidated whether this protein can alter host circadian rhythm-related behaviour, but this could indicate yet another strategy of baculoviruses to manipulate host insect behaviour.

Understanding the molecular mechanisms underlying parasitic manipulation of host behaviour, which is an example of the extended phenotype, is a fascinating new research field, in which knowledge from behavioural genetics, ecology, and parasitology is united. It provides invaluable information on the variety and complexity of host manipulation strategies, and on the evolutionary arms race between parasites and their hosts. The sophisticated ways employed by baculoviruses to manipulate host behaviour provide an excellent starting point for understanding such underlying mechanisms.

Acknowledgements

The authors thank Linda Guarino for kindly providing the AcMNPV Δ*ptp* bacmid, Primitivo Caballero and Delia Muñoz for providing *S. exigua* eggs and pupae, and Salva Herrero for providing the *S. exigua ptp* sequence. Els Roode and Bregje Vrolijk are acknowledged for experimental assistance. We are grateful to Just Vlak for critically reading this manuscript and for useful discussions. Bas Engel is acknowledged for his advice on the statistical analyses.



Chapter 4

Substrate identification of baculovirus protein tyrosine phosphatase: clues for mechanisms underlying virus-induced hyperactive behaviour

Adapted from:

Stineke van Houte, Ambrosius P. Snijders, Esther van Andel, Carmen W.E. Embregts, Just M. Vlak, Monique M. van Oers and Vera I.D. Ros. Substrate identification of baculovirus protein tyrosine phosphatase: clues for mechanisms underlying virus-induced hyperactive behaviour, *manuscript in preparation*.

Abstract

Many parasites alter the behaviour of their host to maximize their transmission and survival. Despite the numerous known examples of parasitic manipulation, the underlying mechanisms are largely enigmatic. Baculoviruses manipulate host behaviour by inducing hyperactivity and climbing behaviour. Previous work demonstrated the involvement of a protein tyrosine phosphatase (PTP) from the baculovirus *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) in the induction of hyperactive behaviour in *Spodoptera exigua* larvae. This finding prompted us to investigate which viral and/or host proteins are targeted by the baculovirus PTP enzyme to accomplish altered host behaviour. Using affinity-tag purification of a substrate-trapping mutant of AcMNPV PTP followed by proteomic analysis, six viral and six host proteins were identified that co-purified with PTP. Several of these proteins are known to be important in cellular signalling and in behaviour in other insects, and are therefore potentially involved in PTP-mediated hyperactivity of infected larvae. For one of these identified host proteins, the 14-3-3 ζ protein, RNA expression levels were found to be significantly higher in AcMNPV WT-infected larvae as compared to AcMNPV Δ*ptp*-infected larvae, indicating that *14-3-3 ζ* expression is dependent on the presence of the baculovirus *ptp* gene. The 14-3-3 ζ protein is known to be important for the synthesis of serotonin and dopamine, which are neurotransmitters that play important roles in many behavioural pathways. We hypothesize that baculovirus *ptp* targets 14-3-3 ζ at the RNA and possible also at the protein level, which ultimately leads to baculovirus-induced hyperactivity.

Introduction

Parasitic manipulation of host behaviour is a commonly observed phenomenon in a wide range of animals and may contribute to transmission or survival of the parasite (reviewed in Hughes et al. (2012); van Houte et al. (2013)). Baculoviruses are arthropod-specific DNA viruses that cause behavioural alterations in their caterpillar hosts upon infection. This is thought to contribute to optimal virus dissemination into the environment. These alterations encompass hyperactivity and climbing behaviour (resulting in 'Wipfelkrankheit' or 'tree-top disease') prior to death (Goulson 1997; Hofmann 1891). Previous studies have reported the involvement of the baculovirus *ptp* gene in inducing hyperactive behaviour in lepidopteran caterpillars (Kamita et al. 2005; van Houte et al. 2012). This gene, which is present in a subset of Alphabaculoviruses named group I nucleopolyhedroviruses (NPVs), encodes a 19 kDa protein tyrosine phosphatase (PTP) enzyme that dephosphorylates tyrosine, serine and threonine residues *in vitro* (Sheng & Charbonneau 1993). PTP not only shows affinity to protein substrates, but also has very high affinity for RNA substrates, suggesting a possible role in baculovirus mRNA capping (Gross & Shuman 1998; Li & Guarino 2008; Takagi et al. 1998).

The *ptp* gene of *Bombyx mori* nucleopolyhedrovirus (BmNPV) is responsible for hyperactivity of infected *B. mori* caterpillars (Kamita et al. 2005), but the phosphatase activity of the encoded PTP enzyme is not necessary to induce this behavioural change (Katsuma et al. 2012). In the related baculovirus *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV), however, phosphatase activity of the viral PTP protein is required for this altered behaviour in infected *Spodoptera exigua* larvae (van Houte et al. 2012). This finding suggests that, at least in the latter virus-host system, a host or viral protein serves as substrate for the AcMNPV PTP enzyme and that dephosphorylation of this substrate consequently leads to the observed change in activity.

In the current study we aimed to identify host and viral proteins that are targeted by the AcMNPV PTP enzyme. To extend the interaction time of potential PTP substrates with the binding pocket of the PTP enzyme, a so-called 'substrate-trapping' mutant of PTP was used (Jia et al. 1995). This is a mutant PTP enzyme in which a catalytic cysteine residue in the PTP-signature motif (Tonks 2006) is mutated into a serine. This mutation ensures proper binding of a PTP substrate, but catalysis does not occur, thus trapping the substrate in the binding pocket of the mutant PTP. Subsequently, these bound substrates are co-purified upon PTP purification (Blanchetot et al. 2005; Liang et al. 2007). Affinity-tag purification of the AcMNPV PTP protein was performed on lysates of infected insect cells, and co-purified proteins were subsequently identified using mass spectrome-

try. Among the co-purified host and viral proteins we identified several proteins that are potentially involved in baculovirus-induced hyperactive behaviour through their interaction with the viral PTP protein. In addition, for one of the identified host proteins, RNA transcript levels were compared using quantitative PCR (qPCR) on uninfected *S. exigua* larvae, AcMNPV WT-infected larvae and larvae infected with a *ptp* deletion mutant of AcMNPV (Δ*ptp*). These data provide additional support that this host protein might be targeted by PTP on both the RNA and protein level.

Materials and methods

Substrate analysis

Insect cell culture

Spodoptera frugiperda 9 (Sf9) cells (Invitrogen) were maintained as monolayers in Sf900II serum-free medium (Invitrogen) supplemented with 5% fetal bovine serum (Invitrogen) and 0.1% gentamycin (Invitrogen). For protein expression experiments using recombinant baculoviruses, suspension cultures of Sf9 cells were grown in Sf900II serum-free medium with 0.1% gentamycin.

Generation of recombinant bacmids

To enable purification of the AcMNPV PTP protein, a recombinant bacmid was created that produced a PTP protein with a C-terminal His-tag. For this construct, the AcMNPV bacmid with a deletion of the *ptp* gene (Δ*ptp*) (van Houte et al. 2012) was used as backbone. The expression of the *ptp-his* open reading frame (ORF) was controlled by the AcMNPV *gp64* promoter (Ac-P_{gp64}) as this promoter is known to be active throughout the infection (Blissard & Rohrmann 1989; Whitford et al. 1989). To this aim, we first amplified the AcMNPV *gp64* (Ac-P_{gp64}) promoter region (AcMNPV nucleotide positions 109720 to 109924) (Blissard & Rohrmann 1991) using primer 1 containing an *Nco*I restriction site (underlined) combined with primer 2 containing an *Nhe*I restriction site (underlined) (Table 1). In this experiment we mutated the Cys residue of the PTP-signature motif into a Ser residue to create a C119S substrate-trapping mutant of PTP. This mutation was created by overlap extension PCR with Phusion polymerase (Finnzymes) using primer 3, which introduced a point mutation at nucleotide (nt) 355 relative to the ATG start codon of PTP, combined with primer 4 that anneals to the 3'-end of the *ptp* ORF (Table 1). The resulting 177 bp product was then used as reverse primer combined with primer 5 annealing to the start of the *ptp* ORF. This ptp^{C119S}

ORF fragment was subsequently used as template for PCR using primer 6, containing an *Nhe*I restriction site (underlined) and encoding an N-terminal Strep-tag (italics) to allow optional tandem purification of the protein, and primer 7, with an *Nsi*I restriction site (underlined) and encoding a 10x His-tag to create a PTP^{C119S} protein with a C-terminal His-tag (italics) (Table 1). The Ac-P_{gp64} and $ptp^{C119S}His$ DNA fragments were cloned as *Nco*I/*Nhe*I fragment and *Nhe*I/*Nsi*I fragment, respectively, into the pFBD-polhΔp10 vector, which is a derivative from pFastBacDual (Invitrogen) in which the AcMNPV polyhedrin (*polh*) ORF was inserted downstream of the *polh* promoter, and the *p10* promoter was removed (Peng et al. 2010; van Houte et al. 2012). In the resulting construct pFBDpolhP_{gp64} -$ptp^{C119S}His$, the *gp64* promoter and $ptp^{C119S}His$ sequence occupy the position of the original *p10* promoter. Bac-to-Bac transposition (Luckow et al. 1993) was performed to integrate the P_{gp64}-$ptp^{C119S}His$ and P_{polh}-*polh* fragments into the AcMNPV Δ*ptp* bacmid (Fig. 1A).

Two different bacmids were used as negative controls (Fig. 1A). The AcMNPV wild type (WT) bacmid (van Houte et al. 2012) that expresses *Ac-polh*, but is not encoding any His-tagged protein, was used as negative control in the purifications to check for background proteins that bind nonspecifically to the Co^{2+}-charged resin of the His-purification column. A second negative control consisted of an AcMNPV WT bacmid expressing a His-tagged eGFP protein to check for proteins that co-purify with His-tagged proteins in a nonspecific manner. This bacmid was originally created by Bac-to-Bac transposition with the pFastBacHTb vector (Invitrogen), in which the eGFP ORF with an N-terminal 6x His-tag was cloned as *Bam*HI/*Sst*I fragment downstream of the AcMNPV *polh* promoter (Kaba et al. 2002). Transfections of Sf9 cells with bacmid DNA were performed with Cellfectin reagent (Invitrogen). Budded virus (BV) stocks for all recombinant viruses were produced in Sf9 cells according to the Bac-to-Bac system (Invitrogen).

Protein purification

Sf9 suspension cultures of 200 mL each (app. $2x10^6$ cells/mL) were infected with either AcMNPV encoding PTP^{C119S}-His (named 'PTP'), AcMNPV encoding eGFP-His (named 'GFP') or AcMNPV WT (named 'WT') using a multiplicity of infection (MOI) of 5 $TCID_{50}$/cell. At 72 hours post-infection (hpi) cells were harvested by centrifugation and resuspended in phosphate-buffered saline (PBS) containing a protease inhibitor cocktail (*cOmplete*, Roche) and 0.1% Nonidet P-40 (AppliChem). Cells were lysed by sonication, and the supernatant was subjected to a 0.5 mL TalonSpin column (ClonTech) to purify the His-tagged proteins according to the manufacturer's instructions. Proteins were eluted with elution buffer containing 300 mM imidazole. SDS-PAGE was

performed on the purified samples to confirm the presence of the His-tagged proteins (or absence in the negative control) by Western blot analysis using anti-His antibodies (Sigma-Aldrich).

Gel electrophoresis, in-gel digestion and LC/MS-MS procedure

Equal volumes of elution fractions of the His-purified samples were separated by SDS-PAGE (12% acrylamide) for approximately 1.4 cm into the gel, which was then stained with Coomassie Brilliant Blue. To prepare the peptides for LC/MS-MS for each of the three samples described above (PTP, GFP, WT), equal portions of the central part of each gel lane were excised. Each gel lane was cut into eight equally sized gel pieces and subjected to in gel trypsin digestion using a Perkin Elmer Janus Automated Workstation. Peptide mixtures were acidified with 0.1% TFA and injected into a nanoACQUITY UPLC (Waters Corporation) coupled to a LTQ-Orbitrap XL (Thermo Fisher Scientific) via an Advion Biosciences Nanomate. Peptides were eluted over a 30 min gradient (5-40% ACN). Data were acquired in a data-dependent fashion using a top 10 method.

Database searching

Tandem mass spectra were extracted by Mascot Distiller. Charge state deconvolution and deisotoping were not performed. Merged peak lists corresponding to each lane were searched using Mascot (Matrix Science, London, UK; version 2.4.1). Mascot was set up to search a subset of the UniProt database supplemented with *S. exigua* 14-3-3 ζ and ε protein sequences (GenBank accession numbers KC188794 (14-3-3 ζ) and KC188795 (14-3-3 ε), experimental procedures on obtaining these sequences are detailed below) and *S. exigua* cGMP-dependent protein kinase (PKG) protein sequences (GenBank accession number FJ606838), and the *Drosophila melanogaster* reference proteome assuming the digestion enzyme trypsin. Mascot Spectra were searched with a fragment ion mass tolerance of 0.80 Da and a parent ion tolerance of 10 PPM. Oxidation of methionine, carbamidomethylation of cysteine and phosphorylation of serine, threonine and tyrosine were specified as variable modifications. Mascot data files were imported into Scaffold for further data analysis.

Criteria for protein identification

Scaffold (version Scaffold_3.6.5, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 90.0% probability as specified by the Peptide Prophet algorithm (Keller et al. 2002). Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least

2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al. 2003). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. A coefficient of variance test was performed in Scaffold to find those proteins that showed significantly more assigned unique spectra in the PTP sample as compared to the GFP and WT control samples.

Gene expression analysis

Insect larvae and virus amplification

Spodoptera exigua larvae were reared on artificial diet at 27 ºC with 50% relative humidity as described before in (Smits et al. 1986), and with a 14:10 h light:dark photoperiod. Virus production, amplification and purification were all done as described in van Houte et al. (2012). Stocks of occlusion bodies (OBs) were stored at 4 ºC.

Infection of larvae

Early 3rd instar *S. exigua* larvae were either infected with wild type (WT) AcMNPV or with an AcMNPV *ptp* deletion mutant (Δ*ptp*) as described previously in van Houte et al. (2012). Per treatment 35-40 larvae were infected with a viral dose of 10^8 OBs/ml (LC_{90-95} dose) using droplet feeding. Mock-infected larvae were used as uninfected controls, and these larvae were droplet fed with a virus-free sucrose solution as described in van Houte et al. (2012). After infection, larvae were placed individually in 12-well plates, provided with a piece of artificial diet (Smits et al. 1986) and reared at 27 ºC.

RNA isolation and reverse transcription

RNA was isolated at one day post-infection (dpi) (10 larvae per treatment), two dpi (10 larvae per treatment) and three dpi (15 larvae per treatment). Single larvae were homogenized in 250 µl Trizol reagent (Invitrogen) and total RNA was isolated following the manufacturer's instructions. The RNA pellet was dissolved in 50 µl water and heated for 10 min at 55 ºC. RNA concentrations were measured using a NanoDrop-1000 spectrophotometer (Thermo Fisher Scientific) and the integrity of the RNA was examined by 1% agarose gel electrophoresis. To remove contaminating DNA, 1500 ng of extracted RNA was treated with DNAse (DNA*free*, Applied Biosystems) according to the company's protocol. After DNAse treatment, 1000 ng extracted RNA was used to produce cDNA using SuperScript III Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol.

Acquiring the *S. exigua 14-3-3 ζ* and *14-3-3 ε* cDNA sequences

RNA was isolated and cDNA obtained from an uninfected larva as described above. RT-PCR was performed using High-Fidelity Phusion polymerase (Fermentas) to acquire the cDNA sequence of i) *14-3-3 ζ* using primers 8-11 (Table 1), hand-designed from available lepidopteran *14-3-3 ζ* sequences (GenBank) and ii) *14-3-3 ε* using primers 12-15 (Table 1), hand-designed from available lepidopteran *14-3-3 ε* sequences (GenBank). PCR products were gel-purified using the PCR DNA and gel band purification kit (GE Healthcare) and sequenced.

qPCR primer design and optimization

Primers for qPCR analysis (primers 16-23, Table 1) were designed for the *S. exigua 14-3-3 ζ* gene and for the viral AcMNPV genes *ptp* and *vp39* (obtained from GenBank accession L22858) using Primer 3 (Untergasser et al. 2012) and mFold (Integrated DNA Technologies) as described in Thornton & Basu (2011). For 28S rRNA (reference gene), qPCR primers from Xue et al. (2010) were used (primers 22-23, Table 1). qPCR conditions were optimized for primer concentration and annealing temperature using IQTM SYBR Green Supermix (Biorad).

Quantitative real time PCR

The obtained cDNA was used for quantitative real time polymerase chain reaction (qPCR) using IQTM SYBR Green Supermix (Biorad). Total reaction volume was 25 μl, including forward and reverse primers at a 100 nM final concentration, and 5 μl of 50x diluted cDNA (final dilution: 250x). Non-template controls were included in each run. For each gene, standard curves were created *in triplo* (final dilutions of 125x, 625x, 3125x, 15625x, 78125x, 390625x). In subsequent runs the 3125x dilution was taken along with the samples and the standard curve was imported. Expression levels of *14-3-3 ζ* and *ptp* genes were measured for each sample *in duplo* (for *ptp* gene expression only samples from WT AcMNPV-infected individuals were included) and were normalized to the 28S rRNA gene expression levels (Salem et al. 2011). For each sample of infected larvae (WT or Δ*ptp*), a PCR was performed to amplify the viral *vp39* gene to confirm successful infection of the larvae. Samples that had no *vp39* expression were excluded from all analyses.

The PCR reactions were performed in a Rotor-Gene 6000 thermal cycler (Corbett Research) and fluorescence was recorded on the FAM channel using automatic first acquisition gain optimization. The program used for all genes was 94 °C for 3 min; 45 cycles of 95 °C for 15 sec, 62 °C for 20 sec, 72 °C for 30 sec (acquisition of fluores-

Table 1. Primers used in this study.

Primer number	Primer name	Sequence (5' to 3')	Function
1	gp64_FW	GCGCCATGGTGTCGACTGAGCGTCCGTGT	amplify AcMNPV *gp64* promoter region
2	gp64_RV	GACGCTAGCTTGCTTGTGTGTTCCTTATTGAAG	amplify AcMNPV *gp64* promoter region
3	ptp_mut_C119S	CCCGGCATGTTGGTGGGCGTGCACAGCACACACGGTATTAATCGC	create C119S mutant of AcMNPV PTP
4	ptp_RV	TTAAATTAATAAATCTTGAAC	amplify AcMNPV *ptp* ORF
5	ptp_FW	ATGTTTCCCGCGCGTTGGCAC	amplify AcMNPV *ptp* ORF
6	Strep-ptp	CAGGCTAGCATG*TGGAGCCACCCCCAGTTCGAAAAG*AGCGCATTTCCCGCGCGTTGGCACAAC	add N-terminal Strep-tag (italics) to PTP
7	ptp-His	GTCATGCATTTA*GTGGTGGTGATGGTGATGATGGTGGTGATG*AATTAATAAATCTTGAACGTAATTTTG	add C-terminal 10x His-tag (italics) to PTP
8	Zeta_F1	GGCATCAGTGAATTARTYCTC	amplify *14-3-3 ζ* cDNA sequence
9	Zeta_F2	TCCACGATGTCCGTCGACAAG	amplify *14-3-3 ζ* cDNA sequence
10	Zeta_R1	GGCGACAACTAATMTCKC	amplify *14-3-3 ζ* cDNA sequence
11	Zeta_R2	GAGAACAAATGCGTGTGC	amplify *14-3-3 ζ* cDNA sequence
12	Epsilon_F1	TGTCATCCACAATGTCGG	amplify *14-3-3 ε* cDNA sequence
13	Epsilon_F2	CCACAATGTCGGAAAGG	amplify *14-3-3 ε* cDNA sequence
14	Epsilon_F3	TGGAGGCGATGAAGAATGTAGC	amplify *14-3-3 ε* cDNA sequence
15	Epsilon_R4	GCATGTCGGAGGTCCACA	amplify *14-3-3 ε* cDNA sequence
16	qPCR_zeta_F1	CTGAGCAATGAGGAAAGGAACC	qPCR primer for *14-3-3 ζ*
17	qPCR_zeta_R1	AATGGAGGAAATGACACGCC	qPCR primer for *14-3-3 ζ*
18	qPCR-ptp-F1	TTGGCACAACTATTTACAATGCG	qPCR primer for *ptp*
19	qPCR-ptp-R1	GTTTTACTATCTGTTCTGCGGTC	qPCR primer for *ptp*
20	qPCR-vp39-F1	ACAACCCGATAAGAAGCAGTGACA	qPCR primer for *vp39*
21	qPCR-vp39-R1	TAAGCGTTCTGTCCAGCTCACG	qPCR primer for *vp39*
22	Sf_28S_F	CGACGTTGCTTTTTGATCCT	qPCR primer for 28S rRNA (reference gene) (Xue et al. 2010)
23	Sf_28S_R	GCAACGACAAGCCATCAGTA	qPCR primer for 28S rRNA (reference gene) (Xue et al. 2010)

cence signal); 60 ºC for 5 min, followed by a melting curve (60 ºC - 99 ºC) to examine amplification specificity. Cycle threshold (Ct) values and PCR efficiencies were calculated by the Rotor-Gene 6000 Series Software 1.7 and obtained using the 'Slope Correct' and 'Dynamic Tube' options. Relative expression levels were calculated using the ΔΔCt method (Livak & Schmittgen 2001; Schmittgen & Livak 2008).

Statistical analysis

Calculated ΔΔCt values were used for statistical analysis. The data were checked for normality and equality of variances by Kolmogorov-Smirnov and Levene's tests, respectively. Data were normalized by log (for *14-3-3 ζ*) or square root (for *ptp*) transformation. One-way ANOVA tests were performed to test for differences between groups. In the case of (near) equality of variances (*14-3-3 ζ*), differences between groups were further examined using Least Significance Difference (LSD) post-hoc pair-wise comparisons. When equality of variances could not be assumed (*ptp*), non-parametric Dunnett's T3 post-hoc pair-wise comparisons were used. Statistical tests were carried out using IBM SPSS Statistics v20.

Results

Substrate analysis

Protein production and His-tag purification

To enable affinity-tag purification of the AcMNPV PTP protein from insect cells, a recombinant bacmid was created that expressed the PTP catalytic mutant C119S with a C-terminal 10xHis-tag (Fig. 1A). As negative controls an AcMNPV WT bacmid expressing polyhedrin (*polh*) (WT) and an AcMNPV bacmid encoding His-eGFP (GFP) were used in the purification and subsequent LC-MS/MS analysis (Fig. 1A). SDS-PAGE followed by Coomassie staining and Western blot analysis using anti-His antibodies were performed on the eluted protein fractions to confirm the presence of His-tagged proteins after column purification (Fig. 1B). On Western blot GFP showed a clear band at around 31 kDa, corresponding to the expected size of the protein. As expected no GFP could be observed in the eluted protein fraction of WT. For PTP a clear band could be observed at around 24 kDa, which is slightly higher than the expected 22 kDa size of the tagged protein (Fig. 1B).

Table 2

(A). Baculovirus (AcMNPV) proteins that co-purified with the AcMNPV PTP protein. MS/MS spectra were searched against the UniProt protein database. Six viral proteins were found to co-purify specifically with PTP. Shown is the number of unique peptides and the % coverage found for each of those proteins.

Viral (AcMNPV) proteins		**UNIPROT**	
Protein name	***ORF number***	***# of unique peptides***	***% coverage***
Protein tyrosine phosphatase (PTP) (input)	*1*	*13*	*77*
DNA polymerase	65	9	17
Probable DNA polymerase sliding clamp (PCNA)	49	6	49
Apoptosis inhibitor 1 (IAP-1)	27	2	10
Serine/threonine-protein kinase 1 (PK-1)	10	4	23
3 kDa early protein homolog	92	2	21
Capsid-associated protein VP91	83	7	15

(B). Host proteins that co-purified with the AcMNPV PTP protein. MS/MS spectra were searched against the UniProt protein database supplemented with three additional protein sequences from *S. exigua*, and against the *D. melanogaster* protein database. Six host proteins were found to co-purify specifically with PTP. For each of those proteins the number of unique peptides and the % coverage is shown per database search.

Host proteins	**UNIPROT**			**DROSOPHILA**	
Protein name	***Organism***	***# of unique peptides***	***% coverage***	***# of unique peptides***	***% coverage***
Caspase-1	*S. frugiperda*	5	24	-	-
Arginine kinase	*Plodia interpunctella*	4	21	2	6
Serine/threonine-protein phosphatase PP1 (catalytic subunit)	*D. melanogaster*	-	-	3	15
Serine/threonine-protein phosphatase PP2A (catalytic subunit)	*D. melanogaster*	-	-	3	14
14-3-3 ε	*S. exigua*	5	35	2	18
14-3-3 ζ	*S. exigua*	3	30	4	15
Chloride intracellular channel (CLIC4)	*D. melanogaster*	-	-	4	25

Proteomic analysis

In total 13 unique peptides were found that matched the input protein AcMNPV PTP with a coverage of 77% (Table 2A). Using the UniProt database to match the MS/MS spectra we found a total of six AcMNPV proteins that co-purified specifically with AcMNPV PTP. These proteins encompass two proteins involved in DNA replication, DNA polymerase (ORF65) and proliferating cell nuclear antigen (PCNA, ORF49), an

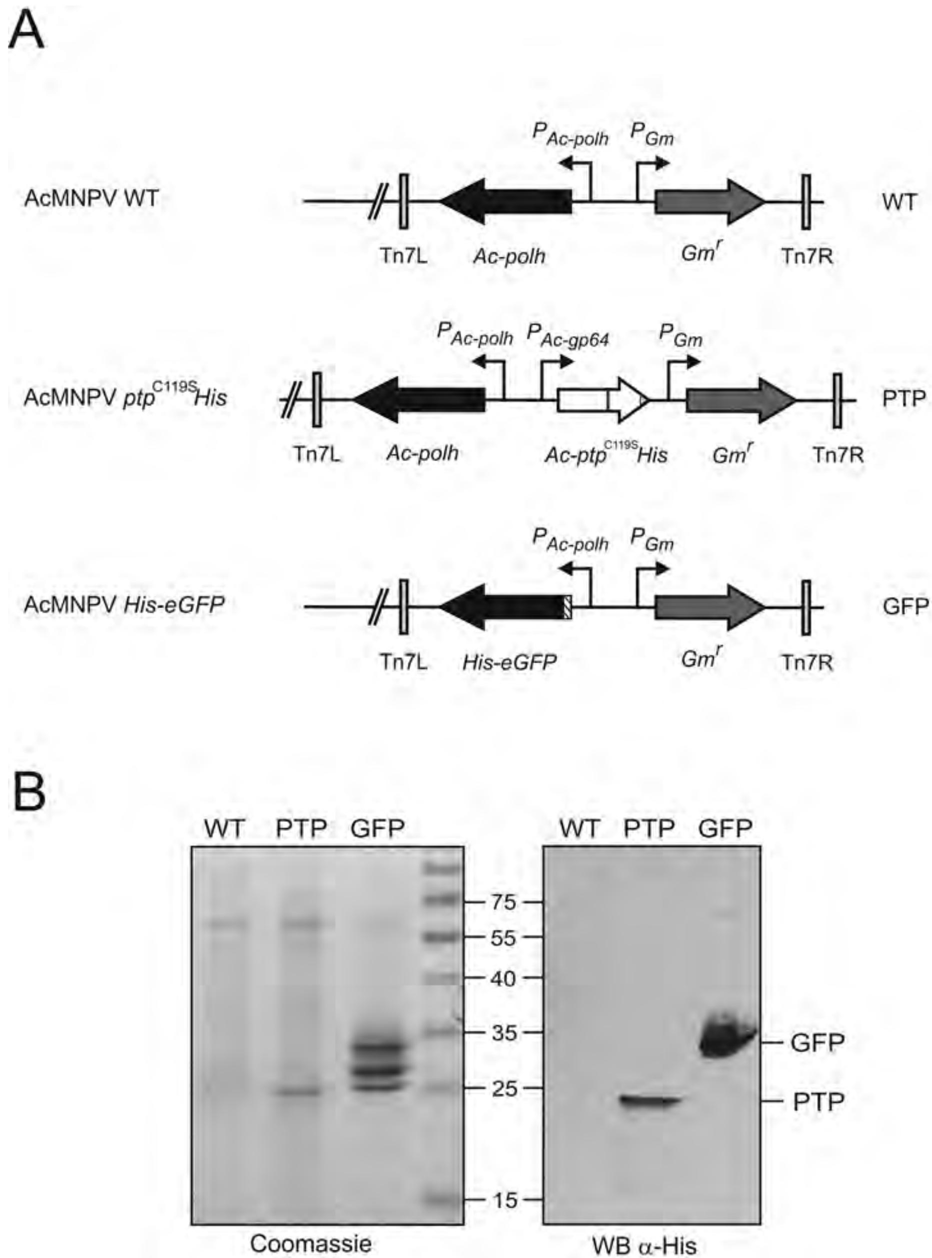


Figure 1. (A) Overview of the recombinant bacmids used for the substrate analysis. The names at the left side represent the full names of the bacmids used in this experiment; at the right hand their short names are indicated. His-tags (C-terminal for *ptp*^{C119S}; N-terminal for *egfp*) are depicted as striped fragments in the respective ORFs. (B) Coomassie staining (left) and Western blot detection (right) on eluted protein fractions from His-tag purifications of Ac-PTP expressed in Sf9 cells. WT: His-purified fraction of AcMNPV WT virus (negative control); PTP: His-purified fraction of AcMNPV PTP^{C119S}-His; GFP: His-purified fraction of AcMNPV eGFP-His (negative control).

inhibitor of apoptosis (IAP-1, ORF27), a viral Ser/Thr kinase (PK-1, ORF10), a sulfhydryl oxidase putatively involved in disulphide bond formation (P33, ORF92), and a structural protein associated with budded viruses (BVs) and occlusion-derived viruses (ODVs) (VP91, ORF83) (Table 2A).

We hypothesized that several host candidate proteins could be targeted by AcMNPV PTP to induce hyperactive behaviour. These proteins are cGMP-dependent protein kinase (PKG) (van Houte et al. 2013), 14-3-3 ζ, and 14-3-3 ε, which are all strong candidates for a possible role in behavioural manipulation by baculoviruses. PKG has been reported to have a conserved function in feeding-related locomotion behaviour in a variety of invertebrate species (reviewed in Kaun & Sokolowski (2009); van Houte et al. (2013)) and may thus be a suitable target for parasites to manipulate behaviour. The 14-3-3 protein, which has two isoforms (ζ and ε) in insects (Aitken 2006), is important in the production of neurotransmitters, which in turn play important roles in behavioural manipulation (Perrot-Minnot & Cézilly 2013; van Houte et al. 2013). As these 14-3-3 protein sequences are not in the UniProt database for *S. exigua*, we used for our proteomic analysis a database search that consists of the UniProt database supplemented with these protein sequences as predicted from cDNA analysis (see Materials and Methods). Using this database and the *D. melanogaster* protein database to match the MS/MS spectra, a total of six host proteins were found that co-purified specifically with AcMNPV PTP (Table 2B). Although for most of these host proteins phosphorylation is known to occur, we did not find any phosphorylated peptides.

Viral proteins co-purifying with the AcMNPV PTP protein

DNA polymerase and proliferating cell nuclear antigen (PCNA)

Two viral proteins involved in DNA replication were found to co-purify with PTP, i.e. DNA polymerase and PCNA. Baculovirus DNA polymerase was originally identified (Tomalski et al. 1988) based on its protein sequence similarity with other viral polymerases, including herpes simplex virus type 1 (HSV-1) and vaccinia virus. The gene is essential for virus replication (Kool et al. 1994; Vanarsdall et al. 2005), transcribed in the early phase of infection and translated into a 114 kDa protein (Hang & Guarino 1999; Mikhailov et al. 1986). Viral PCNA is a protein that associates with the DNA replication site (Iwahori et al. 2004), but is not essential for DNA replication. Cellular PCNA is present in a wide range of organism and functions as a DNA polymerase sliding clamp, which is a ring-shaped protein that encircles DNA and ensures proper sliding of DNA polymerase along the DNA to allow replication (Tsurimoto 1998, 1999). Since host PCNA is able to fully substitute viral PCNA it is hypothesized that baculovirus PCNA

may have a similar function in DNA replication (Iwahori et al. 2004). However, baculovirus PCNA also associates with both BV and ODV particles (Belyavskyi et al. 1998; Wang et al. 2010), and forms a complex with two other proteins; the baculovirus virion protein EC27 and the host cyclin-dependent kinase 6 (cdk6) (Belyavskyi et al. 1998). This complex has been shown to phosphorylate histone H1, which is hypothesized to enhance viral replication (Belyavskyi et al. 1998). The apparent association between AcMNPV PTP and viral proteins involved in DNA replication may imply that PTP may play an, as yet unknown, function in DNA replication.

Protein kinase 1 (PK-1)

AcMNPV protein kinase 1 (*pk-1*) is a gene encoding a 32 kDa protein that shows strong similarity to the catalytic domains of serine/threonine kinases, but lacks the regulatory domain commonly found in the latter kinases (Reilly & Guarino 1994). PK-1 phosphorylates histone H1 *in vitro* (Reilly & Guarino 1994), which might be related to the release of mature virions from infected cells (Reilly & Guarino 1994), or to the abovementioned enhanced viral replication (Van Opdenbosch et al. 2012). PK-1 is required for very late gene expression in baculovirus-infected cells (Fan et al. 1996). It is activated *in vitro* by protein kinase interacting protein (PKIP), a 19 kDa baculovirus-encoded protein (Fan et al. 1998) putatively involved in phosphorylation of two host proteins of unknown identity (McLachlin et al. 1998). A recent study reported that PK-1 is part of a phosphorylated very late transcription initiation complex (Mishra et al. 2008). The discovery that baculoviruses encode both a kinase (PK-1) and a phosphatase (PTP) led to the hypothesis that PK-1 and PTP may act in concert as regulators of host- or virus-induced signalling cascades during infection (Reilly & Guarino 1994). However, experimental data confirming a possible interaction between these two proteins have been lacking so far. The identification of PK-1 as protein co-purifying with PTP sheds new light on this hypothesis.

Inhibitor of apoptosis 1 (IAP1)

Commonly, apoptosis induction is an effective host response to limit virus infection in the initial phase. As a countermeasure, baculoviruses encode several different proteins that have anti-apoptotic activity. Besides the P35 protein found in AcMNPV and the P49 protein from *S. litura* multiple nucleopolyhedrovirus (SpltMNPV) and *S. littoralis* MNPV (SpliMNPV), baculoviruses may also encode one or more inhibitor of apoptosis (IAP) proteins (Clem 2001, 2007). Although for several of these IAP proteins anti-apoptotic activity could be demonstrated (Crook et al. 1993; Liang et al. 2012) many IAPs are thought to have other non-apoptotic functions in cell survival and intracellular

signalling (Liang et al. 2012), (Srinivasula & Ashwell 2008). A recent study showed that the IAP1 protein from several different baculoviruses, including AcMNPV, acts as an inducer of apoptosis rather than an anti-apoptotic protein (Ikeda et al. 2011). This apoptotic response was accompanied by caspase activation. The biological significance of IAP-mediated apoptosis is currently unknown, but it is hypothesized that in the late stage of infection induction of apoptosis may in fact be beneficial to baculovirus egress and spread. While inhibition of apoptosis is crucial in the initial phase of infection to yield enough progeny virus, in the late phase apoptosis may contribute to efficient liberation of occluded virions from infected cells (Ikeda et al. 2011).

Besides the identification of AcMNPV IAP1 as one of the proteins co-purifying with PTP, we also found the host protein *S. frugiperda* Caspase-1 (see below), which is the main effector caspase in cell lines derived from this insect species (LaCount et al. 2000). This observation may indicate that AcMNPV PTP is involved in apoptosis regulation during baculovirus infection. Many parasites utilize host apoptotic pathways to increase parasite replication and dissemination (reviewed in James & Green (2004)), and these strategies may have behavioural consequences that are adaptive for the parasite. Several studies strengthen this hypothesis: during behavioural manipulation of the cricket *Nemobius sylvestris* parasitised by the nematomorph hairworm *Paragordius tricuspidatus* the host anti-apoptotic protein BIR was differentially expressed (Biron et al. 2006) and *Glossina palpalis gambiensis* tsetse flies infected with the protozoan *Trypanosoma brucei brucei* showed differential expression of several proteins involved in the regulation of apoptosis (Lefèvre et al. 2007).

33 kDa early protein homolog

AcMNPV *p33* (ORF92) is a baculovirus essential core gene (Prikhod'ko et al. 1999) that encodes a sulfhydryl oxidase enzyme (Long et al. 2009). As the enzyme was found to be associated with BV and ODV particles, it is hypothesized to play a role in virion assembly (Nie et al. 2011; Wu & Passarelli 2010), possibly by catalysing disulphide bond formation in viral structural proteins (Hakim et al. 2011). Like sulfhydryl oxidase, the PTP enzyme is also associated with BVs and ODVs (Li & Miller 1995a; Wang et al. 2010). The finding that sulfhydryl oxidase co-purifies with PTP may indicate that disulphide bond formation may be important for proper PTP folding or enzyme activation. Redox activation is a common mechanism to regulate enzymatic activity of many cellular protein tyrosine phosphatases (Tonks 2006).

Capsid-associated protein VP91

AcMNPV *vp91* (also referred to as *p95*) is a core gene encoding a 96 kDa protein of unknown function. It is putatively associated with capsids of BVs and ODVs (Braunagel et al. 2003; Russell & Rohrmann 1997; Wang et al. 2010), which may explain its co-purification with PTP, which is also capsid-associated (Li & Miller 1995a; Wang et al. 2010). VP91 contains two chitin-binding domains and is putatively N-glycosylated based on *in silico* predictions. Furthermore, AcMNPV VP91 was found to co-purify with *per os* infectivity factor 1 (PIF1) in a co-immunoprecipitation assay and is a putative component of the *per os* infectivity complex (Peng et al. 2012).

Host proteins co-purifying with the AcMNPV PTP protein

Caspase-1

Caspases are highly conserved aspartic acid proteases that are key players in regulating the apoptotic pathway (Courtiade et al. 2011; Park et al. 2007). They are present in the cell as inactive proenzymes. Apoptotic signals lead to the formation of a protein complex (the apoptosome) that recruits initiator caspases, which are activated by autocatalysis. Initiator caspases subsequently activate downstream caspases by proteolytic cleavage. These effector caspases then target specific substrates, consequently leading to cell destruction, e.g. through cytoskeleton rearrangement, DNA degradation and disintegration of cellular content into apoptotic bodies (Roulston et al. 1999). *Spodoptera frugiperda* Caspase-1 was the first lepidopteran caspase described (Ahmad et al. 1997) and was characterized as an effector caspase, which is probably activated by the initiator caspase SfDronc in the apoptotic pathway (Huang et al. 2013). Caspase-1 is a target of the baculovirus-encoded anti-apoptotic protein P35 (Ahmad et al. 1997). A recent study showed that Caspase-1 is expressed in *B. mori* larvae that are resistant to baculovirus infection, but not in susceptible larvae, suggesting a role in virus resistance (Qin et al. 2012). Co-purification of Caspase-1 with AcMNPV PTP, in addition to a viral inhibitor of apoptosis-1, suggests a possible role for AcMNPV PTP in the apoptotic pathway (see above).

Arginine kinase

Arginine kinase is a phosphotransferase enzyme, which acts as a regulating factor in nitric oxide (NO) synthesis (Dai et al. 2013; Mori & Gotoh 2000). NO is an important neuronal signalling molecule in insects, and is released during immunological responses (Bicker 2001; Rivero 2006). Interestingly, arginine kinase was found to be differentially expressed in the brains of two different gammarid species, *Gammarus insensibilis* and *G. pulex*, infected with the parasitic worms *Microphallus papillorobustus* and *Polymor-*

phus minutes, respectively (Ponton et al. 2006). Both these hosts display aberrant evasive behaviour and negative geotaxis during parasite infection, favouring the hypothesis that parasites may manipulate host behaviour through modulation of host immunological responses (reviewed in Biron & Loxdale (2013)). Furthermore, arginine kinase was differentially expressed in *Spinochordodes tellinii*, a nematomorph hairworm that parasitises the grasshopper *Meconema thalassinum* and causes suicidal behaviour in its host (Biron et al. 2005; Thomas et al. 2002).

Serine/threonine-protein phosphatase PP1-87B, catalytic subunit

The catalytic subunit of serine/threonine protein phosphatase 1 (official name PP1-87B) is the predominant active phosphatase in many *Drosophila* tissues, including larval brains (Asztalos et al. 1993). Mutant flies with defects in the gene encoding PP1-87B showed several behavioural abnormalities, including anomalies in olfactory learning, visual conditioning, flight behaviour and locomotion activity (Asztalos et al. 1993). Later studies demonstrated an important role for PP1-87B in photoreceptor morphology and axon guidance in larval optic lobes through interaction with the Bifocal protein (Bif) (Babu et al. 2005; Helps et al. 2001). The Bif protein itself is activated by receptor tyrosine phosphatase signalling, and in turn modulates phosphatase activity of PP1-87B. Interestingly, another PP1 isoform (PP1-96A), sharing 96% protein similarity with PP1-87B, was found to be differentially expressed in *Nemobius sylvestris* crickets that were parasitised by the Gordian worm *Paragordius tricuspidatus* and that showed behavioural alterations due to infection (Biron et al. 2006).

Serine/threonine-protein phosphatase PP2A, catalytic subunit

Besides the catalytic subunit of protein phosphatase 1 (PP1-87B), as described above, we also identified a catalytic subunit of a different type of protein phosphatase. This subunit is a 35 kDa protein that, together with the 65 kDa regulatory subunit PR65, constitutes the active phosphatase complex PP2A (reviewed in Wera & Hemmings (1995)). Among the numerous phosphatases active in a cell, PP2A is considered one of the most abundant ones, accounting for the major portion of serine/threonine phosphatase activity (reviewed in Sontag (2001)). Although PP2A was originally characterized as a Ser/Thr phosphatase, it also shows Tyr phosphatase activity under certain conditions. This activity is increased by a 37 kDa protein tyrosine phosphatase activator (PTPA) protein through a currently unknown mechanism, which only occurs with the dimeric form of PP2A (Cayla et al. 1990; Cayla et al. 1994).

The identification of PP2A as a potentially interacting protein of AcMNPV PTP is of

interest for several reasons. First, PP2A activity is regulated by cGMP-dependent protein kinase (PKG), which is a well-known behavioural determinant in a wide range of invertebrate species, including *D. melanogaster* and *Caenorhabditis elegans* (reviewed in Kaun & Sokolowski (2009)). It is involved in different behavioural phenotypes, including feeding-related locomotion behaviour, learning, and memory formation. Two recent studies demonstrated an important role for PP2A in *D. melanogaster* in PKG-regulated heat stress tolerance of synaptic transmission and behaviour (Dawson-Scully et al. 2007) and in behavioural tolerance to anoxic stress (Dawson-Scully et al. 2010). At the molecular level, PKG phosphorylates PP2A, which in turn leads to dephosphorylation of potassium channel proteins resulting in increased channel conductance (Zhou et al. 1996). The apparent interaction between PP2A and viral PTP is possibly regulated by PKG, and this is a promising candidate pathway to study in the context of behavioural manipulation of insects by baculoviruses and other parasites (reviewed in van Houte et al. (2013)). However, we could not find PKG itself as an interacting protein of PTP in our analysis.

Secondly, PP2A plays a role in regulating the activity of the transcription factor cAMP-responsive element binding protein (CREB) (reviewed in Benito & Barco (2010)), although conflicting studies claim that not PP2A but PP1 (see above) is the dominant CREB phosphatase (reviewed in Wera & Hemmings (1995) and Lonze & Ginty (2002)). CREB plays a crucial role in learning and memory in both vertebrates and invertebrates (reviewed in Benito & Barco (2010)), and putatively regulates transcription of several genes that are directly or indirectly involved in behaviour, including 14-3-3 (see below), tyrosine hydroxylase (see below) and genes encoding neuropeptide precursors (Lonze & Ginty 2002).

Thirdly, there is the intriguing finding that PP2A is targeted by several different viruses to deregulate signalling pathways in the host cell, thereby increasing viral replication (reviewed in Sontag (2001)). This is achieved by direct interaction between viral proteins and PP2A, resulting in alterations of PP2A activity and substrate specificity. For example, the SV40 small tumour antigen binds PP2A and thereby blocks the inhibitory effect that PP2A normally has on MAPK signalling (Sontag et al. 1993). Deregulation of MAPK pathways by SV40 virus stimulates cell cycle progression and cell survival, and leads to cellular and viral promoter activation. Other viruses that target PP2A include polyomavirus, human papilloma virus and HIV-1 (reviewed in Sontag (2001)). The polyomavirus middle T antigen is known to be part of a larger complex that contains PP2A, several tyrosine kinases and 14-3-3 proteins (see below). Likely these viral strategies are primarily aimed at increasing virus replication, but whether any of these manipulations may also have an effect on host behaviour is unknown to date.

Chloride intracellular channel protein 4 (CLIC4)

CLIC4 is a 30 kDa protein member of the CLIC family of proteins, which have putative intracellular chloride channel activity (Ashley 2003), but are also implicated in several other functions, including regulation of transforming growth factor (TGF)-β signalling (Shukla et al. 2009) and regulation of ryanodine receptors, which are intracellular calcium release channels (Jalilian et al. 2008). A recent study demonstrated a role for the *D. melanogaster* CLIC4 protein in ethanol-induced behaviour. While wild type flies commonly lose negative geotactic behaviour as a result of exposure to ethanol, CLIC4-knockout flies remained negatively geotactic (Bhandari et al. 2012). This role seems conserved across phyla as a similar function in ethanol-related behaviour was also found for CLIC homologs in mice and *C. elegans* (Bhandari et al. 2012). Interestingly, vertebrate CLIC4 is known to form a complex with actin, tubulin and 14-3-3 proteins (see below) (Suginta et al. 2001).

14-3-3 isoforms ζ and ε

The 14-3-3 protein family comprises a group of highly conserved dimeric 30 kDa proteins that are involved in a wide range of cellular processes, including cell cycle progression, cell signalling and transcription (reviewed in Aitken (2006)). While in mammalian brains seven different isoforms have been described, insects only have the ε and ζ isoforms. Although these two isoforms occur in a wide range of tissues, they are most abundant in the central nervous system (CNS). They modulate interactions between proteins, commonly through phosphorylation of the interacting protein, but also through autophosphorylation of 14-3-3. For example, several Ser/Thr kinases are known to regulate interactions between 14-3-3 and its target proteins by phosphorylation of 14-3-3 (Aitken 2006).

The 14-3-3 protein regulates activity of tryptophan hydroxylase and tyrosine hydroxylase, rate-limiting enzymes in the biosynthesis of serotonin and dopamine respectively (Ichimura et al. 1988). This is particularly interesting in the light of behavioural manipulation for several reasons. Both serotonin and dopamine play an important role in immunity and in circadian rhythm, but are also behavioural determinants in many organisms (Perrot-Minnot & Cézilly 2013). In several parasite-host associations serotonin and/or dopamine are thought to be involved in parasite-induced host behavioural changes (van Houte et al. 2013). For example, the trematode *M. papillorobustus* induces positive phototaxis in the gammarid *G. insensibilis* through alterations in serotonin levels in the brain of parasitised insects (Tain et al. 2007; Tain et al. 2006). These parasitised gammarids showed differential expression of an enzyme (aromatic L-amino acid decarboxylase) involved in serotonin synthesis (Ponton et al. 2006). Dopamine was detected

in the venom of the parasitoid wasp *Ampulex compressa*, which induces a zombie-like state in its cockroach host *Periplaneta americana* (Gal et al. 2005). A proteomic analysis on heads of *Glossina palpalis gambiensis* tsetse flies infected with the protozoan *Trypanosoma brucei brucei* showed differential expression of two pyridoxal-dependent decarboxylases, enzymes that catalyse the final step in the synthesis of dopamine and serotonin. This suggests that dopamine and/or serotonin brain levels are altered, which may be related to the behavioural modifications that occur in these infected flies (Lefèvre et al. 2007). Tyrosine hydroxylase, involved in dopamine synthesis, is differentially expressed in *Spinochordodes tellinii*, a Gordian worm that parasitised the grasshopper *Meconema thalassinum* (Biron et al. 2005). Furthermore, 14-3-3 was identified as a differentially expressed protein in *Anopheles gambiae* mosquitoes infected with *Plasmodium berghei*, the causative agent of malaria. It was hypothesized that the observed changes in 14-3-3 expression may indicate the involvement of this protein in host behavioural manipulation by the parasite, which includes increasing biting rates and prolonging feeding and probing times (Lefèvre et al. 2007).

In conclusion, numerous studies show that 14-3-3 is directly or indirectly associated with behavioural manipulation in several different host-parasite systems. The discovery that 14-3-3 ζ and ε co-purify with viral PTP, a parasite protein that is crucial for inducing host hyperactivity (van Houte et al. 2012), may indicate that 14-3-3 ζ and ε are involved in behavioural manipulation by baculoviruses. Strikingly, proteomics analysis showed that 14-3-3 ζ protein associates with AcMNPV BV particles (Wang et al. 2010), which may be connected with its putative interaction with PTP.

Gene expression analysis

The level of *14-3-3 ζ* RNA expression is dependent on AcMNPV *ptp* expression

To obtain more insight in the possible involvement of 14-3-3 ζ in PTP-induced behavioural manipulation, quantitative RT-PCR analysis was performed to compare *14-3-3 ζ* RNA expression levels in AcMNPV WT-infected larvae with AcMNPV Δ*ptp*-infected larvae. In WT-infected larvae, *ptp* expression increased over the course of infection, reaching a maximum level at 3 days post-infection (dpi) (Fig. 2A). *Ptp* expression levels differed significantly over the days ($F_{(2,31)}$=20.92; P<0.001), with expression at 2 and 3 dpi being significantly higher than at 1 dpi (P<0.001 for both), and no significant difference between 2 and 3 dpi (P=0.081). Levels of *14-3-3 ζ* RNA expression at 1 dpi were significantly higher in mock- than in WT- and Δ*ptp*-infected larvae ($F_{(2,27)}$=16.57, P<0.001; post-hoc P<0.001 for mock compared to WT and to Δ*ptp*). However, expression levels of *14-3-3 ζ* were very low at 1 dpi (Fig. 2B). On 2 and 3 dpi, *14-3-3 ζ* expression

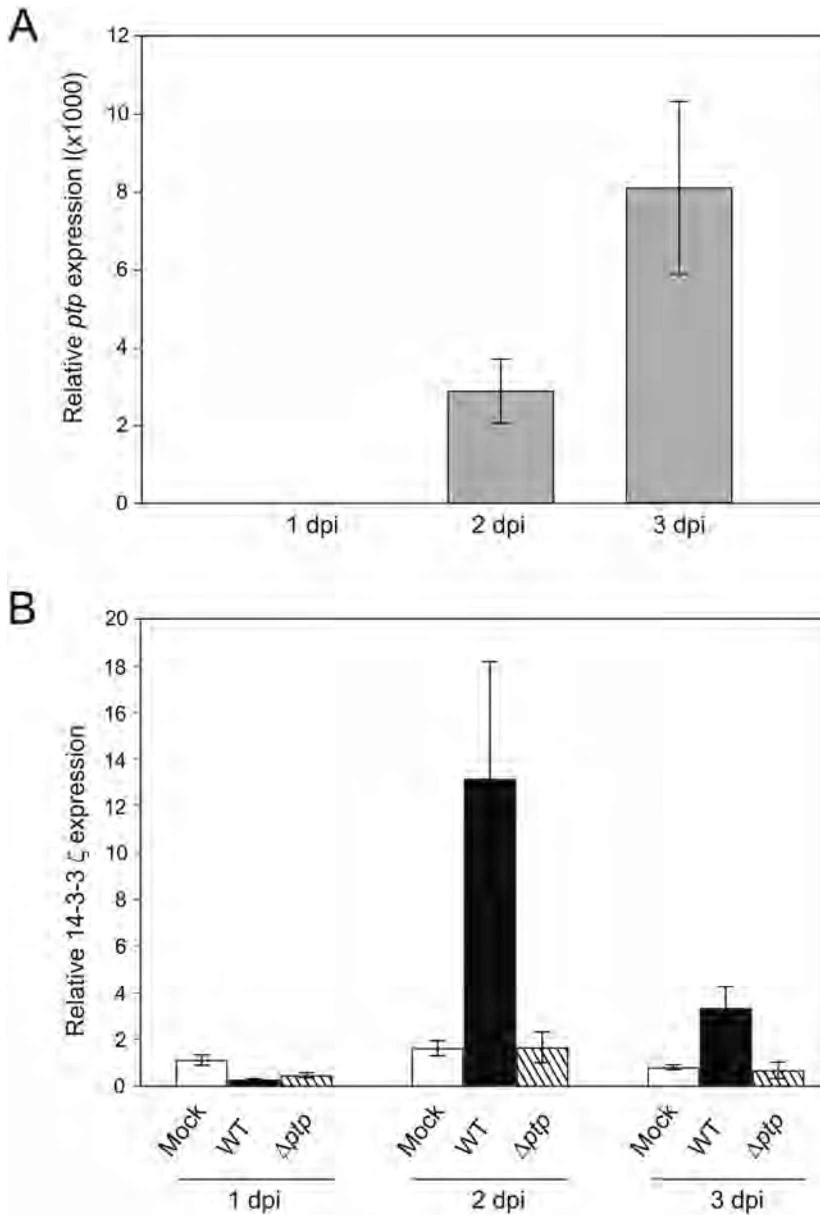


Figure 2. RNA expression levels of *14-3-3 ζ* are significantly upregulated in the presence of AcMNPV *ptp*. (A) AcMNPV *ptp* expression increases over time during the infection, and reaches its maximum at 3 days post-infection (dpi). Gene expression levels at 2 and 3 dpi are relative to the level at 1 dpi. Bars assigned with different letters are significantly different. Error bars indicate standard errors of the mean (SEM). (B) At 2 and 3 dpi host *14-3-3 ζ* levels are significantly higher in *S. exigua* larvae infected with WT AcMNPV compared to *S. exigua* larvae infected with Δ*ptp* AcMNPV or to uninfected larvae. Gene expression levels are relative to the level of mock-infected larvae at 1 dpi. Bars assigned with different letters are significantly different. Error bars indicate SEM.

was significantly higher in WT-infected larvae as compared to mock- and Δ*ptp*-infected larvae (Fig. 2B) (2 dpi: $F_{(2,27)}$=10.06, P=0.001; post-hoc P=0.002 for WT compared to mock and P<0.001 for WT compared to Δ*ptp*; 3 dpi: $F_{(2,41)}$=16.34; P<0.001; post-hoc P<0.001 for WT compared to mock and to Δ*ptp*). These data indicate that the level of *14-3-3 ζ* expression over time is related to increased expression of the baculovirus *ptp* gene. *Ptp* expression seemed maximal at 3 dpi (although the difference with 2 dpi was not significant), and high *14-3-3 ζ* expression levels were already found at 2 dpi. The observed host behavioural change (hyperactivity) is found at 3 dpi in this system (van Houte et al. 2012) and it is possible that the host pathway leading to this behavioural change is already initiated at 2 dpi, explaining why *14-3-3 ζ* expression levels are already high at 2 dpi. It is currently unclear how PTP may affect 14-3-3 ζ at both the RNA and protein level. Possibly, activation of 14-3-3 ζ by PTP induces a positive feedback loop on *14-3-3 ζ* transcription. Alternatively, PP1 and/or PP2A, both of which also co-purified with PTP, may be involved in the regulation of *14-3-3 ζ* expression. As mentioned above, these phosphatases are thought to be important in regulating activity of the transcription factor CREB (Benito & Barco 2010; Lonze & Ginty 2002). CREB is known to regulate transcription of certain isoforms of 14-3-3 in vertebrates (Lonze & Ginty 2002) and a similar effect may occur in insects. Possibly, interaction between PTP and PP1 and/or PP2A affects CREB activation, thereby altering *14-3-3 ζ* expression.

Future studies on the putative role of 14-3-3 ζ in PTP-induced behaviour, for example by comparing protein levels between AcMNPV WT- and Δ*ptp*-infected insect larvae or by investigating direct protein interactions between PTP and 14-3-3 ζ, will shed more light on the role of 14-3-3 ζ in baculovirus manipulation of caterpillar behaviour. Likewise, the other 14-3-3 isoform found to co-purify with PTP (14-3-3 ε) is worth investigating in this context.

Discussion

Our analysis of possible target proteins of the AcMNPV PTP enzyme identified a suite of promising candidate host proteins that are potentially involved in PTP-induced behavioural manipulation. A schematic model encompassing these six host proteins and the cellular processes that these proteins are associated with is depicted in Fig. 3. All of these proteins are known to play a role in host signalling cascades and many of them are associated with parasitic manipulation. This is of importance as a previous study on BmNPV PTP, a homolog of AcMNPV PTP, suggested that the function of BmNPV PTP in altering host behaviour was merely based on its presence as a protein in the baculovirus virion rather than as an enzyme. BmNPV PTP induces enhanced locomotion activity in

B. mori host larvae (Kamita et al. 2005), but PTP phosphatase activity was not necessary to induce this behavioural change (Katsuma et al. 2012). As AcMNPV PTP phosphatase activity is crucial to induce hyperactivity, distinct mechanisms must exist that underlie PTP-induced behaviour in BmNPV and AcMNPV (van Houte et al. 2012). Although no phosphorylated peptides were detected for any of the host proteins that co-purified with PTP, the fact that these proteins are all involved in cell signalling is in accordance with the hypothesis that AcMNPV PTP alters host behaviour by regulating the phosphorylation status of target proteins.

Aside from the proteins that were found to co-purify with AcMNPV PTP, we were also able to extract additional information by looking at proteins that, in contrast to our expectancy, did not co-purify with PTP. For example, a protein that does not co-purify with PTP in our experiments is the baculovirus phosphoprotein 78/83 (PP78/83), a Wiskott-Aldrich syndrome protein (WASP)-like protein involved in nuclear actin assembly to support virion movement through the cytoplasm during baculovirus infection (Goley et al. 2006; Ohkawa et al. 2010; Wang et al. 2008). This protein was found to interact with BmNPV PTP protein in a yeast-2-hybrid screening (Katsuma et al. 2012). The finding that PP78/83 did not co-purify with AcMNPV PTP in the current

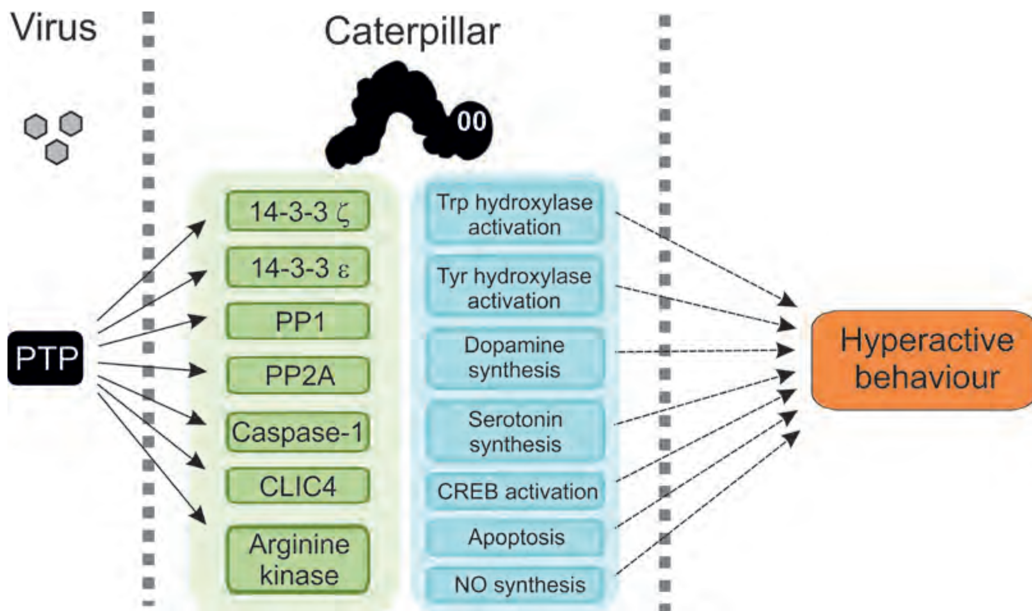


Figure 3. Schematic model depicting all the host proteins that putatively interact with the baculovirus PTP enzyme as found in this study. Depicted are also some of the biological process(es) that each of these proteins is involved in, as these processes may be an important link in the pathway of hyperactivity induced by PTP.

analysis may indicate that the function of PTP as a capsid protein is different between BmNPV and AcMNPV. This latter hypothesis could be of importance for the apparent difference in the PTP-mediated mechanism that these two baculovirus species employ to alter host behaviour (Katsuma et al. 2012; van Houte et al. 2012).

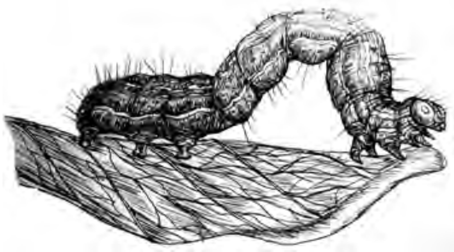
Other proteins that did not co-purify with PTP in our experiments are proteins with predicted functions in RNA processing. Based on structural similarity to cellular RNA capping enzymes and its high affinity *in vitro* for RNA substrates, PTP was originally hypothesized to act as an RNA phosphatase rather than as a protein phosphatase (Gross & Shuman 1998; Takagi et al. 1998). The predicted role of PTP as RNA phosphatase, involved in capping of baculovirus mRNAs, could never be demonstrated *in vivo* (Li & Guarino 2008) and our analysis does not provide any support for this theory either. However, it is still possible that PTP exerts its function in behavioural manipulation through dephosphorylation of an RNA substrate, and it would be interesting to search specifically for such RNA targets of PTP in future experiments.

Furthermore, the aforementioned cGMP-dependent protein kinase (PKG), one of the host candidate proteins hypothesized to be a target of PTP, was not found to co-purify with PTP in our experiments. Although it cannot be excluded that PKG is still indirectly affected by PTP, our observations could indicate that PKG is not involved in PTP-induced hyperactivity.

Two important limitations of our study are (i) the use of cell cultures instead of whole insects or tissues to study interactions between PTP and other (host) proteins and (ii) the lack of a large protein database of the host organism being studied, i.e. *S. exigua*. Cell culture has the big advantage that only one cell type is present so that interacting proteins will co-purify in amounts high enough to enable LC-MS/MS detection. At the same time, though, this is also the main drawback: cell cultures may be derived from a tissue distinct from the one in which PTP finds its target or partner protein to induce behavioural manipulation. As a consequence, interactions may be found that are not biologically relevant while true interactions may be missed, for example when the interacting protein is not expressed in the particular cell line studied. Nevertheless, it is surprising to identify so many (host) proteins that are specifically associated with signalling pathways and behavioural traits. Searching for PTP interacting proteins in the whole insect or in specific tissues thereof would be an exciting next step to take. Exponentially growing data based on economically important insects such as *S. exigua* and other phytophagous lepidopterans will further aid in the identification of additional targets of baculovirus PTP.

Acknowledgements

SVH and VIDR are both supported by the Program Strategic Alliances of the Royal Dutch Academy of Sciences (project 08-PSA-BD-01) and VIDR is supported by a VENI grant of the Netherlands Organisation for Scientific Research (project 863.11.017).



Chapter

5

Functional characterization of *Spodoptera exigua* multiple nucleopolyhedrovirus protein tyrosine phosphatase 2 (PTP2)

Adapted from:

Stineke van Houte, Ambrosius P. Snijders, Vera I.D. Ros, Han Yue, Susan van Aalst, Monique M. van Oers. Functional characterization of *Spodoptera exigua* multiple nucleopolyhedrovirus protein tyrosine phosphatase 2 (PTP2), *manuscript in preparation*.

Abstract

Baculoviruses are DNA viruses that infect only the larval stages of mainly lepidopteran hosts. A subset of baculoviruses carries a protein tyrosine phosphatase (*ptp*) gene. This gene was previously shown to be necessary for the induction of hyperactive behaviour in infected host caterpillars. Besides *ptp*, a second *ptp* gene (*ptp2*) is present in members of the family *Baculoviridae*. This *ptp2* gene is phylogenetically unrelated to *ptp*, but also encodes a phosphatase. It contains a functional domain that is characteristic for mitogen-activated protein kinase (MAPK) phosphatases, which are important proteins in the regulation of cellular processes such as apoptosis and immune responses. The *ptp2* gene is present in a subset of baculoviruses, some of which also carry *ptp*. In contrast to *ptp*, the function of *ptp2* during virus infection is unknown. Although phylogenetically unrelated to baculovirus *ptp2*, *ptp* genes are abundantly present in polydnaviruses and several of them are pro-apoptotic and suppress immune cell activity. In this study the biological function of the *ptp2* gene (ORF26) from the baculovirus *Spodoptera exigua* multiple nucleopolyhedrovirus (SeMNPV) was analysed. It was found that *ptp2* induced mild apoptosis in insect cells upon transient expression, an effect which may be related to suppression of host immune responses during virus infection. By substrate analysis of the SeMNPV PTP2 protein five host proteins that are implicated in the apoptotic response and/or MAPK signalling were found to co-purify with PTP2. These proteins may thus be related to the putative role of SePTP2 in apoptosis. In addition, three co-purified host proteins were found that are involved in vesicular trafficking, which may indicate a role for PTP2 in trafficking during virus infection, for example for virion transport through the cell.

Introduction

Protein tyrosine phosphatases (PTPs) form a large family of proteins that, in conjunction with protein tyrosine kinases, regulate the phosphorylation status of target proteins. Characteristic for all members of the PTP superfamily is the active site His-Cys (HC) signature motif, in which the cysteine residue confers catalytic activity (Neel & Tonks 1997; Tonks 2006). Several different subgroups of PTPs exist, which exert distinct functions in cellular signalling (Neel & Tonks 1997; Stoker 2005; Tonks 2006). While classical PTPs are restricted to tyrosine dephosphorylation, dual-specificity phosphatases (DUSPs) have the ability to dephosphorylate not only tyrosine residues, but also serine and threonine residues, which allows these enzymes to exert a much broader range of regulatory functions. Many DUSPs are important in the regulation of mitogen-activated protein kinase (MAPK) cascades, which are highly conserved signalling pathways that mediate a wide variety of cellular processes, including cell proliferation, inflammation and apoptosis (Lang et al. 2006; Owens & Keyse 2007; Theodosiou & Ashworth 2002).

Within the family *Baculoviridae*, large DNA viruses that cause lethal infections in arthropod hosts, two different genes are found that encode proteins belonging to the DUSP superfamily. However, these genes are phylogenetically unrelated, and share only 13% amino acid similarity. The *ptp* gene encodes a protein tyrosine phosphatase that is important in behavioural manipulation of baculovirus-infected caterpillars (Kamita et al. 2005; van Houte et al. 2012). This gene is present in a subset of evolutionarily related baculoviruses belonging to the group I nucleopolyhedroviruses (NPVs) in the genus *Alphabaculovirus* (Jehle et al. 2006; van Houte et al. 2012). The second gene, named *ptp2*, is present in several group I and group II NPVs in the genus *Alphabaculovirus* and in two members of the genus *Betabaculovirus* (van Houte et al. 2012), and encodes a 19 kDa protein. The baculovirus PTP2 proteins carry a C-terminal consensus sequence that is characteristic for MAPK phosphatases (MKPs). This highly conserved C-terminal domain is required for MKP catalytic activity, and consists of the consensus sequence **D**X26(V/L)X(V/I)H**C**XAG(I/V)S**R**SXT(I/V)XXAY(L/I)M, in which X can be any amino acid and catalytic residues are in bold. Classical MKPs also carry an N-terminal domain that confers substrate specificity (Patterson et al. 2009). In contrast, the so-called atypical DUSPs, to which baculovirus PTP2 belongs, lack this N-terminal domain. Although they commonly have highly divergent substrate specificities and cellular functions, several atypical DUSPs are known to regulate MAPK signalling and to be important regulators of apoptosis (reviewed in Patterson et al. (2009)).

A recent study showed the involvement of a bracovirus protein belonging to the PTP

superfamily in the induction of apoptosis (Suderman et al. 2008). Inducing apoptosis is thought to be a viral strategy to evade the host immune response by specifically targeting immune cells (haemocytes) that are involved in antiviral immunity.

In this study, we analysed the biological function of the baculovirus *ptp2* gene (ORF26, IJkel et al. (1999)) during baculovirus infection, using the group II NPV *S. exigua* MNPV (SeMNPV) infecting *S. exigua* larvae. Based on the observation that the baculovirus *ptp* and *ptp2* genes are not phylogenetically related, that *ptp2* has a conserved MAPK phosphatase domain and on the pro-apoptotic effects of a polydnavirus *ptp* gene, it was hypothesized that SeMNPV *ptp2* may not function as a 'behavioural' gene but rather as a pro-apoptotic gene targeting haemocytes. We thus investigated whether the SeMNPV *ptp2* gene is involved in the induction of apoptosis in insect cells. If so, this may indicate that SeMNPV *ptp2* functions as an MKP during virus infection, as apoptosis is known to be tightly controlled by MAPK signalling (Theodosiou & Ashworth 2002; Zhang & Liu 2002). To obtain additional insight in the putative role of SeMNPV *ptp2*, we performed a substrate analysis on purified SeMNPV PTP2 protein.

Materials and methods

Apoptosis assays

Construction of plasmids for transient expression assays

The pIB-DEST plasmid (Invitrogen) was used as an expression vector for transient expression assays in Sf21 cells. This plasmid contains the OpIE2 early promoter from *Orgyia pseudotsugata* (Op) MNPV for constitutive expression of the gene of interest. For each of the genes to be tested, the eGFP open reading frame (ORF) and a foot and mouth disease 2A autoprotease site (FMDV2A) were fused to the 5' end of the target ORF (Fig. 1A). EGFP was used to monitor the transfection efficiency. FMDV2A is a 17 amino acids long (NFDLLKLAGDVESNPGP) ribosome-skipping element that allows co-translational cleavage between the eGFP protein and the expressed protein of interest (Luke et al. 2008; Ryan & Drew 1994). In total, five different pIB-DEST plasmids were created: i) pIB-eGFP coding only for eGFP; ii) pIB-Ac*ie1*, expressing eGFP and the AcMNPV immediate early 1 (IE1) protein, which has been shown to induce apoptosis in Sf21 cells upon transient expression (Prikhod'ko & Miller 1996); iii) pIB-Se*ptp2*, encoding eGFP and SeMNPV PTP2; iv) pIB-Se*ptp2mut*, encoding eGFP and a catalytic C110S mutant of SeMNPV PTP2; and v) pIB-Ac*ptp*, coding for eGFP and AcMNPV PTP, to compare the effect of transient expression of a different protein tyrosine phos-

phatase in Sf21 cells on apoptosis.

The AcMNPV *ie1* and *ptp* ORFs were amplified using primers 6 and 7, and 8 and 9 respectively (Table 1) from the AcMNPV E2 bacmid (Luckow et al. 1993; Smith & Summers 1979) as a PCR template. The Se*ptp2* ORF was amplified using primers 10 and 11 (Table 1) with the SeMNPV bacmid SeBAC10 (Pijlman et al. 2002) as a PCR template. The C110S catalytic mutant of Se*ptp2* was amplified using primers 10 and 11 (Table 1) with a plasmid containing the *ptp2*^{C110S} fragment as a template. In this fragment the Cys110 residue in the Se PTP2 catalytic site was mutated to Ser110. This mutation was originally created using primer 1 (Table 1), which introduced point mutations at nucleotides 328 and 330 (shown in bold) relative to the ATG start codon, combined with primer 2 (Table 1), which annealed to the 3' end of the *ptp2* ORF. The resulting 198 bp PCR product was then used as reverse primer combined with primer 3 (Table 1) annealing to the start of the *ptp2* ORF. The resulting *ptp2*^{C110S} fragment was cloned and sequenced in a pJET1.2 cloning vector (Fermentas) for further cloning purposes.

In all primers, an attB1 site (italics) and a *Hind*III restriction site (underlined) were included in the forward primer, while an attB2 site (italics) was included in the reverse primer to enable Gateway® cloning (Invitrogen) of the PCR fragments. All PCRs were performed with the proofreading Phusion polymerase (Finnzymes). The resulting PCR fragments were cloned into the pDONR207 donor plasmid (Invitrogen), sequenced, and subsequently recombined into pIB-DEST. The EGFP-FMDV2A construct was obtained as *Hind*III fragment as described in Fros et al. (2010) and inserted into *Hind*-III-linearized pIB-DEST plasmids containing the genes of interest.

Insect cell culture and transfections

Spodoptera frugiperda 21 (Sf21) cells were maintained as monolayers at 27 °C in Grace's supplemented medium in the presence of 10% fetal bovine serum (FBS) and 0.1% gentamycin. These cells and medium components were all obtained from Invitrogen. Sf21 cells were seeded at a confluence of 25-30% in 6-well plates and incubated overnight. Two hours prior to transfection, medium was replaced with Grace's unsupplemented Insect Medium (Sigma-Aldrich) without antibiotics. Transfections were performed with 4 µg of plasmid DNA. Cellfectin II (Invitrogen) was used as transfection reagent according to the manufacturer's instructions.

Microscopy analysis and caspase assays

Transfections were performed as described above with the five different expression

Table 1. Primers used in this study.

Primer number	Primer name	Sequence (5' to 3')	Function
1	Septp2-C110Smut	GGCGAGGGCAAAGGGTGTACGT-TCAT**A**G**C**CATGCGGGCGTGTCG	Create C110S mutation in SePTP2 catalytic site
2	Septp2-RV	TCAAAATTCCATTTGTGATTC	Amplify SeMNPV *ptp2* ORF
3	Septp2-FW	ATGCATACTAACGACGACAAC	Amplify SeMNPV *ptp2* ORF
4	NheI-start-Strep-Septp2-FW	CAGGCTAGCATG*TGGAGC-CACCCCCAGTTCGAAAAG*AGCGCA-CATACTAACGACGACAACTTTAC	Amplify SeMNPV *ptp2* ORF encoding N-terminal Strep-tag (italics) and insert *Nhe*I site (underlined)
5	Septp2-His-stop-NsiI-RV	GTCATGCATTTA*GTGGTGGTGATG-GTGATGATGGTGGTGATG*AAATTC-CATTTGTGATTCGGCC	Amplify SeMNPV *ptp2* ORF encoding C-terminal His-tag (italics) and insert *Nsi*I site (underlined)
6	attB1-HindI-II-Acie1-FW	GGGG*ACAAGTTTGTACAAAAAAG-CAGGCT*GGGAAGCTTATGACG-CAAATTAATTTTAACGC	Amplify AcMNPV *ie1* ORF with attB1 and *Hind*III site
7	Acie1-attB2-RV	GGGGA*CCACTTTGTACAAGAAAGCT-GGGT*TTAATTAAATTCGAAT-TTTTTATATTTACAATTT	Amplify AcMNPV *ie1* ORF with attB2 site
8	attB1-HindI-II-Acptp-FW	GGGG*ACAAGTTTGTACAAAAAAG-CAGGCT*GGGAAGCTTATGT-TTCCCGCGCGT	Amplify AcMNPV *ptp* ORF with attB1 and *Hind*III site
9	Acptp-attB2-RV	GGGG*ACCACTTTGTACAAGAAAGCT-GGGT*TTAAATTAATAAATCTT-GAACGTAATTTTGTC	Amplify AcMNPV *ptp* ORF with attB2 site
10	attB1-HindI-II-Septp2-FW	GGGG*ACAAGTTTGTACAAAAAAG-CAGGCT*AAGCTTATGCAT-ACTAACGACGACAAC	Amplify SeMNPV *ptp2* ORF with attB1 and *Hind*III site
11	Septp2-attB2-RV	GGGG*ACCACTTTGTACAAGAAAGCT-GGGT*TCAAAATTCCATTTGTGAT-TCGGC	Amplify SeMNPV *ptp2* ORF with attB2 site

constructs. Actinomycin D (ActD, Sigma-Aldrich), a chemical inducer of apoptosis, was added to the medium 8 hours post-transfection (hpt) at a final concentration of 0.25 µg/ml. Cells were observed daily using fluorescence light microscopy to observe eGFP expression and to screen for the formation of apoptotic bodies.

For the caspase assays, transfections were performed in 24-well plates. Transfection and apoptosis induction/inhibition procedures were as described above, but each of the reagent volumes were four times smaller. All treatments were done *in duplo*. At 48 hpt cells were resuspended in culturing medium for the caspase assay. In a white 96-well plate 25 µl of caspase-glo 3/7 substrate (Promega) was mixed thoroughly with 25 µl cell

suspension of the different samples. The plate was incubated in the dark for 30 minutes at room temperature (RT) and subsequently luminescence was measured in a Fluometer (Optima). ANOVA (SPSS) analysis was performed to determine statistically significant differences in caspase activity between the different treatments.

Substrate analysis

Generation of recombinant bacmids

To enable purification of the SeMNPV PTP2 protein, a recombinant bacmid was created that produced a catalytic mutant of the PTP2 protein with a C-terminal His-tag (Fig. 3A). For this construct, the AcMNPV bacmid with a deletion of the *ptp* gene (Δ*ptp*) (van Houte et al. 2012) was used as backbone. The above described *ptp2*^{C110S} fragment cloned in pJET1.2 was used as a PCR template. This *ptp2*^{C110S} ORF fragment was subsequently used as template for PCR using primer 4 (Table 1), containing an *Nhe*I restriction site (underlined) and encoding an N-terminal Strep-tag (italics) to allow optional tandem purification of the protein, and primer 5 (Table 1), containing an *Nsi*I restriction site (underlined) and encoding a 10x His-tag (italics) to create a PTP2^{C110S} protein with a C-terminal His-tag. The expression of the *ptp2*^{C110S}-*His* open reading frame (ORF) was controlled by the AcMNPV *gp64* promoter (Ac-P_{gp64}). To this aim, the Ac-P_{gp64} promoter region was amplified as described in Chapter 4.

The Ac-P_{gp64} and *ptp2*^{C110S}*His* DNA fragments were cloned as *Nco*I/*Nhe*I fragment and *Nhe*I/*Nsi*I fragment, respectively, into the pFBDpolhΔp10 vector. This plasmid contains the AcMNPV *polh* ORF downstream of the AcMNPV *polh* promoter (P_{polh}-*polh*), while the *p10* promoter was removed, as described in Peng et al. (2010). Bac-to-Bac transposition (Luckow et al. 1993) was performed to integrate the plasmid fragment containing P_{gp64}-*ptp2*^{C110S}*His* and P_{polh}-*polh* into the AcMNPV Δ*ptp* bacmid. Transfections of Sf9 cells with bacmid DNA were performed with Cellfectin II reagent (Invitrogen) and budded virus (BV) stocks were produced according to the Bac-to-Bac system (Invitrogen).

The AcMNPV wild type (WT) bacmid (van Houte et al. 2012) that expresses Ac-*polh*, but does not encode any His-tagged protein, was used as negative control (Fig. 3A). A second negative control consisted of an AcMNPV WT bacmid expressing a His-tagged eGFP protein, as described in Chapter 4 (Fig. 3A). This bacmid was used to check for proteins that co-purify with His-tagged proteins in a nonspecific manner.

Insect cell culture and protein purification

S. frugiperda 9 (Sf9) cells (Invitrogen) were maintained as monolayers in Sf900II serum-free medium (Invitrogen) supplemented with 5% fetal bovine serum (FBS, Invitrogen) and 0.1% gentamycin (Invitrogen). For the protein expression experiments, 200 ml cell suspension cultures (app. $2x10^6$ cells/ml) were grown in Sf900II serum-free medium with 0.1% gentamycin. These cultures were infected with either AcMNPV encoding SeMNPV $PTP2^{C110S}$-His (named 'PTP2'), AcMNPV encoding eGFP-His (named 'GFP') or AcMNPV WT (named 'WT') using a multiplicity of infection of 5 $TCID_{50}$/cell. Cell harvest, lysis, and His-purification procedures were all performed as described in Chapter 4.

Gel electrophoresis, in-gel digestion and LC-MS/MS procedure, database searching and criteria for protein identification

Electrophoresis, trypsin digestions, LC-MS/MS procedures and database searching were performed as described in Chapter 4. A coefficient of variance test was performed in Scaffold to find proteins that showed significantly more assigned unique spectra in the SePTP2 sample as compared to the GFP and WT control samples.

Results and Discussion

Apoptosis assays

SeMNPV *ptp2* expression leads to the formation of apoptotic bodies

Given the fact that a bracovirus *ptp* gene (*ptp-H2*) was shown to be pro-apoptotic in lepidopteran cells (Suderman et al. 2008), we hypothesized that SeMNPV *ptp2* (Se*ptp2*) might induce apoptosis in a similar way. The apparent similarity of Se*ptp2* with MAPK phosphatases provides additional support for this hypothesis, as many MAPK phosphatases play important roles in regulation of cell survival (Owens & Keyse 2007; Patterson et al. 2009). To investigate pro-apoptotic effects of Se*ptp2* we performed transient expression assays in lepidopteran cells. The following five expression plasmids were used: pIB, pIB-Ac*ie1*, pIB-Se*ptp2*, pIB-Se*ptp2mut* and pIB-Ac*ptp* (Fig. 1A). Based on counting of GFP expressing cells, transfection efficiencies were estimated to be 50-60% for each of the expression plasmids. Cells transfected with pIB, expressing only eGFP appeared to be healthy (Fig. 1B). However, if these cells were subsequently treated with ActD, a strong inducer of apoptosis, extensive membrane blebbing and the formation of apoptotic bodies could be observed (indicated with white arrows) (Fig. 1B). Expression of

Ac*ie1* also caused membrane blebbing and the formation of apoptotic bodies, although the effect was less severe than with the ActD treatment (Fig. 1B). In a similar fashion, membrane blebbing and apoptotic body formation were observed upon transient expression of Se*ptp2* (Fig. 1B), although to a lesser extent than Ac*ie1* expression and ActD treatment. In contrast, when we expressed the catalytically inactive mutant Se*ptp2mut* no signs of apoptosis were observed (Fig. 1B), comparable to the effect of expression of only eGFP. Ac*ptp* transient expression did not cause signs of apoptosis either; no membrane blebbing or apoptotic bodies were observed (Fig. 1B). These data indicate that Se*ptp2* acts as a pro-apoptotic gene in Sf21 cells. However, in these experiments the effect is mild as compared to the effect of the known pro-apoptotic gene Ac*ie1*. The efficiency of the FMDV2A protease to cleave the polypeptide into eGFP and the protein of interest was 100% for each of the expression plasmids when analysed by Western blotting (data not shown). Similarly, comparable levels of eGFP protein were observed for each of the expression plasmids using Western blotting (data not shown).

SeMNPV *ptp2* expression in Sf21 cells induces effector caspase activity

To quantify the pro-apoptotic effect of Se*ptp2* in insect cells, we measured the activity of the effector caspases 3 and 7 at 48 hpt. Caspase activity was measured as a luminescent signal expressed in relative light units (RLU). Cells transfected with pIB, only expressing eGFP, showed a caspase activity of app. 270,000 relative light units (RLUs) (Fig. 2), while treatment with ActD or expression of Ac*ie1* activates caspases to app. 880,000 and 630,000 RLUs, respectively (Fig. 2). Expression of Se*ptp2* showed caspase activity levels of app. 390,000 RLUs (Fig. 2), which is significantly lower than Ac*ie1* expression and ActD treatment (both $P<0.0001$), but significantly higher than pIB-eGFP expression ($P=0.015$). The catalytic mutant Se*ptp2mut* caused a caspase activity of app. 230,000 RLUs which was significantly lower than Se*ptp2* ($P=0.002$), but not significantly different from the negative pIB-eGFP control ($P=0.368$). Furthermore, Ac*ptp* expression showed caspase levels of app. 260,000 RLUs, which was significantly lower than Se*ptp2* ($P=0.008$), but equivalent to pIB-eGFP or Se*ptp2mut* ($P=0.741$ and $P=0.564$ respectively).

Overall, these data provide evidence that Se*ptp2* has a mild pro-apoptotic effect upon transient expression in insect cells. Furthermore, as this effect appears to be absent for the *ptp2* catalytic mutant, we conclude that the phosphatase activity of the encoded PTP2 protein is important for this pro-apoptotic effect. This pro-apoptotic effect seems to be specific for SePTP2, since expression of a different tyrosine phosphatase, AcMNPV PTP, does not induce apoptosis (Fig. 2) in Sf21 cells, excluding the possibility that apoptosis would be an overall effect of transiently expressed PTPs. Although the underlying mecha-

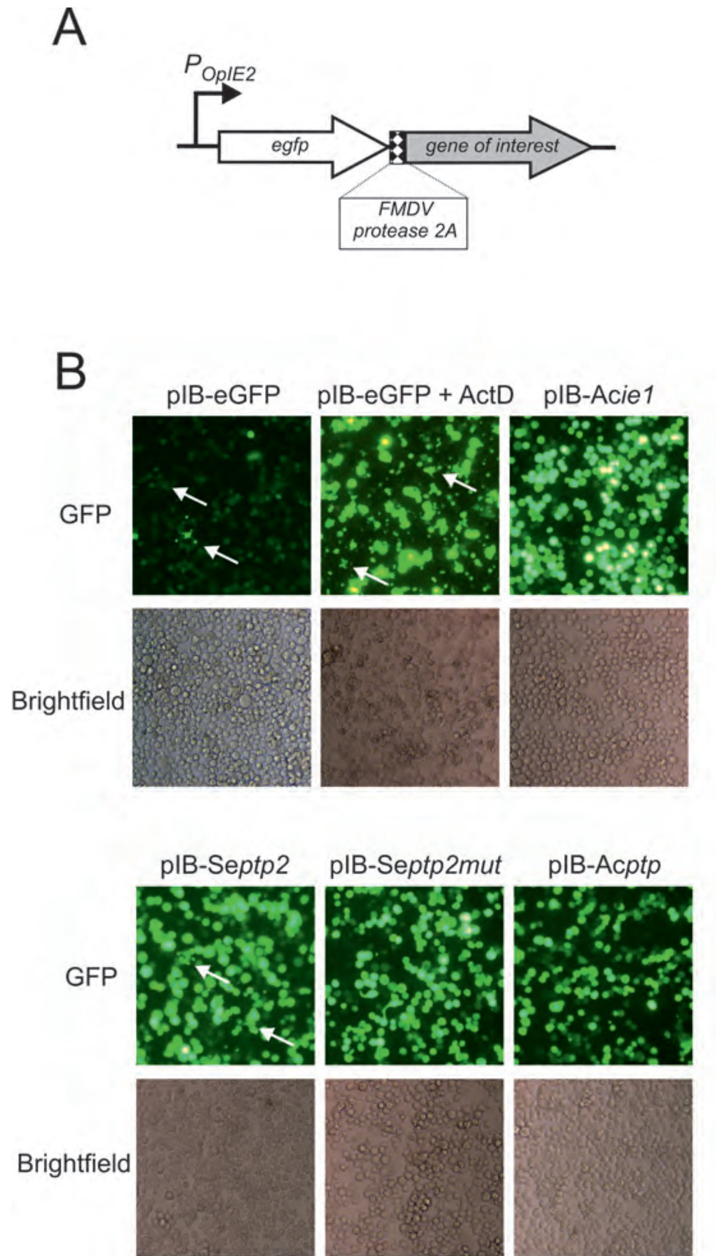


Figure 1. (A) Overview of part of the pIB-DEST expression plasmid containing the OpIE2 promoter (P_{OpIE2}), the FMDV autocleaving protease 2A, and the gene of interest. (B) Microscopy analysis of transfected Sf21 cells at 72 hours post-transfection (hpt), showing GFP expression and the corresponding brightfield image. Treatments: pIB, pIB + ActD, pIB-Ac*ie1*, pIB-Se*ptp2*, pIB-Se*ptp2mut* and pIB-Ac*ptp*. Transient expression of SeMNPV *ptp2* in Sf21 cells causes the formation of apoptotic bodies, as indicated with white arrows.

nism is unknown, it is possible that regulation of MAPK activity in cells might be fundamental to the effect of PTP2 on apoptosis. Apoptosis is known to be tightly controlled by MAPK signalling (Theodosiou & Ashworth 2002; Zhang & Liu 2002). Extracellular signal-regulated kinase (ERK), Janus-kinase (JNK) and p38, the major players in the three major MAPK pathways, are all involved in regulating apoptosis (Kim et al. 2008; Liu et al. 2001; Porras et al. 2004; Wada & Penninger 2004; Xia et al. 1995). Interestingly, a study on the baculovirus *Bombyx mori* NPV (BmNPV) demonstrated that the MAPKs ERK and JNK were activated during the late stage of virus infection, and that downregulation of MAPK signalling affected virus production, as seen by reduced budded virus BV and occlusion body (OB) production (Katsuma et al. 2007).

Why would a virus encode a protein that induces apoptosis? The answer to this question might be related to suppression of the host immune system. The *ptp-H2* gene from *Microplitis demolitor* bracovirus (MdBV) causes apoptosis both in Sf21 cells and in immune cells (haemocytes) (Suderman et al. 2008). Furthermore, two *ptp* genes from the related bracovirus *Cotesia plutellae* bracovirus (CpBV) were shown to have immunosuppressive effects on insect immune cells (haemocytes) (Ibrahim & Kim 2008). By doing so, it is hypothesized that bracoviruses can inhibit host defence responses (Suderman et al. 2008). Host immune suppression by inducing apoptosis is an effective way of

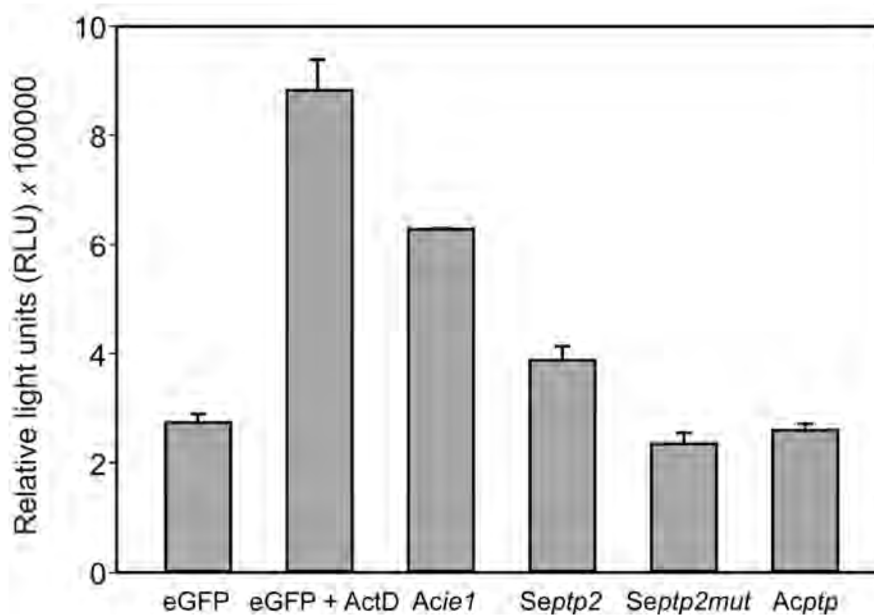


Figure 2. SeMNPV *ptp2* expression in insect cells induces activation of effector caspases. Caspase 3/7 activity in transfected Sf21 cells was measured as luminescence (RLU*100000) at 48 hpt, relative to blank consisting of only cell medium. Error bars represent the standard error of the mean.

establishing a successful virus infection. Investigating the effect of SePTP2 expression specifically in immune cells (haemocytes) could shed more light on such a tissue-specific function. Alternatively, apoptosis may facilitate virus dissemination from cells that have entered the late stage of virus infection (Bideshi et al. 2005; Imajoh et al. 2004; Roulston et al. 1999; Wurzer et al. 2003). For example, *S. frugiperda* ascovirus (SfAV), a lepidopteran-specific DNA virus that is transmitted via oviposition by parasitic wasps, encodes a caspase that induces apoptosis in insect cells. During virus infection this caspase promotes virus dissemination by inducing the formation of apoptotic bodies which can be utilized as large vesicles in which virion assembly can take place (Bideshi et al. 2005). Whether such a dissemination route occurs during baculovirus infection remains to be established. Finally, SePTP2 may have a cellular function different from inducing apoptosis, and the pro-apoptotic effect seen in Sf21 cells may just be a side-effect. Exploring the pro-apoptotic activity of SePTP2 in other lepidopteran cell lines could provide additional insight into the relevance of our observations in Sf21 cells.

It is interesting to note that SeMNPV PTP2 may also be a virion protein. A proteomic analysis on *Chrysodeixis chalcites* NPV (ChchNPV), which is also a group II NPV, showed the presence of ChchNPV PTP2 protein in occlusion-derived virions (ODVs) (Xu et al. 2011). This is compatible with the notion that SeMNPV *ptp2* is expressed late after infection (IJkel et al. 1999), at the time when virions are assembled. SeMNPV PTP2 may therefore also function very early in infection when it is associated with virions. Since baculovirus ODVs are only infectious for midgut epithelial cells and therefore cannot target haemocytes, it can be hypothesized that PTP2 should also be present as a virion protein of budded viruses.

Substrate analysis

To obtain further insights in the putative role of Se*ptp2* during virus infection, a substrate analysis on His-purified SePTP2 protein from baculovirus-infected cells was performed by analyzing the identity of co-purified proteins using LC-MS/MS.

Protein production and His-tag purification

To enable affinity-tag purification of SeMNPV PTP2 protein from insect cells, a recombinant bacmid was created that expressed the PTP2 catalytic mutant C110S encoding a C-terminal 10xHis-tag (Fig. 3A). An AcMNPV WT bacmid expressing polyhedrin (WT) and an AcMNPV bacmid encoding His-eGFP (GFP) were used as negative controls (Fig. 3A). To confirm the presence of His-tagged proteins after column purification, a Coomassie staining and Western blot analysis using anti-His antibodies were

performed on the eluted protein fractions (Fig. 3B). His-purified GFP showed a protein band at approximately 31 kDa both using Coomassie staining and Western blot analysis (Fig. 3B). Coomassie staining also showed two other bands at app. 29 and 26 kDa that may represent GFP cleavage products. The sample with purified PTP2 showed a band at approximately 23 kDa for both Coomassie staining and Western analysis, which is slightly higher than the expected 21 kDa size of the tagged protein. The intensity of the band after Coomassie staining indicates that the amount of PTP2 produced and the yield after subsequent His-purification were relatively low (Fig. 3B).

Proteomic analysis

In total four unique peptides were found that matched the input protein SeMNPV PTP2, with a coverage of 22%. Using the UniProt database to match the LC-MS/MS spectra, in total 13 host proteins were found, that co-purified specifically with SeMNPV PTP2 (Table 2). Four of these proteins also co-purified with AcMNPV PTP (Chapter 4). These proteins are 14-3-3 ε; 14-3-3 ζ; arginine kinase and serine/threonine protein phosphatase 1 (PP1-87B). While these proteins are briefly discussed below, a detailed description can be found in Chapter 4. The remaining nine proteins only co-purifying with SeMNPV PTP2 are discussed below.

Proteins that co-purify with SeMNPV PTP2

14-3-3 ζ and ε, arginine kinase and serine/threonine protein phosphatase 1

These four host proteins were found to co-purify both with AcMNPV PTP and SeMNPV PTP2. Serine/threonine protein phosphatase 1 (PP1) is present in a wide variety of tissues (Asztalos et al. 1993) and is an important regulator of MAPK signalling (Chocarro-Calvo et al. 2012; Jaumot & Hancock 2001; Manfroid et al. 2001). It dephosphorylates MAPK kinase MEK and MEK-kinase, both of which are upstream kinases in the ERK pathway that stimulates expression of the transcription factor c-fos (Manfroid et al. 2001). Furthermore, PP1 was shown to be involved in Raf-1 activation, which in turn phosphorylates and activates MEK (Jaumot & Hancock 2001). The similarity of SePTP2 with known DUSP/MAPK phosphatases may indicate that PTP2 and PP1 act in concert to regulate MAPK signalling. Interestingly, in human cell lines the abovementioned Raf-1 forms a complex with the 14-3-3 protein, one of the other proteins found to co-purify with SeMNPV PTP2 and AcMNPV PTP. In this complex, 14-3-3 is hypothesized to shield Raf-1, thereby preventing its dephosphorylation by PP1 (Jaumot & Hancock 2001). Arginine kinase is implicated in the synthesis of nitric oxide (NO), which is an important neuronal signalling molecule in insects that is released during immunological responses (Bicker 2001; Rivero 2006). This may be related to the

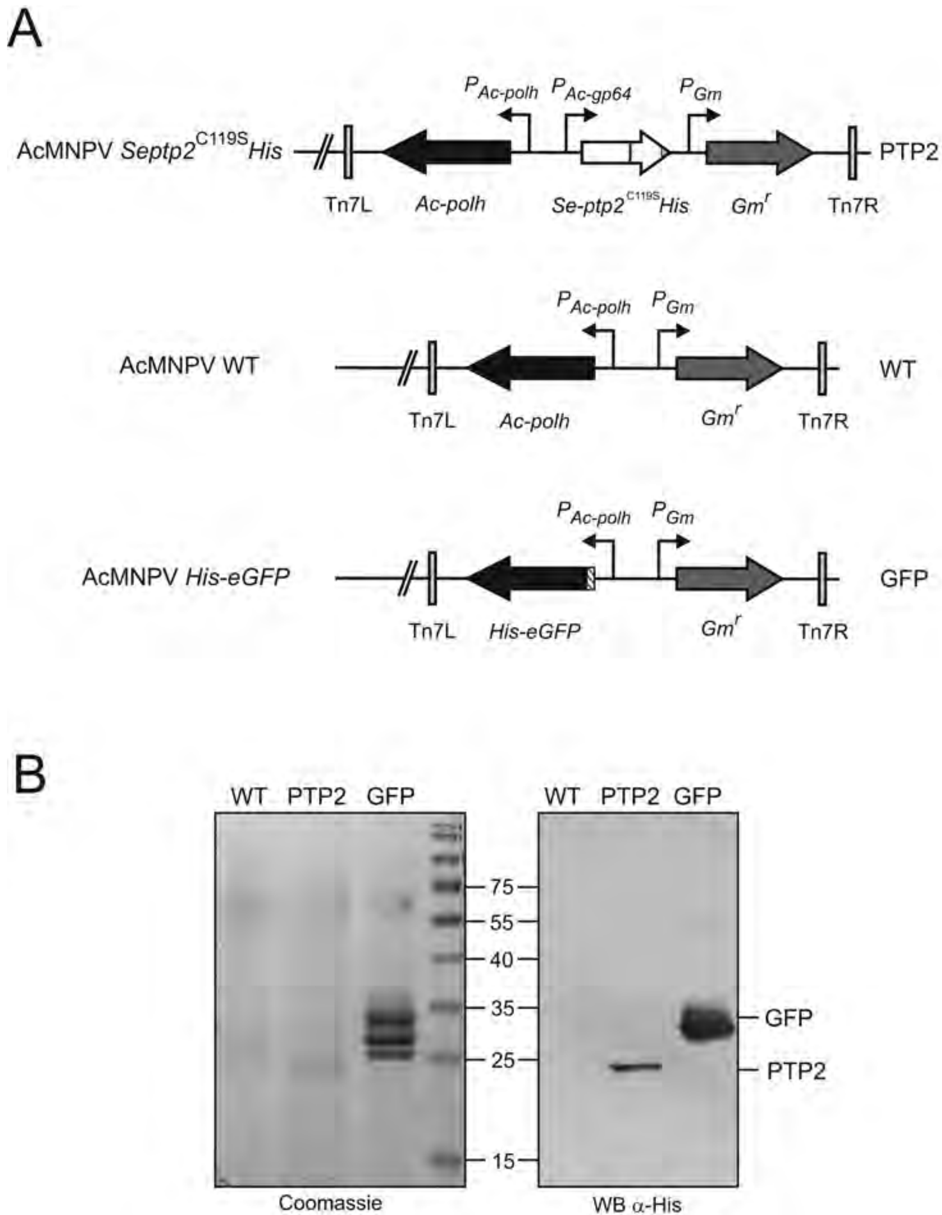


Figure 3. (A) Overview of the recombinant bacmids used for the substrate analysis. The names at the left side represent the full names of the bacmids used in this experiment; at the right hand their short names are indicated. His-tags (C-terminal for *ptp2*C110S, N-terminal for *egfp*) are depicted as striped fragment in the respective ORFs. (B) Coomassie staining (left) and Western blot detection using anti-His antibodies (right) on eluted protein fractions from His-tag purifications of SePTP2 expressed in Sf9 cells. Cells were infected with three different viruses; WT: His-purified fraction of AcMNPV WT virus (negative control), PTP2: His-purified fraction of SeMNPV PTP2C110S-His, and GFP: His-purified fraction of AcMNPV eGFP-His (negative control).

Table 2. Host proteins found to co-purify with SeMNPV PTP2. LC-MS/MS spectra were searched against the UniProt protein database supplemented with three additional protein sequences from *S. exigua* (14-3-3 ζ, 14-3-3 ε, and cGMP-dependent protein kinase (PKG)) and against the *D. melanogaster* protein database. In total, 18 host proteins were found to co-purify specifically with PTP2. For each of those proteins the number of unique peptides and the % coverage is shown per database search.

Host proteins	**UNIPROT**			**DROSOPHILA**	
Protein name	***Organism***	***# of unique peptides***	***% coverage***	***# of unique peptides***	***% coverage***
14-3-3 ζ	*S. exigua*	6	66	4	23
14-3-3 ε	*S. exigua*	8	47	7	33
Arginine kinase	*Plodia interpunctella*	5	33	3	12
Serine/threonine-protein phosphatase PP1	*D. melanogaster*	-	-	4	17
26S protease regulatory subunit 8	*Manduca sexta*	6	26	4	19
GTP-binding nuclear protein Ran	*D. melanogaster*	5	27	4	21
Selenide, water dikinase	*D. melanogaster*	3	13	5	15
Protein Mo25	*D. melanogaster*	3	11	3	11
GDP dissociation inhibitor	*D. melanogaster*	-	-	8	24
Eb1, isoform F	*D. melanogaster*	-	-	5	12
CG8549 – Shwachman-Bodian-Diamond Syndrome-like protein	*D. melanogaster*	-	-	4	16
CG6359 – sorting nexin 3	*D. melanogaster*	-	-	3	14
Isocitrate dehydrogenase (NADP)	*D. melanogaster*	-	-	4	10

aforementioned suggestion that SePTP2 might be involved in suppression of the host immune response.

26S protease regulatory subunit 8

This protein is a 26S proteasome subunit and is part of the AAA protein family, which are ring-shaped proteins that generate mechanical force through ATP hydrolysis (Bar-Nun & Glickman 2012). This force can be used to induce conformational changes of target molecules and is also named 'unfoldase' activity. The 26S proteasome, which is a large multi-protein complex that degrades ubiquitinated proteins, requires such an activity as only properly unfolded proteins can be delivered to the proteases (Bar-Nun & Glickman 2012). Proteasomal ATPase activity is known to be involved in apoptosis during meta-

morphosis in *Manduca sexta* (Sun et al. 1996).

GTP-binding nuclear protein Ran

Ras-related nuclear protein (Ran) is a 25 kDa protein that mediates transport of cargo proteins and RNA molecules in and out of the nucleus, via the nuclear pore complex (NPC) (reviewed in Pemberton & Paschal (2005)). Ran can bind either GDP or GTP, whereby the GTP-bound form can freely bind to cargo molecules. Inside the nucleus, RanGDP is converted to RanGTP by the Ran Guanine Exchange Factor RanGEF. RanGTP binds to importin protein that has shuttled cargo from cytoplasm to nucleus through the NPC, causing the cargo to be released in the nucleus. RanGTP can also form a complex with exportin proteins, which can then transport cargo molecules from the nucleus to the cytoplasm. In the cytoplasm exchange of GTP to GDP takes place through the action of the Ran GTPase activating protein RanGAP. Recycling of Ran occurs by translocation of RanGDP from the cytoplasm to the nucleus by a nuclear transport factor. Interestingly, a recent study reported the identification of a micro RNA (miRNA) expressed by BmNPV that specifically targets Ran expression (Singh et al. 2012). Expression of this viral miRNA lead to lower levels of host miRNAs, and greatly enhanced virus proliferation. Since Ran, together with exportin-5, is needed for translocation of newly synthetized host miRNAs from the nucleus to the cytoplasm, this viral strategy is thought to avoid downregulation of viral genes by host miRNAs (Singh et al. 2012).

Selenide water dikinase

Selenide water dikinase, also named selenophosphate synthetase, is an enzyme that catalyses the ATP-dependent conversion of selenide to selenophosphate, which is a necessary step in the formation of selenocysteine. Proteins containing selenocysteine (selenoproteins) have anti-oxidative activity and are thus important in counteracting reactive oxygen species (ROS)-induced stress (Morey et al. 2003). In *D. melanogaster*, mutations in the gene encoding selenophosphate synthetase affect selenoprotein synthesis and cause ROS accumulation (Alsina et al. 1999). This leads to stabilization of the tumour suppressor protein p53, which in turn activates transcription of the pro-apoptotic gene *reaper* (*rpr*), thereby inducing an apoptotic response (Morey et al. 2003).

Protein Mo25

MO25 is a 39 kDa protein that is highly conserved in both vertebrates and invertebrates (Karos & Fischer 1999; Nozaki et al. 1996), and is important for cell polarization

(Baas et al. 2004; Mendoza et al. 2005). In humans, MO25 is associated with a protein complex consisting of the pseudokinase STRADα and the serine/threonine kinase LKB1 (Boudeau et al. 2003; Milburn et al. 2004). This complex is hypothesized to play a role in, amongst others, cell polarity and apoptosis. A more recent study demonstrated that MO25 regulates activity of multiple kinases belonging to the sterile 20 (STE20) kinase family, which act upstream of the MAPK pathway (Filippi et al. 2011). Although the function of MO25 in *D. melanogaster* is still elusive, a recent genome-wide screening study demonstrated a significant up-regulation of *mo25* transcripts upon Stat hyperactivation in fruit flies, implying a possible link with JAK/STAT signalling (Flaherty et al. 2009).

GDP dissociation inhibitor

GDP dissociation inhibitors (GDIs) are important regulatory proteins of Rho and Rab GTPases, which are essential components of vesicular trafficking pathways (DerMardirossian & Bokoch 2005). These GTPases act as signalling switches by alternating between an inactive GDP-bound state and an active GTP-bound state. While the inactive state is mainly cytosolic, active GTPases are generally membrane-associated, where their target proteins commonly reside. GDIs modulate Rho/Rab GTPase activity in multiple ways; they inhibit the GDP dissociation from these GTPases, and they prevent GTP hydrolysis by interacting with GTP-bound GTPases. It is thought that GDI phosphorylation is an important regulatory mechanism to modulate interactions between GTPases and GDIs, and therefore to modulate Rho/Rab GTPase activation (DerMardirossian & Bokoch 2005; DerMardirossian et al. 2004).

Several studies report the involvement of GDI in virus infections. GDI was shown to hamper HIV-1 replication, possibly by inhibiting RhoA and Rac1 (Watanabe et al. 2012). These GTPases are known to promote virus entry by enhancing the efficiency of virus-cell membrane fusion (del Real et al. 2004; Watanabe et al. 2012). Tobacco mosaic virus (TMV), which replicates on vesicle-like membrane bodies, encodes a protein that interacts with and alters the localization of GDI protein during infection (Kramer et al. 2011). Silencing of GDI also enhanced susceptibility for TMV, indicating that TMV infection alters vesicular trafficking through interaction with GDI (Kramer et al. 2011).

Eb1, isoform F

Microtubule end-binding protein 1 (EB1) is a 33 kDa dimeric protein that is highly conserved among animals, plants and fungi (Jiang & Akhmanova 2011). EB1 binds to the growing end of microtubules, where it seems necessary as docking site for plus-end

tracking proteins (+TIPs), a large group of cellular factors that accumulate at growing microtubule ends and play important roles in regulating microtubule dynamics and interactions between microtubules and cellular structures (Akhmanova & Steinmetz 2008). Hijacking (parts of) the cytoskeleton is a very important strategy of viruses to successfully complete the viral life cycle. For example, endocytosis, transport of progeny virus particles, virion assembly and cell-to-cell spread all require different aspects of fine-tuned cytoskeleton functioning (Radtke et al. 2006). While baculoviruses are well known to rely on actin remodelling for efficient transport of capsids to the nucleus (Charlton & Volkman 1993; Lanier & Volkman 1998; Ohkawa et al. 2010; van Loo et al. 2001), nucleocapsid morphogenesis (Ohkawa & Volkman 1999), and transport of nucleocapsids to the nuclear periphery (Marek et al. 2011), their potential interaction with microtubules is less well studied. However, two studies suggested that baculoviruses utilize microtubules to induce cell lysis (Patmanidi et al. 2003) and to allow efficient egress of nucleocapsids from the nucleus (Fang et al. 2009). Interestingly, it is becoming increasingly clear that viruses manipulate the microtubule network not only for transport purposes, but also to regulate apoptosis and innate immune signalling (Brice & Moseley 2013). For example, HIV1 induces an apoptotic response through binding of HIV1 Tat protein to microtubules (Chen et al. 2002).

CG8549 – Shwachman-Bodian-Diamond Syndrome-like protein

CG8549 is a 29 kDa protein that contains a Shwachman-Bodian-Diamond Syndrome (SBDS) protein domain. SBDS proteins are highly conserved across all kingdoms of life and play a role in RNA metabolism and ribosome maturation (Menne et al. 2007; Savchenko et al. 2005). In humans, an autosomal recessive disorder causes SBDS protein deficiency, which subsequently leads to bone marrow failure and leukemia predisposition (Menne et al. 2007).

CG6359 – sorting nexin 3

Sorting nexins are cytoplasmic proteins involved in membrane trafficking and protein sorting (Worby & Dixon 2002). Sorting nexin 3 (SNX3) is a 19 kDa protein that is essential for Wnt signalling. Wnt signalling is an important signal transduction pathway involved in many developmental processes, such as embryonic axis formation, cell migration and proliferation (Niehrs 2012). Specialized cells secrete Wnt proteins, which then bind to specific Wnt receptors on the membranes of receiving cells to induce targeted gene expression. Wnt secretion is mediated by Wntless (Wls), a Wnt-binding protein that translocates Wnt from the Golgi network to the cell membrane for secretion. Recycling of Wls is mediated by a retromer pathway that shuttles Wls between the cell membrane and

the Golgi network through endosomal transport, a process which is SNX3-dependent (Harterink et al. 2011; Johannes & Wunder 2011; Zhang et al. 2011). Wnt signalling is hypothesized to play a role in behavioural manipulation of grasshoppers and crickets by Gordian worms (Biron et al. 2005; Biron et al. 2006). Besides significantly higher host Wnt protein levels in the brains of manipulated hosts as compared to uninfected ones, also Wnt-like proteins were found in the parasite proteome, suggesting that these parasites hijack Wnt signalling pathways to alter host behaviour. Recently, SNX3 was also shown to inhibit phagocytosis in dendritic cells, possibly by competition with other proteins involved in phagocytosis for binding to phagosomes (Chua & Wong 2013). Furthermore, SNX3 seems to be involved in the spread of *Salmonella* infections, as it binds to and possibly aids in transport of *Salmonella*-containing vacuoles (Braun et al. 2010).

Isocitrate dehydrogenase (NADP)

Isocitrate dehydrogenase (IDH) is a 53 kDa protein that catalyzes the oxidative decarboxylation of isocitrate, resulting in the production of α-ketoglutarate, NADPH and CO2 (Losman & Kaelin 2013). Three different isoforms are known to be expressed in eukaryotes. Isoforms IDH2 and IDH3 both localize to the mitochondrial matrix where they play a role in mitochondrial respiration and modulation of isocitrate and α-ketoglutarate levels (Losman & Kaelin 2013). IDH1 localizes in the cytoplasm and peroxisomes where it, besides regulating isocitrate and α-ketoglutarate levels, promotes activity of other enzymes that utilize α-ketoglutarate as substrate. The *D. melanogaster* IDH homolog identified here shows highest similarity (87%) with the IDH1 isoform. Activation of IDH is regulated by the isocitrate dehydrogenase kinase/phosphatase AceK, which is able to both phosphorylate and dephosphorylate its substrate (Zheng et al. 2012).

Conclusion

The data presented here indicate that SeMNPV *ptp2* may function as a pro-apoptotic gene in infected hosts. However, in our experiments this effect was only tested in cells derived from *S. frugiperda*. Therefore a relevant next step would be to investigate the pro-apoptotic effect of *ptp2* also in cells derived from *S. exigua*, which is the only known host susceptible for SeMNPV. The substrate analysis on SeMNPV PTP2 protein revealed several proteins that are somehow connected to apoptosis (see above). These proteins include the 26S protease regulatory subunit 8, selenide water dikinase, SNX3 and PP1. However, additional experiments are needed to confirm that the interaction between these proteins and PTP2 is part of the pro-apoptotic effect.

The pro-apoptotic effect of SePTP2 may be related to its putative activity as MAPK phosphatase. In the substrate analysis two proteins were identified that are known to affect MAPK signalling: PP1 and Mo25. Possibly, the interaction between PTP2 and these proteins relates to the hypothesized role of PTP2 as MAPK phosphatase. *In vitro* assays to test the specificity of SePTP2 for MAPK substrates could shed more light on this. The fact that only a subset of baculoviruses carry the *ptp2* gene (van Houte et al. 2012) suggests that this MAPK phosphatase activity is only relevant for this subset of baculoviruses.

In addition, the substrate analysis revealed three proteins that are implicated in cellular trafficking. GDI and SNX3 are both involved in vesicular trafficking, while Ran is involved in shuttling cargo between the nucleus and the cytoplasm. It would be interesting to see whether PTP2 plays any role in cellular trafficking in virus-infected cells, for example for the purpose of transportation of baculovirus nucleocapsids to or from the nucleus. In this perspective the interaction between PTP2 and EB1 is also relevant, since virion transport presumably relies on the cytoskeleton.

There are two important limitations of this study that should be kept in mind. Firstly, cell cultures were used instead of whole insects or insect tissues to study interactions between SePTP2 and host proteins. This may lead to interactions between PTP2 and host proteins that are biologically meaningless if they would never occur in the insect due to spatio-temporal constraints. Secondly, a large protein database of the host organism is lacking. Presumably, data from whole genome sequencing projects of economically important insects such as *S. exigua* will improve the identification of additional proteins that interact with baculovirus PTP2.

Although both PTP and PTP2 are members of the DUSP family of phosphatases, the results on SeMNPV PTP2 presented here strongly suggest that this protein has a function at the cellular level, which possibly involves a pro-apoptotic response. Besides this effect, it is currently unknown whether PTP2 is also involved in behavioural manipulation, a function which could even be related to the pro-apoptotic effect observed in insect cells. Further research will shed more light on the cellular function of PTP2 during baculovirus infection and on a possible role in behavioural manipulation.

Acknowledgements

Just Vlak is acknowledged for critically reading this manuscript and useful discussion. SVH and VIDR are both supported by the Program Strategic Alliances of the Royal

Dutch Academy of Sciences (project 08-PSA-BD-01) and VIDR is supported by a VENI grant of the Netherlands Organisation for Scientific Research (project 863.11.017).

Chapter 6

Baculovirus-induced hyperactivity and climbing behaviour are governed by independent mechanisms

Adapted from:

Stineke van Houte, Vera I.D. Ros and Monique M. van Oers. Baculovirus-induced hyperactivity and climbing behaviour are governed by independent mechanisms, *in revision*.

Abstract

Although many parasites are known to manipulate behaviour of their hosts, the mechanisms underlying such manipulations are largely unknown. Baculoviruses commonly manipulate host behaviour by increasing the locomotion activity of their caterpillar hosts and by inducing climbing behaviour leading to death at elevated positions (tree-top disease or Wipfelkrankheit). These manipulations likely increase virus dispersal into the environment. In a recent study we demonstrated the involvement of the protein tyrosine phosphatase (*ptp*) gene from *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) in the induction of hyperactive behaviour in *Spodoptera exigua* larvae. Here we show that AcMNPV *ptp* is not required for AcMNPV-induced tree-top disease, indicating that baculovirus-induced hyperactivity and death at elevated positions are independent behavioural phenotypes that are governed by distinct mechanisms. Furthermore, a moulting-dependent difference in the height at death of infected larvae was found, which may relate to ecological differences between moulted and unmoulted larvae.

Introduction

Like many parasites, baculoviruses cause host behavioural changes to enhance transmission of progeny virions in the environment. Baculoviruses induce hyperactive behaviour in lepidopteran larvae, which is thought to increase virus dispersal over a larger area (Goulson 1997; Kamita et al. 2005; van Houte et al. 2012). In addition, baculoviruses can alter host climbing behaviour leading to host death at elevated positions (Goulson 1997; Hoover et al. 2011), a phenomenon known as 'tree-top disease' or 'Wipfelkrankheit'. This manipulative strategy most likely aids in optimal virus dispersal on plant foliage after liquefaction of the host.

Previously, we demonstrated the requirement of the AcMNPV protein tyrosine phosphatase (*ptp*) gene to induce hyperactive behaviour in *S. exigua* (van Houte et al. 2012). The *ptp* gene appears to play a conserved role in altering host locomotion activity in group I NPVs of the genus *Alphabaculovirus* (Kamita et al. 2005; van Houte et al. 2012) This gene is absent in baculoviruses infecting lepidopteran insects belonging to group II NPVs (*Alphabaculovirus*) or to the genus *Betabaculovirus*, which harbours the granuloviruses (van Houte et al. 2012). Here we tested the hypothesis that the AcMNPV *ptp* gene, besides being a key player in inducing hyperactive behaviour, is also involved in inducing tree-top disease in *S. exigua* larvae. Our results indicate that hyperactivity and tree-top disease are two distinct strategies of baculoviruses to manipulate their caterpillar hosts that are induced by different mechanisms.

Materials and methods

Insect larvae and virus amplification

Spodoptera exigua (Hübner) larvae were reared as described in van Houte et al. (2012). Virus production, amplification and purification were all done as described in van Houte et al. (2012). Stocks of occlusion bodies (OBs) were stored at 4 ºC.

Behavioural assays

Early 3rd instar *S. exigua* larvae were either infected with wild type (WT) AcMNPV or with an AcMNPV *ptp* deletion mutant (Δ*ptp*) by droplet feeding as described in van Houte et al. (2012). Per treatment 24-30 larvae were infected with a viral dose of 10^8 OBs/ml, which corresponds to a lethal concentration of 90-95% (LC_{90-95}). Mock-infected larvae were used as uninfected controls and these were droplet fed with a virus-

free sucrose solution as described in van Houte et al. (2012). Larvae were placed individually in sterile glass jars (120 mm tall x 71 mm wide) with metal lids (with small holes for fresh air). The jars were lined with sterile mesh wire to facilitate climbing and a block of artificial diet (approximately 3.5 cm^3) was placed at the bottom. The jars were incubated in climate-controlled incubators under the same temperature (27 ^oC) and light-dark conditions (14:10 light/dark) at which the larvae were reared. The vertical position of the larvae was monitored every morning and evening, starting from one day post-infection (dpi) until all larvae were either dead or had pupated. The experiment was performed twice (indicated as replicate 1 and replicate 2). Larvae that did not die due to virus infection (died of other causes or survived despite being droplet fed with virus) were excluded from further analyses. The relation between height at death and treatment (WT/Δ*ptp*) was analysed using a general linear model in the software package R (R Core Team 2013), with treatment (WT/Δ*ptp*), stage (died as L3/L4) and experiment number as fixed effects.

Results and Discussion

To determine whether the AcMNPV *ptp* gene plays a role in climbing behaviour in *S. exigua* larvae, we performed climbing studies with mock-, AcMNPV WT- and AcMNPV Δ*ptp*-infected *S. exigua* larvae. Mock-infected control larvae started climbing upwards from day 1 (around 24 hours post-infection (hpi)) (Fig. 1A and 2A). A small peak in height at day 2 (48 hpi) coincided with the larval moult from the 3rd to the 4th instar. From day 3 (70 hpi) control larvae again started to climb upwards, and had moulted from the 4th to the 5th instar at day 4 (100 hpi). After that, larvae tended to stay high up in the jar until the onset of pupation, corresponding to approximately day 6 (140 hpi). At this moment the larvae gradually descended and burrowed themselves in the piece of diet at the bottom of the jar for pupation.

In contrast, virus-infected larvae showed a climbing pattern that was dependent on the developmental stage of the larvae (Fig. 1B and 2B). WT-infected 3rd instar larvae that moulted to 4th instar during the infection (so died as 4th instars) generally died at elevated positions in the jar (Exp. 1: 75 mm ± 15.3 (Fig. 1B), Exp. 2: 80 mm ± 7.1 (Fig. 2B)), while larvae that did not undergo a moult during infection (so died as 3rd instars) showed downward movement during the infection and died at much lower positions in the jar (Exp. 1: 13 mm ± 9.5 (Fig. 1B), Exp. 2: 9 mm ± 0 (Fig. 2B)). WT-infected larvae started dying from 85 hpi onwards and at 140 hpi all larvae had succumbed to baculovirus infection (Fig. 1C and 2C).

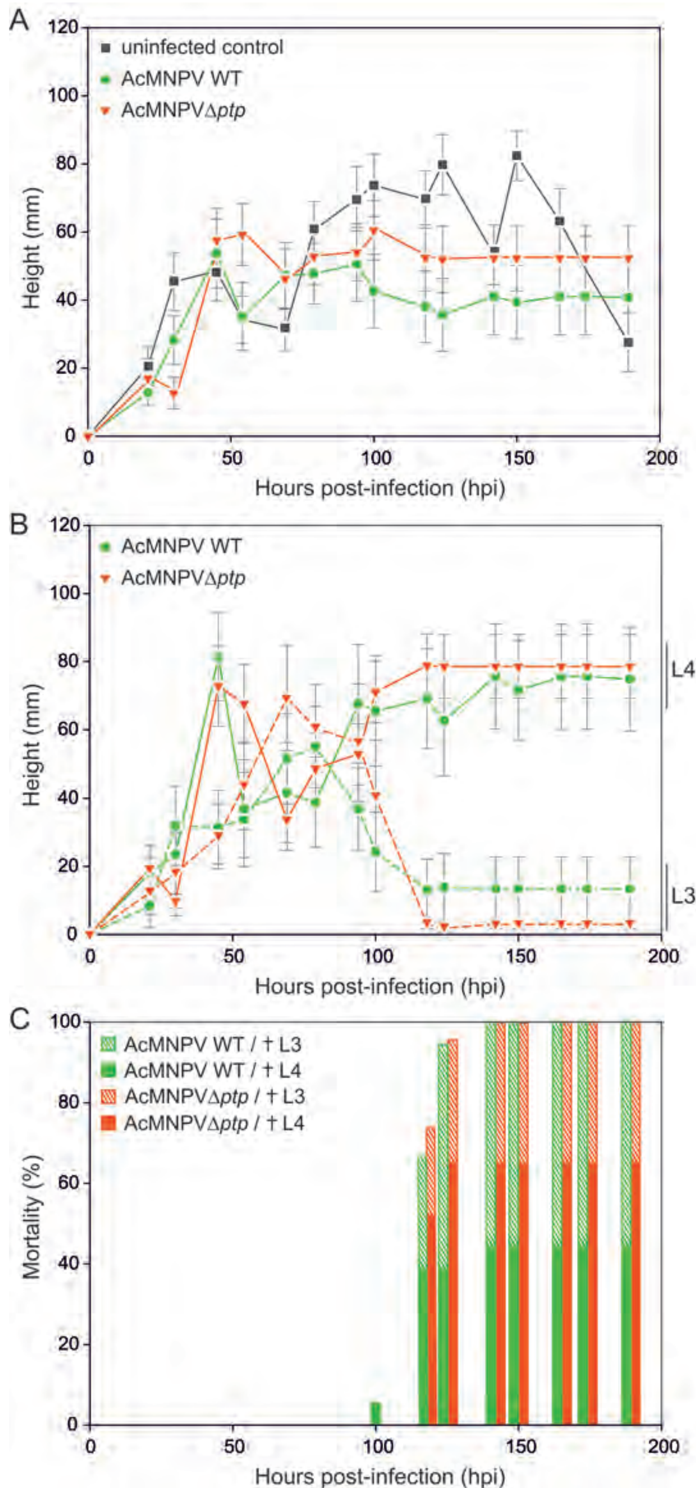
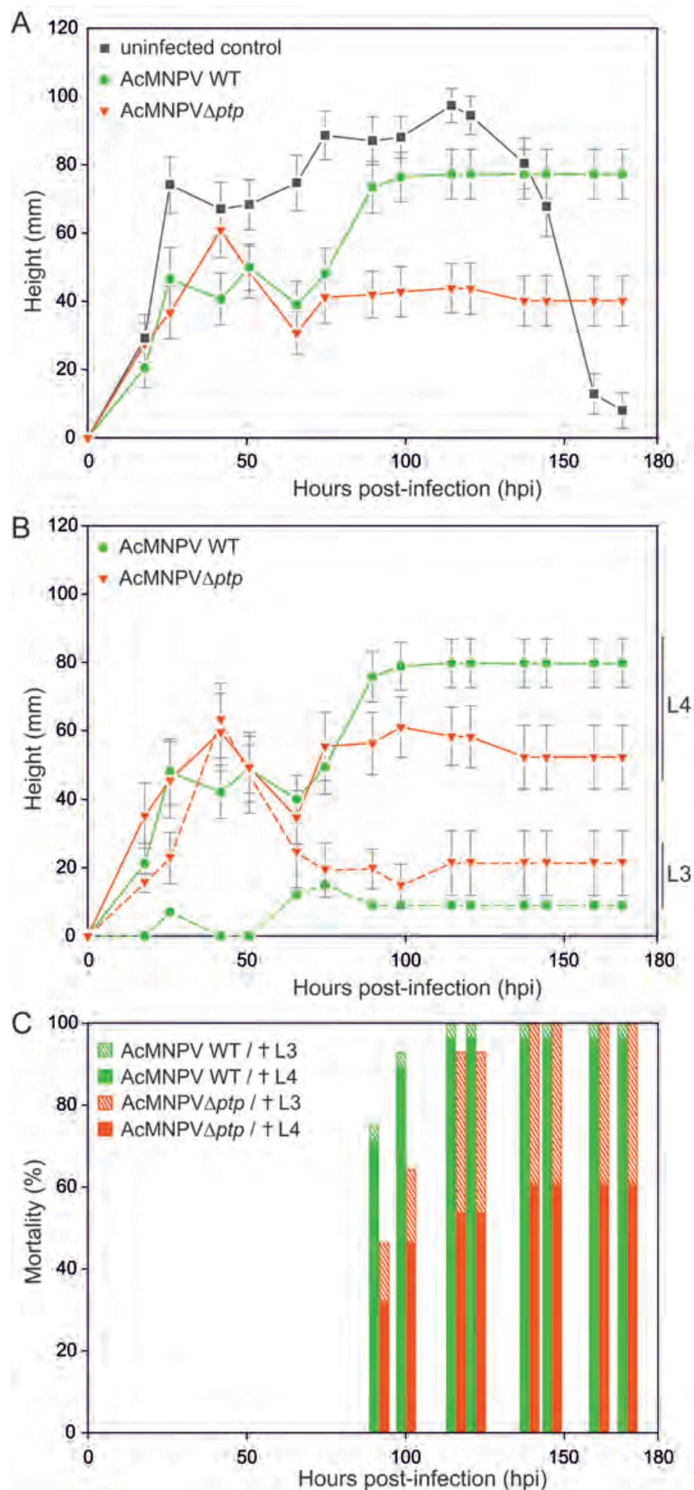


Figure 1. AcMNPV-induced tree-top disease in *S. exigua* is independent of the *ptp* gene (replicate experiment 1). (A) Vertical position (mm) of mock-infected larvae (black squares, n=29), WT-infected larvae (green dots, n=18) and Δ*ptp*-infected larvae (orange triangles, n=23) at different time points after infection (hours post-infection (hpi)). Error bars represent standard error of the mean (SEM). (B) Vertical position (mm) of WT-infected larvae that died as 3rd instars (L3) (green dots, dotted line, n=10), WT-infected larvae that died as 4th instars (L4) (green dots, solid line, n=8), Δ*ptp*-infected larvae that died as 3rd instars (L3) (orange triangles, dotted line, n=8) and Δ*ptp*-infected larvae that died as 4th instars (L4) (orange triangles, solid line, n=15). Error bars represent SEM. (C) Cumulative mortality (%) of WT- and Δ*ptp*-infected larvae. Striped green bars represent WT-infected larvae that died as 3rd instars (†L3), solid green bars represent WT-infected larvae that died as 4th instars (†L4). Striped orange bars represent Δ*ptp*-infected larvae that died as 3rd instars (†L3), solid orange bars represent Δ*ptp*-infected larvae that died as 4th instars (†L4).

Figure 2. AcMNPV-induced tree-top disease in *S. exigua* is independent of the *ptp* gene (replicate experiment 2). (A) Vertical position (mm) of mock-infected larvae (black squares, n=29), WT-infected larvae (green dots, n=28) and Δ*ptp*-infected larvae (orange triangles, n=26) at different time points after infection (hours post-infection (hpi)). Error bars represent standard error of the mean (SEM). (B) Vertical position (mm) of WT-infected larvae that died as 3rd instars (green dots, dotted line, n=1), WT-infected larvae that died as 4th instars (green dots, closed line, n=27), Δ*ptp*-infected larvae that died as 3rd instars (orange triangles, dotted line, n=12) and Δ*ptp*-infected larvae that died as 4th instars (orange triangles, closed line, n=14). Error bars represent SEM. (C) Cumulative mortality of WT- and Δ*ptp*-infected larvae. Striped green bars represent WT-infected larvae that died as 3rd instars (†L3), solid green bars represent WT-infected larvae that died as 4th instars (†L4). Striped orange bars represent Δ*ptp*-infected larvae that died as 3rd instars (†L3), solid orange bars represent Δ*ptp*-infected larvae that died as 4th instars (†L4).



Throughout the measurements no differences were observed in climbing behaviour of WT- and Δ*ptp*-infected larvae (Fig. 1 and 2). The height at death of Δ*ptp*-infected larvae that moulted to the 4th instar was similar to WT-infected larvae (Exp. 1: 79 mm ± 9.4 (Fig. 1B), Exp. 2: 56 mm ± 9.4 (Fig. 2B)). In contrast, Δ*ptp*-infected larvae that died as 3rd instars died at low positions (Exp. 1: 3 mm ± 1.4 (Fig. 1B), Exp. 2: 21 mm ± 9.5 (Fig. 2B. Like WT-infected larvae, Δ*ptp*-infected larvae started dying from 85 hpi onwards and at 140 hpi all larvae had succumbed to the infection (Fig. 1C and 2C).

When the results of the two replicate experiments were combined, WT-infected larvae 4th instar larvae died on average at 79 mm ± 6.5 and Δ*ptp*-infected larvae 4th instars at 66 mm ± 6.9 (Fig. 3). WT-infected 3rd instar larvae died on average at 13 mm ± 8.6, and Δ*ptp*-infected larvae 3rd instars at 14 mm ± 5.9 (Fig. 3). No significant difference was found between the two experiments ($F_{(3,94)}$= -1.359; P=0.8612) and treatment (WT/Δ*ptp*) was not significantly different either ($F_{(3,94)}$= -8.795; P=0.2440). However, the larval instar at death (died as 3rd or as 4th instar) was highly significant ($F_{(3,94)}$= 58.183; P<0.001). As the average time to death (LT_{50}) was previously reported to be similar between WT- and

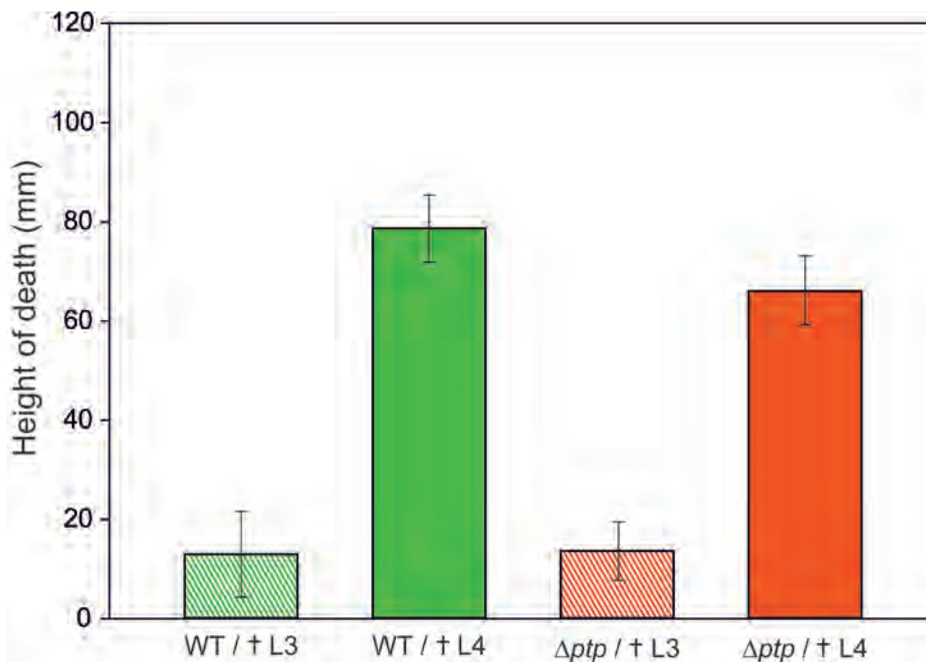


Figure 3. Average height at death (mm) of WT- and Δ*ptp*-infected larvae that died either as 3rd or 4th instars. Combined data of two replicate experiments. Striped green bars represent WT-infected larvae that died as 3rd instars (n=11) (†L3), solid green bars represent WT-infected larvae that died as 4th instars (n=35) (†L4). Striped orange bars represent Δ*ptp*-infected larvae that died as 3rd instars (n=20) (†L3), solid orange bars represent Δ*ptp*-infected larvae that died as 4th instars (n=29) (†L4). Error bars represent SEM.

Δ*ptp*-infected larvae (van Houte et al. 2012), no statistical analysis on the time to death was carried out in this study.

The results indicate that the AcMNPV *ptp* gene does not play a role in baculovirus-induced tree-top disease in *S. exigua* larvae. However, we found that the height at death is dependent on whether hosts had moulted during the infection or not. The reason for this stage-dependent difference in height at death is currently unclear and requires further analysis. Interestingly, downward movement is observed in larvae of the winter moth *Operophtera brumata* infected with *O. brumata* NPV (OpbuNPV) descended from the foliage to the lower tree stems to die there (Raymond et al. 2005). Since OBs were shown to persist better on stems than on foliage, this behavioural change was hypothesized to contribute to virus persistence (Raymond et al. 2005). The differences observed in this study in height at death between the stages may also be related to differences in the ecology of 3rd and 4th instar larvae. The 1st and 2nd instars of *S. exigua* larvae are gregarious, while during the 3rd developmental stage a transition occurs towards solitary behaviour. Larvae of the 4th and 5th instar are completely solitary feeders (Smits et al. 1987). Whether the differences observed in in our climbing assays are related to such a transition from gregarious to solitary behaviour is unclear and requires further research.

The main finding of this study is that the AcMNPV *ptp* gene does not play a role in determining the height at which infected *S. exigua* larvae die. Combining this with the previous findings on the conserved role of this gene in hyperactivity (van Houte et al. 2012) leads to the conclusion that AcMNPV utilizes independent mechanisms to alter locomotion activity and tree-top disease. This implies that baculoviruses may have evolved multiple strategies to alter host behaviour, probably by manipulating distinct host signalling pathways. Although we here exclude a role for *ptp* in climbing in AcMNPV-infected *S. exigua* hosts, it is unknown whether another specific viral gene is responsible for tree-top disease in *S. exigua*. A recent study by Hoover et al. (2011) demonstrated that tree-top disease in *Lymantria dispar* larvae infected with *L. dispar* MNPV (LdMNPV) requires the *egt* gene, which encodes ecdysteroid UDP-glucosyl transferase. This enzyme is known to inhibit the activity of host ecdysteroid hormones, thereby blocking the larval moult (O'Reilly 1995). *Egt* is a highly conserved gene among Lepidoptera-infecting baculoviruses and may also play a conserved role in tree-top disease. A next step would be to investigate whether the AcMNPV *egt* gene is also responsible for tree-top disease in infected *S. exigua* larvae. It is also possible that climbing behaviour upon AcMNPV infection is not induced by a specific viral genetic factor *per se*. For example, virus infection as such could indirectly affect host behavioural gene expression, e.g. as a result of metabolic changes or immunological responses.

Acknowledgements

The authors thank Linda Guarino of Texas A&M University for kindly providing the AcMNPV Δ*ptp* bacmid. Just Vlak is acknowledged for critically reading this manuscript and for useful discussions. SVH and VIDR are both supported by the Program Strategic Alliances of the Royal Dutch Academy of Sciences (project 08-PSA-BD-01) and VIDR is supported by a VENI grant of the Netherlands Organisation for Scientific Research (project 863.11.017).

Chapter

7

Baculovirus-induced tree-top disease: how extended is the role of *egt* as a gene for the extended phenotype?

Adapted from:

Vera I.D. Ros*, Stineke van Houte*, Lia Hemerik, Monique M. van Oers. Baculovirus-induced tree-top disease: how extended is the role of *egt* as a gene for the extended phenotype?, *under review*.

* These authors contributed equally to this paper

Abstract

Many parasites alter host behaviour to enhance their chance of transmission. Recently, the ecdysteroid UDP-glucosyl transferase (*egt*) gene from the baculovirus *Lymantria dispar* multiple nucleopolyhedrovirus (LdMNPV) was identified to induce tree-top disease in *L. dispar* larvae. This behaviour causes the gypsy moth larvae to die at elevated positions (hence the term tree-top disease), which is thought to promote dissemination of the virus to lower foliage. Here, we studied tree-top disease induced by *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) in two different host insects, *Trichoplusia ni* and *Spodoptera exigua*, and we investigated the role of the viral *egt* gene therein. While infected *T. ni* larvae died at elevated positions, we found a moulting-dependent effect on the position at death in *S. exigua* larvae. Those undergoing a larval moult during the infection process died at elevated positions, while larvae that did not moult after infection died at low positions. The AcMNPV *egt* gene had no effect on the position where *S. exigua* and *T. ni* larvae died. Therefore, we conclude that the *egt* gene has no conserved role in inducing tree-top disease in lepidopteran larvae.

Introduction

Manipulation of host behaviour is a common strategy exploited by parasites to increase their transmission (Hughes et al. 2013; Libersat et al. 2009; Moore 2002; van Houte et al. 2013). Exquisite examples of such changes in host behaviour are known, including climbing to elevated positions, as is seen for ants infected by *Ophiocordyceps* fungi (Hughes et al. 2011) or by lancet liver flukes (*Dicrocoelium dendriticum*) (Libersat et al. 2009) and for caterpillars infected by baculoviruses (Goulson 1997; Hoover et al. 2011; Vasconcelos et al. 1996). After infection, caterpillars climb to the top of plants, where they eventually die and liquefy, releasing new virus particles (Goulson 1997; Kamita et al. 2005; Smirnoff 1965; Vasconcelos et al. 1996). This behaviour has been described as 'Wipfelkrankheit' or 'tree-top disease' (Evans 1986; Hofmann 1891; Smirnoff 1965) and is thought to enhance virus dissemination (Goulson 1997; Vasconcelos et al. 1996).

The underlying mechanisms of parasite-induced behavioural changes are poorly understood (Hughes 2013; van Houte et al. 2013). Baculoviruses are the first parasites for which 'genes for the extended phenotype' (parasite genes that have phenotypic effects in other organisms (Dawkins 1982)) have been identified (Hoover et al. 2011; Kamita et al. 2005; van Houte et al. 2012). Hoover et al. (2011) showed that the *egt* gene of *Lymantria dispar* multiple nucleopolyhedrovirus (LdMNPV), encoding ecdysteroid UDP-glucosyl transferase (EGT) induces tree-top disease in *L. dispar* caterpillars. Wild type (WT) LdMNPV-infected larvae died at elevated positions, while larvae infected with an LdMNPV virus from which the *egt* gene had been removed (Δ*egt*) died at low positions. Behaviour of WT-infected and Δ*egt*-infected larvae differed during the last stage of infection, when WT-infected larvae stayed at elevated positions and died, while Δ*egt*-infected larvae moved downwards and died there.

The baculovirus *egt* gene has been studied since 1989, when it was found to affect the hormonal regulation of host development (O'Reilly & Miller 1989). EGT catalyses the transfer of a sugar molecule (UDP-glucose or UDP-galactose) to the ecdysteroid insect moulting hormones, a process that inactivates these ecdysteroids (O'Reilly 1995; O'Reilly & Miller 1989). As a consequence, larval moulting (between the five larval stages) or larval pupation (transfer from 5th instar larva to pupa) is blocked. As larvae normally stop feeding prior to moulting, it is hypothesized that *egt* expression causes the host insect to continue feeding after infection, leading to bigger larvae that produce more progeny baculoviral occlusion bodies (OBs). O'Reilly & Miller (1991) showed that 4th and 5th instars of *Spodoptera frugiperda* infected with AcMNPV Δ*egt* fed less and died earlier than those infected with the WT virus. This indicates that *egt* extends the time until the

host dies, and also extends the host's feeding period, maintaining the host in an actively feeding state. In 5th instars of *S. frugiperda* infected with AcMNPV, an increased yield of OBs was recorded (O'Reilly & Miller 1991), as was also found for AcMNPV-infected *Heliothis virescens* 5th instars (O'Reilly et al. 1998). However, an extension of the time to death through *egt* is not universal, as in some virus-host systems or in certain larval stages no increase in time to death was found (Bianchi et al. 2000; Slavicek et al. 1999).

Analysis of complete genome sequences shows that the *egt* gene is found in almost all lepidopteran baculoviruses, except for one clade of granuloviruses (Genbank search and Ahn et al. (2012)). To see whether AcMNPV induces tree-top disease, and whether the baculoviral *egt* gene has a conserved role in this behavioural manipulation of the caterpillar host, we studied the behaviour and position at death for two different host species, *Trichoplusia ni* and *Spodoptera exigua*, upon infection with either AcMNPV WT or an AcMNPV mutant lacking the *egt* gene. We show that AcMNPV induces tree-top disease in *T. ni* larvae and in *S. exigua* larvae that undergo a moult during the infection process. Additionally, we show that the *egt* gene is not involved in inducing tree-top disease in these two host species.

Materials and methods

Insect cells and larvae

Spodoptera frugiperda Sf9 cells (Invitrogen) were cultured as monolayers in Sf900II serum-free medium (Invitrogen) supplemented with 5% fetal bovine serum (Invitrogen) and 0.1% gentamycin (Invitrogen). *Spodoptera exigua* larvae were reared on artificial diet at 27 °C with 50% relative humidity as described before (Smits et al. 1986), and a 14:10 h light:dark photoperiod. *Trichoplusia ni* larvae were reared at 22 °C, other conditions were the same as for *S. exigua*. The *T. ni* larvae originated from a culture maintained at the Centre for Ecology and Hydrology, Oxford (kindly provided by T. Carty).

Generation of recombinant bacmids

The AcMNPV E2 strain-derived bacmid was used as WT virus in this study (Luckow et al. 1993; Smith & Summers 1979). An AcMNPV bacmid with a deletion of the *egt* gene (Δ*egt*) was created by λ RED recombination (Datsenko & Wanner 2000). A fragment from nucleotides 37 to 1497 of the *egt* open reading frame (ORF) was replaced with the chloramphenicol resistance gene (*cat*) flanked by mutant *loxP* sites (Suzuki et al. 2005). To this aim, primers with 50-bp overhangs homologous to flanking regions of the viral *egt* gene (primers 1 and 2, Table S1) were used to amplify the *cat* gene with Phusion poly-

merase (Finnzymes), using the plasmid pCRTopo-lox-cat-lox (L. Galibert, Généthon, France) as a template. The recombination was performed in AcMNPV bacmid-containing *Escherichia coli* MW003 cells (MW001 cells (Westenberg et al. 2010) with the *bla* gene removed), in which the λ RED recombination was activated by heat-induction at 42 ºC for 10 min. After transformation of the cells with the amplified *cat* fragment, cells were grown at 30 °C in the presence of chloramphenicol (6.8 μg/ml). To remove the *cat* gene, the cells were transformed with the temperature sensitive plasmid pCRE-ts, from which Cre-recombinase was expressed at 30 °C. Finally, the cells were incubated at 37 °C to remove pCRE-ts. To create pCRE-ts, the cassette containing Cre-recombinase under control of the lacZ promotor was isolated as *Pvu*II fragment from the plasmid pUC21-Cre (J. Louwerse, LUMC, The Netherlands) and combined with the ampicillin resistance marker and a temperature-sensitive origin of replication (*ori*), amplified from the pKD46 plasmid (Datsenko & Wanner 2000).

The resulting AcMNPV Δ*egt* bacmid was purified and used to transform DH10BacΔTn7 cells (Airenne et al. 2003). To enable oral infection of larvae, the AcMNPV polyhedrin (*polh*) ORF was reintroduced into the WT and Δ*egt* genomes. For this purpose, Bac-to-Bac transposition (Luckow et al. 1993) was performed with a modified pFastBacDual vector (pFBDpolhΔp10), in which the AcMNPV *polh* ORF was cloned downstream of the *polh* promoter, and from which the *p10* promoter was removed, both as described in Peng et al. (2010).

Amplification and purification of virus

To produce recombinant viruses, Sf9 cells were transfected with the above described bacmids (WT, Δ*egt*) using CellFectin II transfection reagent (Invitrogen), and the resulting budded virus (BV) stock was amplified once in cells. The OBs generated in these infected cells were amplified in *S. exigua* 3rd instar larvae. OBs from infected larvae were purified by grinding deceased larvae in water and filtering through a double layer of cheese cloth. The suspension was first centrifuged at 500x*g*, after which the supernatant was centrifuged at 4000x*g* to sediment OBs. Finally, OBs were resuspended in water and stored at 4 ºC.

Infectivity assays

Infectivity assays were performed to determine the 50% lethal virus concentration (LC_{50}) for each virus and for each host species, as described in van Houte et al. (2012). Newly moulted 3rd instars of *T. ni* (moulted overnight, and starved in the morning for 3-4 h) and *S. exigua* (moulted and starved overnight for 16 h) were infected using droplet

Table S1. Primers used in this study.

Primer number	Primer name	Sequence (5' to 3')	Function
1	loxcat_ 50bp_ EGT_F	*TCGGTTTGAAGCAAAATCACTAT-TCTCTGCTGGCTTGCACTGCTGTC-TACG*GCTCGGATCCACTAGTAACG	primer with 50bp overhang (italics) to amplify a product for homologous recombination to remove *egt*
2	loxcat_50bp_ EGT_R	*ATGGTGAATAACATTTATTGACG-TAATTAAAAGTGTGTTAAGTGAT-TCAT*CCTCTAGATGCATGCTCG	primer with 50bp overhang (italics) to amplify a product for homologous recombination to remove *egt*
3	EGT_F	GGCAATGTTTAGAAAGCGCGGAGT	RT-PCR on AcMNPV *egt*
4	EGT_R	ACAAGATGGATTCCTCCGCCAAGA	RT-PCR on AcMNPV *egt*
5	Ac-ie1 FW	TAAGAATTCGTTGGGCGAAA-GAAAATGT	RT-PCR on AcMNPV *ie1*
6	Ac-ie1 RV	TAAAAGCTTCGCCAGAAATC-CAATAAACT	RT-PCR on AcMNPV *ie1*
7	Se- eIF5A F	GCCATGGCTGACATCGAGGATAC	RT-PCR on *S. exigua* and *T. ni* (host) *eIF5A*
8	Se- eIF5A R	GCGGTACCGGTTTATTTGTCGA-GAGC	RT-PCR on *S. exigua* and *T. ni* (host) *eIF5A*

feeding with WT and Δ*egt* viruses, using virus dilutions ranging from 10^2 to 10^9 OBs/ml. Mock-infected larvae, which were droplet fed with a virus-free sucrose solution as described in van Houte et al. (2012), were used as controls. Once a day, larvae were scored for mortality until all larvae were either dead or had pupated. These infectivity assays were performed twice for each host species. Median LC_{50} values were determined by Probit analysis using SPSS v19.0.0.1.

Behavioural assays

Newly moulted 3rd instars of *T. ni* and *S. exigua* were infected with an LC_{90-95} dose (10^8 OBs/ml) of virus (WT and Δ*egt*) using droplet feeding as described in van Houte et al. (2012). Mock-infected larvae were used as controls (see above). Larvae were placed individually in sterile glass jars (120 mm tall x 71 mm wide) with metal lids (with small holes for fresh air). The jars were lined with sterile mesh wire to facilitate climbing and a block of artificial diet (approximately 3.5 cm^3) was placed on the bottom of the jar. The position and mortality of the larvae were determined twice per day, starting from one day post-infection (dpi) until all larvae were dead or had pupated. Experiments were performed in controlled climate incubators, under the same conditions at which the larvae were reared. All experiments were repeated at least once. Larvae that did not die due to virus infection (died of other causes or survived despite being droplet fed with

virus) were excluded from analyses.

Statistical analysis

To analyse the position at death, a linear regression model (lm) analysis was performed in the program R v3.0.0 (R Core Team 2013), for each of the host species separately. Stage (whether the larvae died as 3rd or 4th instar), treatment (WT or Δ*egt*) and experiment (four replicates for *S. exigua*; two replicates for *T. ni*) were used as covariates and it was determined whether these affected the position at death.

The effects of the covariates (stage, treatment, experiment) on time to death were analysed using Cox's proportional hazards model (Cox 1972), for each host species separately. The model (eqn. 1) is given for *T. ni* and is formulated in terms of the hazard rate $h(t,z)$, which describes the probability per unit time that a certain event (here: death) occurs depending on covariate values z_i (i= 1,2,3).

$$h(t,z_1,z_2,z_3)=h_0(t)exp(b_1 z_1 + b_2 z_2 + b_3 z_3 (t)) \quad (1)$$

Here, the hazard rate $h_0(t)$ is the baseline mortality rate at time t. The exponential factor describes the effects of the covariates on this baseline mortality rate. The effects are $\exp(\beta_i)$ (for a detailed description of the model with time-dependent covariates see Koenraadt et al. (2004)). Here, the first two covariates are fixed (not changing in the course of the experiment), namely z_1 treatment with wild type (WT) as baseline versus Δ*egt*, and z_2 experiment number with experiment 1 as baseline (for *T. ni* there were two experiments, and for *S. exigua* four). Experiment number was analysed as a factor. The 3rd covariate $z_3(t)$ was the larval stage of the caterpillar under observation (3rd instar (L3) as baseline versus 4th instar (L4)) and because this changed in the course of the experiment this is a time-dependent covariate. Analyses were performed using the program R v3.0.0 (R Core Team 2013).

RNA isolation and RT-PCR

Newly moulted 3rd instars of *T. ni* and *S. exigua* were infected by droplet feeding with a viral dose of 10^8 OBs/ml virus (WT and Δ*egt*) as in van Houte et al. (2012). Mock-infected larvae were used as controls. At two dpi total RNA from single larvae was extracted by homogenizing each larva in 250 µl Trizol reagent (Invitrogen) and total RNA was isolated following the manufacturer's instructions. The RNA pellet was dissolved in 100 µl water and heated for 10 min at 55 °C. Any contaminating DNA was removed with the DNA*free* kit (Applied Biosystems) according to the company's protocol. Production of

cDNA was performed using SuperScript III Reverse Transcriptase (Invitrogen) according to the company's protocol. RT-PCR was performed to amplify i) 543 bp within the AcMNPV *egt* ORF (primers 3 and 4, Table S1), ii) 512 bp within the AcMNPV *ie1* ORF (primers 5 and 6), and iii) 486 bp of the *T. ni* resp. *S. exigua* host translation initiation factor *eIF5A* ORF (primers 7 and 8). For each RT sample, a control sample was run in which the RT step was omitted (non-RT) to check for DNA contamination. In addition, a negative control without template was processed for each primer pair.

Results

Viral infectivity is not affected by deletion of the AcMNPV *egt* gene

To see whether removal of the viral *egt* gene of AcMNPV affected viral infectivity, we compared the lethal concentration (LC_{50}) of WT and Δ*egt* virus stocks within both *T. ni* and *S. exigua* 3rd instars. The LC_{50} values were $10^{5.1}$ (WT) and $10^{4.8}$ (Δ*egt*) OBs/ml for *T. ni* and $10^{7.2}$ (WT) and $10^{6.7}$ (Δ*egt*) OBs/ml for *S. exigua*. These have overlapping 95% confidence intervals indicating no significant difference between the viruses within each host species (Table 1). This indicates that the infectivity of AcMNPV is not affected by the absence of the *egt* gene. This is in correspondence with findings in a range of other studies, for AcMNPV in *S. frugiperda* (Eldridge et al. 1992b; O'Reilly & Miller 1991), in *T. ni* (Eldridge et al. 1992a), and in *S. exigua* (Bianchi et al. 2000), for SfMNPV in *S. frugiperda* (Simón et al. 2012), for LdMNPV in *L. dispar* (Slavicek et al. 1999) and for HearNPV in *H. armigera* (Georgievska et al. 2010). Only one study found that the infectivity of AcMNPV Δ*egt* was higher than that of AcMNPV WT, in 5th instars of *T. ni* (Cory et al. 2004).

To confirm the expression of *egt* in WT-infected larvae, and the absence of *egt* expression in Δ*egt*-infected larvae, RT-PCR was performed on total RNA isolated from mock-, WT- and Δ*egt*-infected larvae at two dpi. As expected, the AcMNPV *egt* gene was expressed in WT-infected larvae, but not in mock- and Δ*egt*-infected larvae (Fig. 1). The AcMNPV *ie1* gene, included as a control for virus infec-

Table 1. Dose-mortality response (log LC_{50}) of *S. exigua* and *T. ni* 3rd instars infected with WT and Δ*egt* virus.

Host	Virus	Log LC50 (OBs/ml)	95% confidence limits	
			lower	*upper*
T. ni	WT	5.1	4.1	6.1
	Δ*egt*	4.8	3.9	5.7
S. exigua	WT	7.2	6.6	7.7
	Δ*egt*	6.7	6.1	7.2

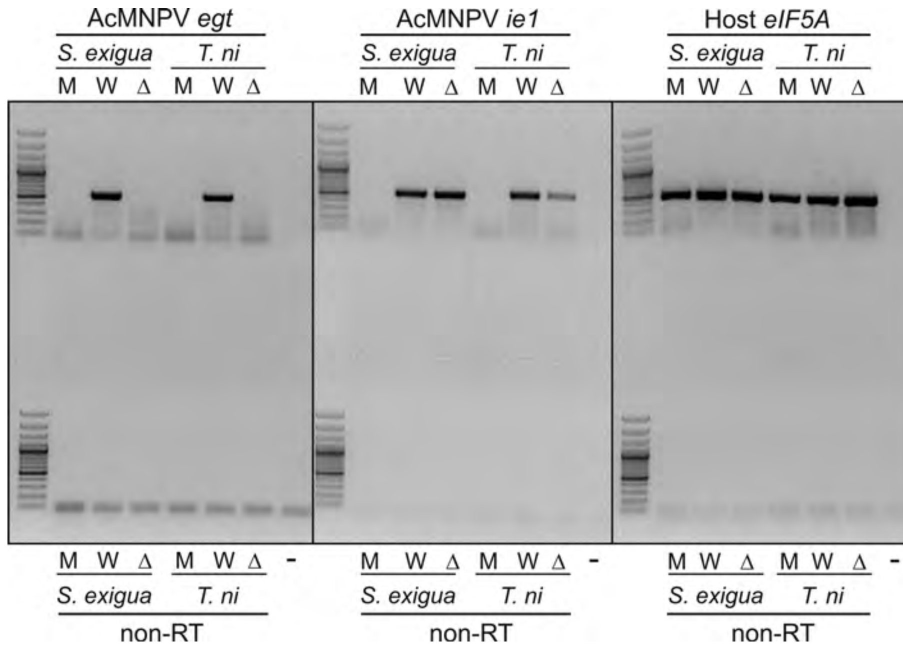


Figure 1. The AcMNPV *egt* gene is expressed in WT-infected larvae and not in Δ*egt*-infected larvae. RT-PCR analysis on mock- (M), WT- (W) and Δ*egt*-infected (Δ) larvae. Expression of the AcMNPV *egt* gene, the AcMNPV *ie1* gene and the host *eIF5A* gene was analysed. For each RT sample, a PCR without RT step (non-RT) was performed in parallel. For each primer pair, a no-template control was processed (-). The GeneRuler 100 bp ladder (Fermentas) was included in the agarose gel to estimate PCR fragment sizes.

tion, was expressed in all virus-infected larvae. The host e*IF5A* gene, encoding eukaryotic translation initiation factor 5A (van Oers et al. 1999) was included as a host control and showed expression in both mock- and virus-infected larvae of both *T. ni* and *S. exigua*.

The AcMNPV *egt* gene affects the time to death differently in *T. ni* and *S. exigua* larvae

We performed a survival analysis to investigate the effects of the covariates (stage, treatment, experiment) on time to death. For *T. ni*, the rate at which larvae die was significantly affected by the treatment, stage of the larvae and the experiment. Cox's model determines a hazard rate (probability to die) for the covariate values. The hazard ratio between larvae infected with AcMNPV Δ*egt* and with WT was 2.42, meaning that the mortality rate was more than twice as large for Δ*egt*- than for WT-infected larvae irrespective of the larval stage. The mortality rate for 4th instars was 0.22 times less than for 3rd instars (i.e. 3rd instars died earlier than 4th instars). There was also a difference between the two experiments: in the 2nd experiment, the mortality rate was 3.30 times larger than in

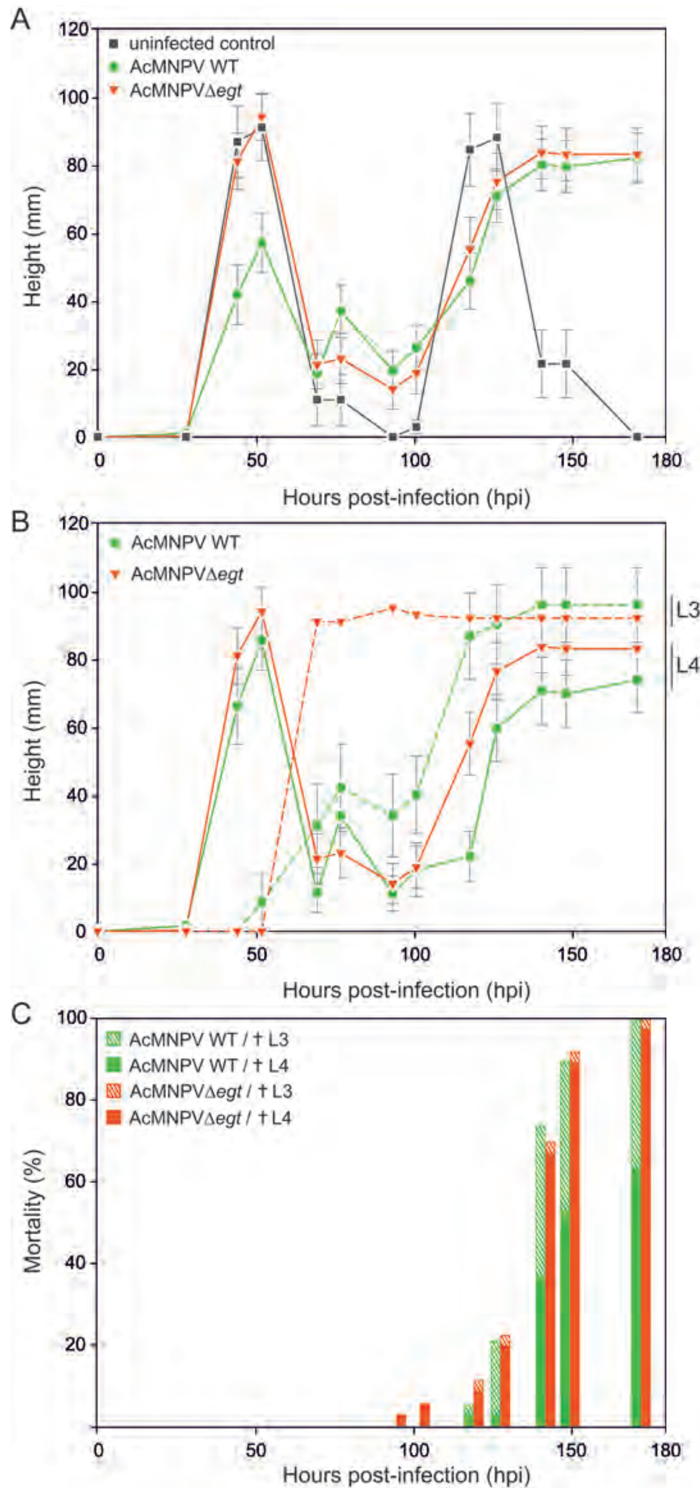
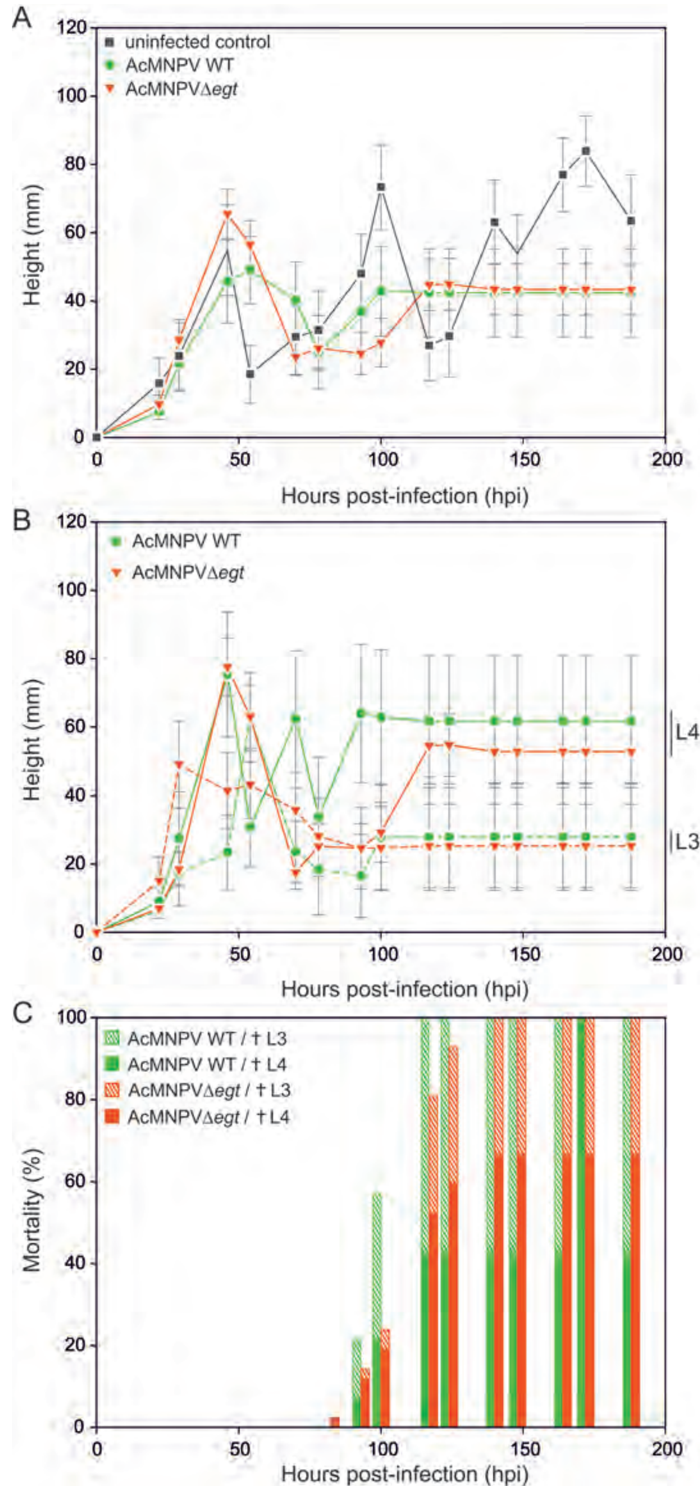


Figure 2. AcMNPV induces tree-top disease in *T. ni* larvae, but this is not affected by the *egt* gene. (A) Vertical position (mm) of mock-infected larvae (black squares, n=22), WT-infected larvae (green dots, n=38) and Δ*egt*-infected larvae (orange triangles, n=36) at different time points after infection (hours post-infection (hpi)). Error bars represent the standard error of the mean (SEM). (B) Vertical position (mm) of WT-infected larvae that died as 3rd instars (L3) (green dots, dotted line, n=14), WT-infected larvae that died as 4th instars (L4) (green dots, solid line, n=24), Δ*egt*-infected larvae that died as 3rd instars (L3) (orange triangles, dotted line, n=1) and Δ*egt*-infected larvae that died as 4th instars (L4) (orange triangles, solid line, n=35). Error bars represent SEM. (C) Cumulative mortality (%) of WT- and Δ*egt*-infected larvae at different hpi. Striped green bars represent WT-infected larvae that died as 3rd instars (†L3), solid green bars represent WT-infected larvae that died as 4th instars (†L4). Striped orange bars represent Δ*egt*-infected larvae that died as 3rd instars (†L3), solid orange bars represent Δ*egt*-infected larvae that died as 4th instars (†L4).

Figure 3. AcMNPV induces tree-top disease in *S. exigua* larvae that undergo a moult after infection, but this is not affected by the *egt* gene. (A) Vertical position (mm) of mock-infected larvae (black squares, n=16), WT-infected larvae (green dots, n=14) and Δ*egt*-infected larvae (orange triangles, n=42) at different time points after infection (hours post-infection (hpi)). Error bars represent the standard error of the mean (SEM). (B) Vertical position (mm) of WT-infected larvae that died as 3rd instars (L3) (green dots, dotted line, n=8), WT-infected larvae that died as 4th instars (L4) (green dots, solid line, n=6), Δ*egt*-infected larvae that died as 3rd instars (L3) (orange triangles, dotted line, n=14) and Δ*egt*-infected larvae that died as 4th instars (L4) (orange triangles, solid line, n=28). Error bars represent SEM. (C) Cumulative mortality (%) of WT- and Δ*egt*-infected larvae at different hpi. Striped green bars represent WT-infected larvae that died as 3rd instars (†L3), solid green bars represent WT-infected larvae that died as 4th instars (†L4). Striped orange bars represent Δ*egt*-infected larvae that died as 3rd instars (†L3), solid orange bars represent Δ*egt*-infected larvae that died as 4th instars (†L4).



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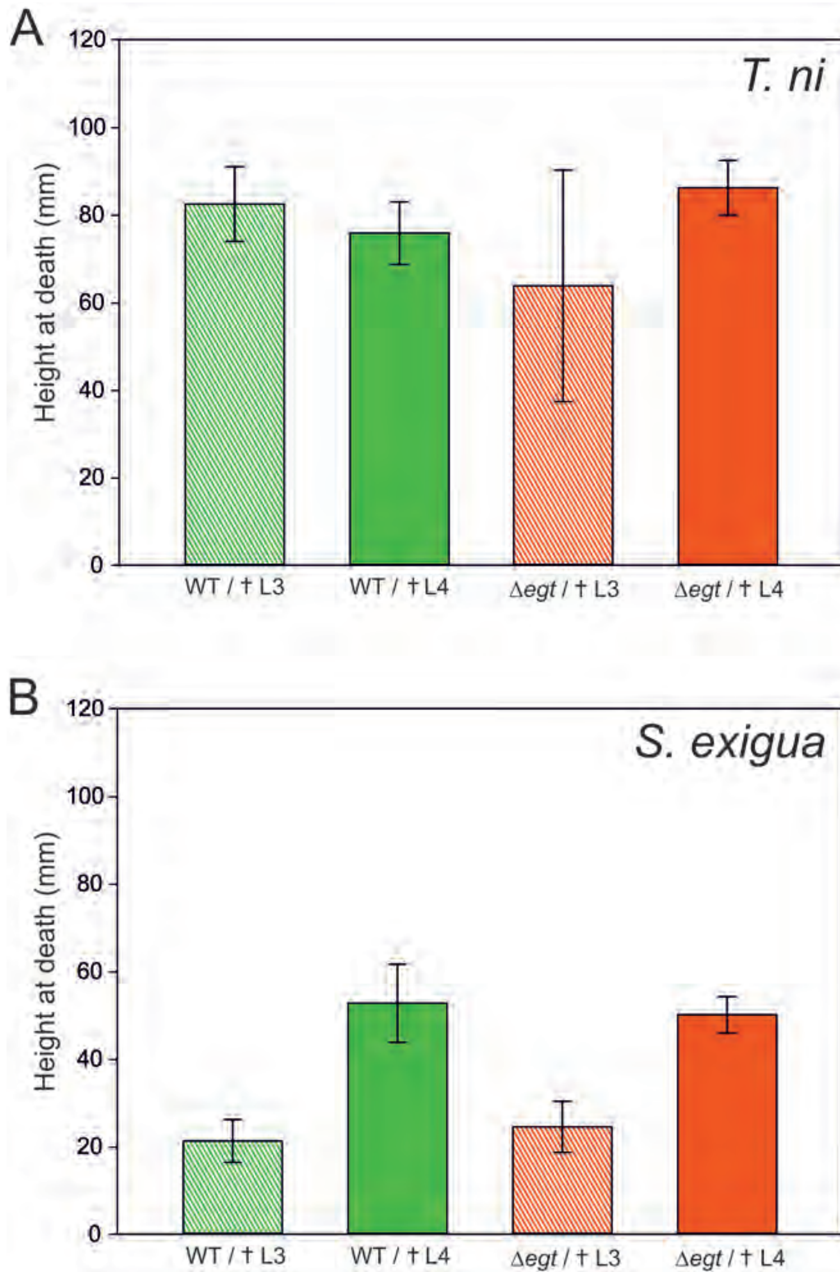


Figure 4. Position at death of AcMNPV-infected *T. ni* (A) and *S. exigua* (B) larvae. Average height at death (mm) of all replicates of WT- and Δ*egt*-infected larvae that died as 3rd instar or as 4th instar. Striped green bars represent WT-infected larvae that died as 3rd instars (n=32), solid green bars represent WT-infected larvae that died as 4th instars (n=46). Striped orange bars represent Δ*egt*-infected larvae that died as 3rd instars (n=5), solid orange bars represent Δ*egt*-infected larvae that died as 4th instars (n=53). Error bars represent SEM.

the 1st experiment (i.e. larvae died earlier in the 2nd experiment).

For *S. exigua*, an effect of treatment on the hazard rate was seen, but the stage of the larvae did not affect the hazard rate. However, the effect of treatment was opposite to that seen for *T. ni*: for *S. exigua* the hazard ratio between larvae infected with AcMNPV Δ*egt* was 0.58 compared to larvae infected with AcMNPV WT, implying that the average time to death was 1.72 times larger for Δ*egt*- than for WT-infected individuals. There was a difference between the experiments in the rate at which larvae died (with the shortest time to death for the 4th replicate (Fig. S4) and the longest for the 3rd replicate (Fig. S3), with the 1st and 2nd replicate (Fig. 3 and Fig. S2) being in between). Within each experiment, the same trend was seen (WT-infected larvae dying earlier than Δ*egt*-infected larvae).

AcMNPV induces tree-top disease in *T. ni*, but this is not affected by the *egt* gene

Our behavioural assays showed that AcMNPV induced tree-top disease in *T. ni* larvae: virus-infected larvae died at elevated positions (Fig. 2A, Fig. 4A and Fig. S1A). Surprisingly, the *egt* gene did not affect the position where the larvae died, as both WT and Δ*egt*-infected larvae died at elevated positions (T-test = 0.715; P = 0.48) (Fig. 2A, Fig. 4A and Fig. S1A). However, we did see an effect of the *egt* gene on pre-moult climbing behaviour. In our setup, *T. ni* larvae climbed prior to moulting, as can be seen in Fig. 2A and Fig. S1A. Mock-infected 3rd instars climbed to elevated positions during 28 to 52 hours post infection (hpi), where they moulted into 4th instars. As 4th instars the larvae returned to the food source at the bottom of the jar. The same pattern was seen when 4th instars moulted into 5th instars (climbing between 98-118 hpi), and when 5th instars started to pupate (climbing starting around 200 hpi) (Fig. 2A and Fig. S1A). Δ*egt*-infected 3rd instars also showed the first pre-moult climbing peak, since they (almost) all moulted into 4th instars, at the same time as the mock-infected larvae (Fig. 2B and Fig. S1B). For WT-infected larvae, however, a reduced pre-moult climbing peak was seen (Fig. 2A and Fig. S1A). This is due to the fact that *egt* expression resulted in suppression of moulting in a subset of WT-infected larvae. Those WT-infected larvae that did not moult into 4th instars (so stayed 3rd instars) did not show a climbing peak at two dpi (Fig. 2B and Fig. S1B). The same holds for one single Δ*egt*-infected larvae that did not moult into 4th instar (Fig. 2B). Those WT-infected larvae that did moult into 4th instars showed the same peak as mock-infected and Δ*egt*-infected larvae (Fig. 2B and Fig. S1B). All WT-infected larvae (those that did moult into 4th instars, and those that did not moult) died at elevated positions, indicating that tree-top disease and climbing prior to moulting are two distinct phenomena. No significant difference in position were the larvae died was found between larvae that died as 3rd or 4th instar (T-test = -0.327; P = 0.74), nor

between the two experiments (T-test = -0.359; P = 0.72).

Tree-top disease in *S. exigua* is moulting dependent, but is not affected by the *egt* gene

Upon infection with AcMNPV, a different behavioural pattern was seen for *S. exigua* larvae than for *T. ni* larvae. In *S. exigua*, the position where the larvae died was dependent on whether the larvae moulted into 4th instars or not. Those larvae that moulted into 4th instars died at elevated positions, while those that did not moult (stayed 3rd instars) died at low positions (T-test = 4.403; P < 0.001) (Fig. 3B, Fig. 4B, Fig. S2B, Fig. S3B and Fig. S4B). This pattern was the same for WT-infected and Δ*egt*-infected larvae, so also here the *egt* gene did not affect the position where the larvae died (T-test = 0.388; P = 0.70). The pre-moult climbing peaks as seen for *T. ni* were not as obvious in *S. exigua*, although some climbing prior to moulting could be observed between 42 and 54 hpi, when moulting from 3rd into 4th instar occurred (Fig. 3, Fig. S2, Fig. S3 and Fig. S4). No differences in position where the larvae died were observed among the different experiments (all three T-test values in absolute value < 1.47; P > 0.14 for all).

Discussion

Manipulation of host behaviour is a common strategy of parasites to enhance their transmission and the mechanisms underlying such manipulations are beginning to be unravelled. With the finding that the LdMNPV *egt* gene induces tree-top disease in *L. dispar* larvae, a 'gene for an extended phenotype' was identified, in this case a parasite gene of which the behavioural phenotype is expressed in its host (Hoover et al. 2011). The *egt* gene is present in the majority of lepidopteran baculoviruses and we investigated whether the baculoviral *egt* gene has a conserved role in tree-top disease. We showed that the baculovirus AcMNPV induced tree-top disease in both *T. ni* larvae and *S. exigua* larvae, although in *S. exigua* a moulting-dependent effect was seen (only those larvae that moulted into 4th instar after infection died at elevated positions). Surprisingly, infection with a mutant AcMNPV lacking the *egt* gene did not change the position were the larvae died, demonstrating that the AcMNPV *egt* gene does not have a crucial role in inducing tree-top disease in these two host species.

A possible explanation is that *egt* does not directly induce tree-top disease, but might exert an effect indirectly, through an effect on moulting and/or the time to death. *Egt* has been reported to suppress moulting of larvae, and to extend the time to death (Cory et al. 2004; O'Reilly & Miller 1991; Wilson et al. 2000). Prior to moulting, larvae stop

feeding, so it was hypothesized that suppression of moulting and extending the time to death ensures prolonged feeding, resulting in bigger larvae and a larger viral OB yield (O'Reilly 1999; Slavicek et al. 1999). In larvae, moulting is often preceded by behavioural changes. *Lymantria dispar* larvae normally reside up in the tree to feed and move downwards to moult. When infected with the LdMNPV WT virus, the moulting is suppressed and larvae continue feeding up in the tree, while larvae infected with the LdMNPV Δ*egt* virus stop feeding and crawl down the tree to moult (Hoover et al. 2011). If the moment of death of the larvae coincides with the onset of moulting, or occurs just after moulting, LdMNPV Δ*egt*-infected larvae might be found dead at the place they (intended to) moult (at low positions), while the LdMNPV WT-infected larvae died at high positions. In this case, the effect of *egt* on tree-top disease is in fact a consequence of its inhibitory effect on moulting and therefore also on moulting-related behaviour. In our experiments concerning AcMNPV in *T. ni* and *S. exigua*, the death of *T. ni* as well as *S. exigua* larvae occurred at least 25 (*T. ni*) to 35 (*S. exigua*) hours after the time moulting occurred (although not all larvae moult, see below). *Trichoplusia ni* larvae, and to a lesser extent *S. exigua* larvae, climbed to elevated positions to moult, after which they moved down again. From 25 (*T. ni*) to 35 (*S. exigua*) hours after moulting they climb up again to die at elevated positions. The *egt* gene has no effect on this climbing prior to death (WT- as well as Δ*egt*-infected larvae died at elevated positions), although it does affect the climbing seen prior to moulting from 3rd to 4th instar, especially in *T. ni* larvae. Larvae in which moulting is suppressed by *egt* do not climb upwards prior to moulting. So, while AcMNPV *egt* affects moulting and moulting-related climbing behaviour in *T. ni* and *S. exigua* larvae, it does not affect tree-top disease, which occurs much later in time.

Another striking observation was that in *S. exigua*, tree-top disease appeared to be moulting dependent: those larvae that moulted into 4th instar died at elevated positions, while those that did not moult (died as 3rd instar) died at low positions. Why this movement prior to death is dependent on the stage of the larvae needs further investigation. Although moulting-dependent tree-top disease has not been reported before, downward movement prior to death has been observed for other baculovirus-host interactions. Larvae of *Operophtera brumata* infected with *O. brumata* NPV (OpbuNPV) moved downwards on trees prior to death, where OBs were shown to persist better than on foliage (Raymond et al. 2005). In this case, the tree stems might serve as an important between-year reservoir of the viral OBs.

In our experiments, the effect of *egt* expression on moulting in *T. ni* as well as *S. exigua* was partial. The majority of AcMNPV Δ*egt*-infected 3rd instars of *T. ni* and *S. exigua* moulted into 4th instars. In AcMNPV WT-infected *T. ni* and *S. exigua* larvae, moulting was suppressed in part of the larvae. Such partial suppression was observed in other

studies as well (Bianchi et al. 2000; O'Reilly et al. 1998). Possibly, EGT must accumulate to some threshold level within the infected insect to successfully prevent moulting (O'Reilly 1995). Furthermore, the timing of EGT accumulation in relation to the ecdysteroid peak in the larvae might be important (O'Reilly et al. 1998). These aspects are influenced by the viral dose used, and the developmental stage of the host (timing of infection) (Cory et al. 2004; O'Reilly et al. 1998).

In the present study, AcMNPV *egt* was found to extend the time to death for *T. ni* larvae, while it reduced the time to death for *S. exigua* larvae. When comparing studies performed on the influence of the *egt* gene on the time to death, outcomes seem to depend on several factors, including the virus-host interaction studied, the timing of infection, the viral dose used and the larval stage studied (Cory et al. 2001). For example, Bianchi et al. (2000) found no difference in time to death between AcMNPV WT- and AcMNPV Δ*egt*-infected 2nd and 4th instars of *S. exigua*, while Cory et al. (2004) found that the time to death between AcMNPV WT- and AcMNPV Δ*egt*-infected *T. ni* larvae differed for all instars, with large differences in some instars, to hardly any difference in other instars. The observation that *egt* shortens the time to death, like was seen for *S. exigua* larvae in this study, has not been reported before.

In conclusion, our study demonstrates that there is no conserved role for the baculovirus *egt* gene in inducing tree-top disease, since in two different host species tree-top disease was induced by AcMNPV irrespective of the presence of the *egt* gene. However, *egt* did affect pre-moult climbing behaviour, which may be a clue to *egt*'s effect on tree-top disease in other virus-host systems, including LdMNPV-infected *L. dispar* larvae (as was outlined above). Likely, a different viral gene is inducing the climbing prior to death (tree-top disease), at least in the host-virus interactions investigated in this study. The outcome of the virus-host interaction and the effect the virus has on the behaviour of the host is a consequence of a long-term arms race between the virus and their host. Baculoviruses have co-evolved with their lepidopteran hosts for over 300 million years (Thézé et al. 2011). In different baculovirus-host interactions the outcome of this arms race might be different, and is likely the result of a complex interplay between the virus and host studied, depending on many factors including the intrinsic behaviour of the host species, the host developmental stage, the viral dose and the timing of infection.

Acknowledgements

We thank Laura de Hoon for her assistance with generating recombinant bacmids. Els Roode is thanked for her help with cell culture and rearing of larvae, and Kelli Hoover for setting up the behavioural assays for the climbing experiments. Thanks to David Cohen for developing the pCRE-ts plasmid. We thank Just Vlak for reviewing the manuscript. SVH and VIDR are both supported by the Program Strategic Alliances of the Royal Dutch Academy of Sciences (project 08-PSA-BD-01) and VIDR is supported by a VENI grant of the Netherlands Organisation for Scientific Research (project 863.11.017).

Supplementary figures

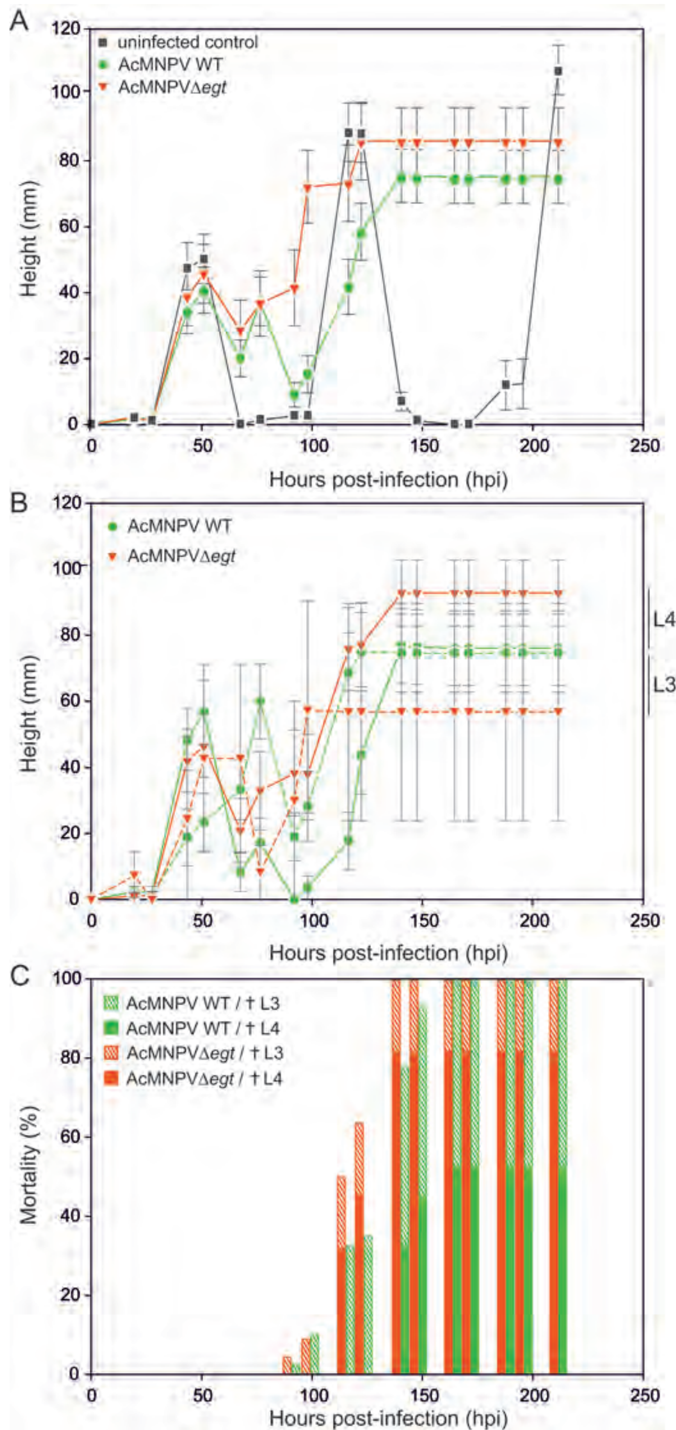
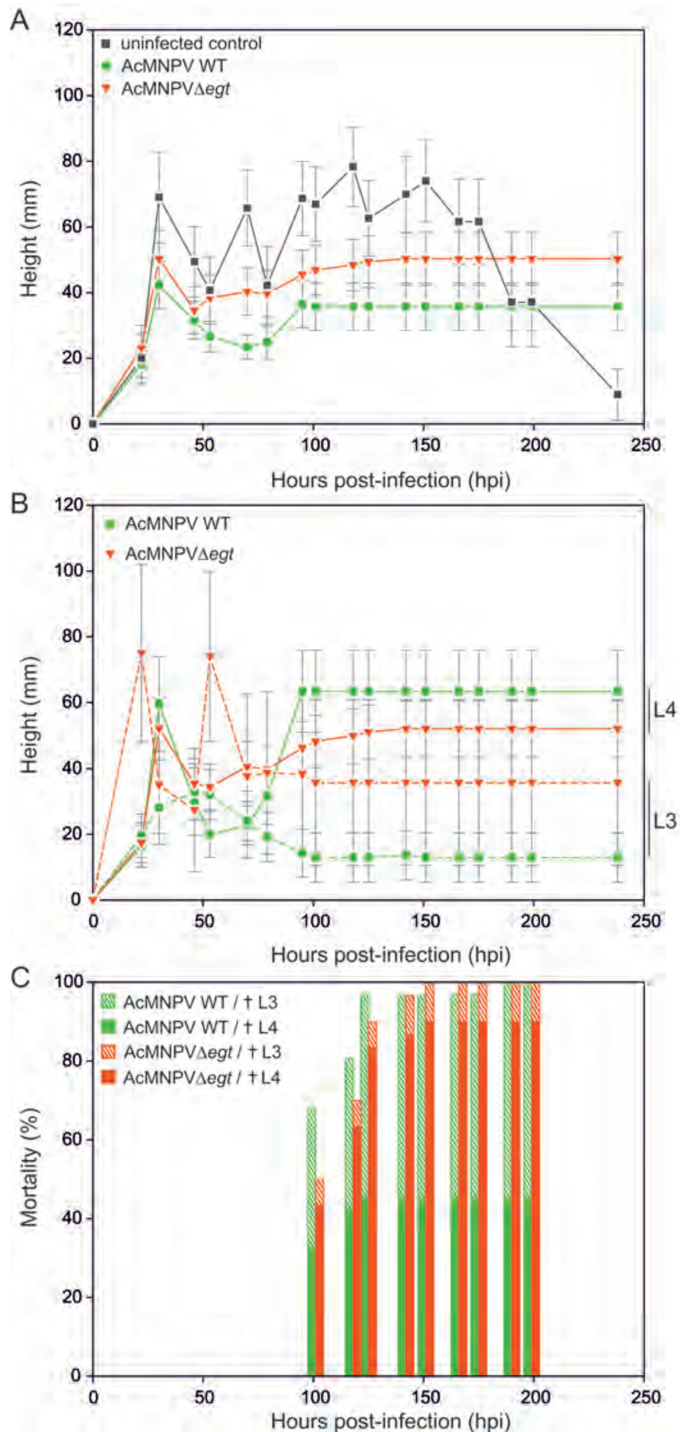


Figure S1. Repetition of the experiment presented in Figure 2. AcMNPV induces tree-top disease in *T. ni* larvae, but the *egt* gene does not affect the position where the larvae die. (A) Vertical position (mm) of mock-infected larvae (black squares, n=22), WT-infected larvae (green dots, n=40) and Δ*egt*-infected larvae (orange triangles, n=22) at different time points after infection (hours post-infection (hpi)). Error bars represent the standard error of the mean (SEM). (B) Vertical position (mm) of WT-infected larvae that died as 3rd instars (L3) (green dots, dotted line, n=19), WT-infected larvae that died as 4th instars (L4) (green dots, solid line, n=21), Δ*egt*-infected larvae that died as 3rd instars (L3) (orange triangles, dotted line, n=4) and Δ*egt*-infected larvae that died as 4th instars (L4) (orange triangles, solid line, n=18). Error bars represent SEM. (C) Cumulative mortality (%) of WT- and Δ*egt*-infected larvae at different hpi. Striped green bars represent WT-infected larvae that died as 3rd instars (†L3), solid green bars represent WT-infected larvae that died as 4th instars (†L4). Striped orange bars represent Δ*egt*-infected larvae that died as 3rd instars (†L3), solid orange bars represent Δ*egt*-infected larvae that died as 4th instars (†L4).

Figure S2. Repetition 1 of the experiment presented in Figure 3. AcMNPV induces tree-top disease in *S. exigua* larvae that undergo a moult after infection, but the *egt* gene does not affect the position where the larvae die. (A) Vertical position (mm) of mock-infected larvae (black squares, n=13), WT-infected larvae (green dots, n=41) and Δ*egt*-infected larvae (orange triangles, n=30) at different time points after infection (hours post-infection (hpi)). Error bars represent the standard error of the mean (SEM). (B) Vertical position (mm) of WT-infected larvae that died as 3rd instars (L3) (green dots, dotted line, n=17), WT-infected larvae that died as 4th instars (L4) (green dots, solid line, n=14), Δ*egt*-infected larvae that died as 3rd instars (L3) (orange triangles, dotted line, n=3) and Δ*egt*-infected larvae that died as 4th instars (L4) (orange triangles, solid line, n=27). Error bars represent SEM. (C) Cumulative mortality (%) of WT- and Δ*egt*-infected larvae at different hpi. Striped green bars represent WT-infected larvae that died as 3rd instars (†L3), solid green bars represent WT-infected larvae that died as 4th instars (†L4). Striped orange bars represent Δ*egt*-infected larvae that died as 3rd instars (†L3), solid orange bars represent Δ*egt*-infected larvae that died as 4th instars (†L4).



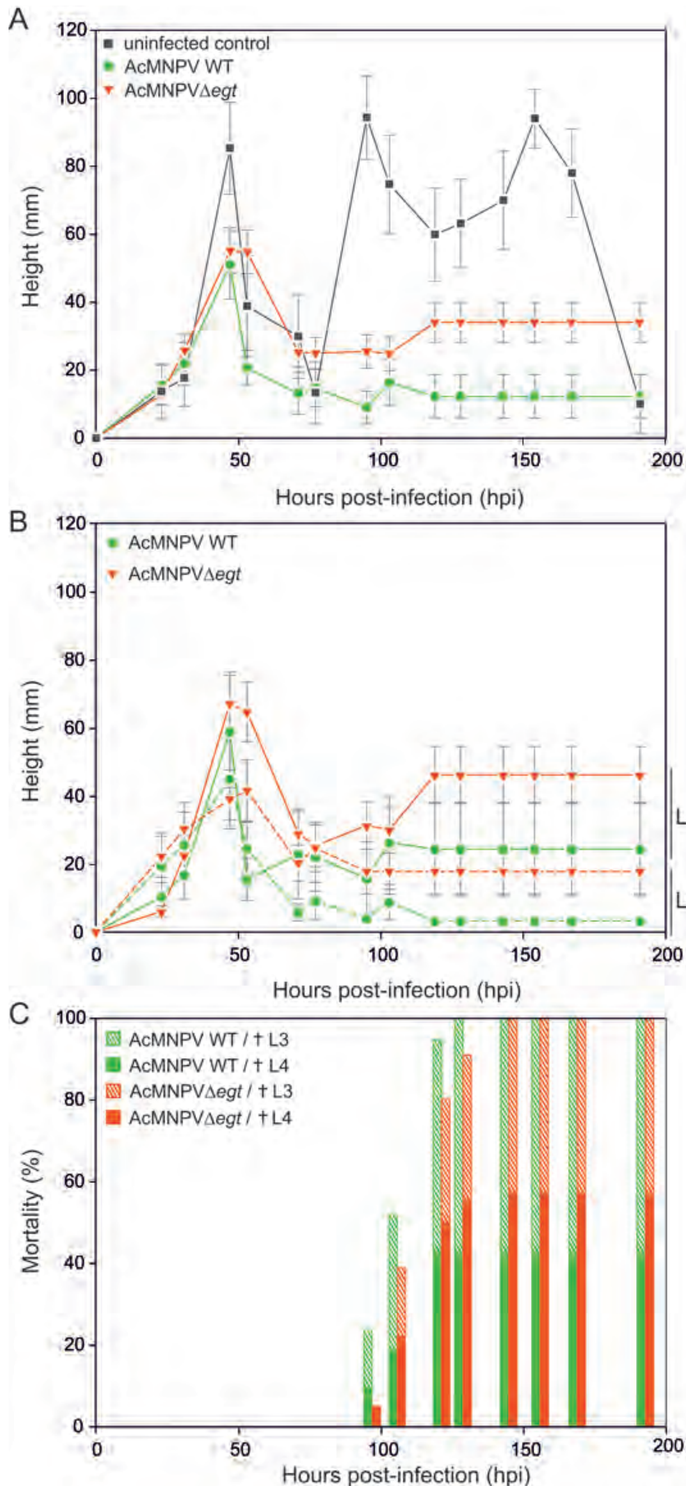
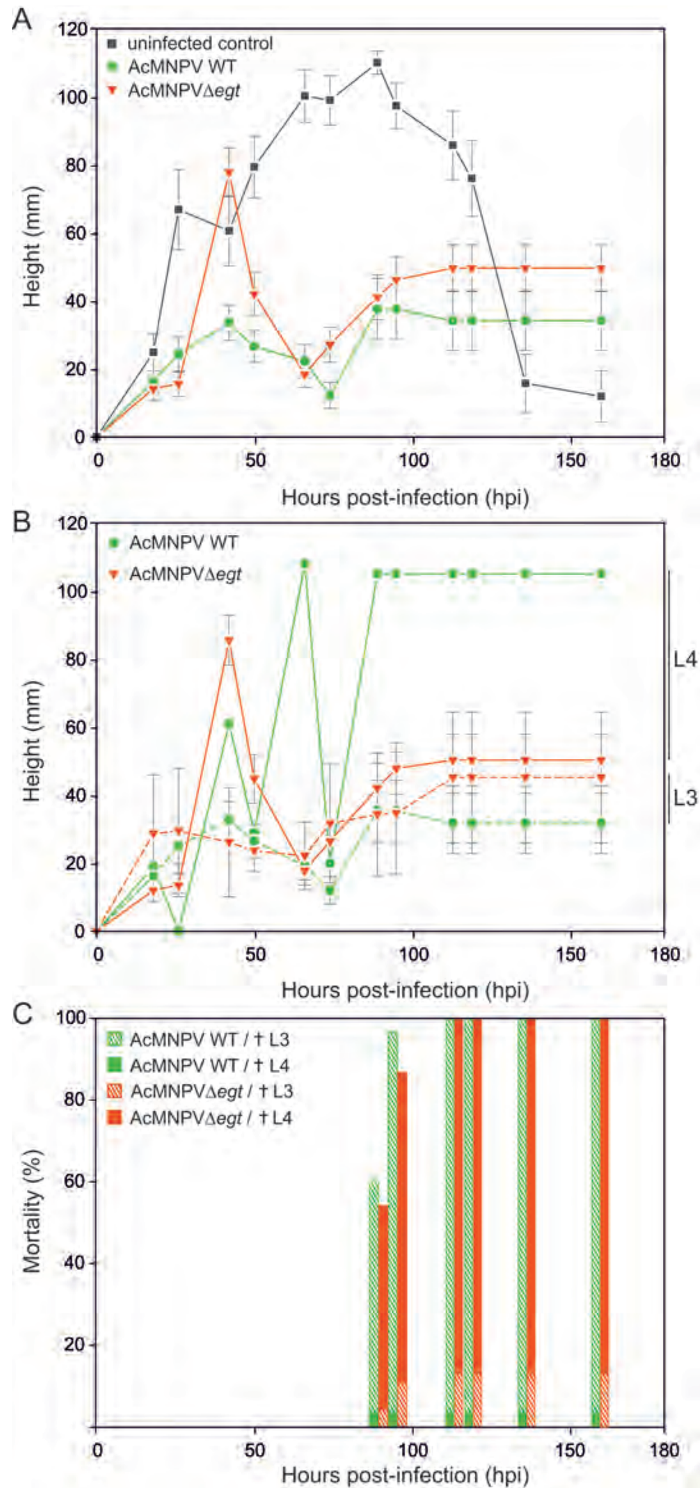


Figure S3. Repetition 2 of the experiment presented in Figure 3. AcMNPV induces tree-top disease in *S. exigua* larvae that undergo a moult after infection, but the *egt* gene does not affect the position where the larvae die. (A) Vertical position (mm) of mock-infected larvae (black squares, n=12), WT-infected larvae (green dots, n=21) and Δ*egt*-infected larvae (orange triangles, n=59) at different time points after infection (hours post-infection (hpi)). Error bars represent the standard error of the mean (SEM). (B) Vertical position (mm) of WT-infected larvae that died as 3rd instars (L3) (green dots, dotted line, n=12), WT-infected larvae that died as 4th instars (L4) (green dots, solid line, n=9), Δ*egt*-infected larvae that died as 3rd instars (L3) (orange triangles, dotted line, n=25) and Δ*egt*-infected larvae that died as 4th instars (L4) (orange triangles, solid line, n=34). Error bars represent SEM. (C) Cumulative mortality (%) of WT- and Δ*egt*-infected larvae at different hpi. Striped green bars represent WT-infected larvae that died as 3rd instars (†L3), solid green bars represent WT-infected larvae that died as 4th instars (†L4). Striped orange bars represent Δ*egt*-infected larvae that died as 3rd instars (†L3), solid orange bars represent Δ*egt*-infected larvae that died as 4th instars (†L4).

Figure S4. Repetition 3 of the experiment presented in Figure 3. AcMNPV induces tree-top disease in *S. exigua* larvae that undergo a moult after infection, but the *egt* gene does not affect the position where the larvae die. (A) Vertical position (mm) of mock-infected larvae (black squares, n=18), WT-infected larvae (green dots, n=30) and Δ*egt*-infected larvae (orange triangles, n=46) at different time points after infection (hours post-infection (hpi)). Error bars represent the standard error of the mean (SEM). (B) Vertical position (mm) of WT-infected larvae that died as 3rd instars (L3) (green dots, dotted line, n=29), WT-infected larvae that died as 4th instars (L4) (green dots, solid line, n=1), Δ*egt*-infected larvae that died as 3rd instars (L3) (orange triangles, dotted line, n=6) and Δ*egt*-infected larvae that died as 4th instars (L4) (orange triangles, solid line, n=40). Error bars represent SEM. (C) Cumulative mortality (%) of WT- and Δ*egt*-infected larvae at different hpi. Striped green bars represent WT-infected larvae that died as 3rd instars (†L3), solid green bars represent WT-infected larvae that died as 4th instars (†L4). Striped orange bars represent Δ*egt*-infected larvae that died as 3rd instars (†L3), solid orange bars represent Δ*egt*-infected larvae that died as 4th instars (†L4).



Chapter

8

Baculovirus triggers a positive phototactic response to induce tree-top disease in caterpillars

Adapted from:

Stineke van Houte, Vera I.D. Ros, Just M. Vlak and Monique M. van Oers. Baculovirus triggers a positive phototactic response to induce tree-top disease in caterpillars, *manuscript in preparation*.

Abstract

Many parasites manipulate host behaviour to enhance their own transmission and survival. An exquisite example is the case of baculoviruses, which induce death at elevated positions of caterpillar hosts ('tree-top disease'). To date, little is known about the underlying processes leading to tree-top disease. Here we show that the baculovirus *Spodoptera exigua* multiple nucleopolyhedrovirus (SeMNPV) triggers a positive phototactic response in *S. exigua* larvae prior to death. We also show that movement of uninfected caterpillars during larval development is light-independent. We hypothesize that SeMNPV hijacks a host pathway involved in phototaxis and/or light perception to induce this remarkable behavioural change.

Introduction

Animals experience continuous invasions by parasites and are abused for replication and spread of the invaders. To enhance transmission, many parasites alter the behaviour of their hosts. The field of parasitic manipulation of host behaviour has attracted much attention in the last decade. Notable examples include alteration of mosquito blood feeding behaviour by malaria parasites (Koella et al. 1998) and suicidal behaviour of crickets infected with hairworms (Thomas et al. 2002). Baculoviruses are also known to alter the behaviour of their caterpillar hosts (Goulson 1997; Hoover et al. 2011; Kamita et al. 2005; van Houte et al. 2012). The first report on atypical behaviour displayed by baculovirus-infected caterpillars dates from 1891, when Hofmann describes larvae that had died from an unknown cause and were hanging upside down in the plant canopy (Hofmann 1891). He named this phenomenon `Wipfelkrankheit´, today more commonly known as 'tree-top disease'. Later studies showed that baculovirus-infected caterpillars migrate to the top of the plant prior to death (Evans 1986; Goulson 1997; Smirnoff 1965). The liquefaction of the larvae at these elevated positions probably ensures optimal dissemination of progeny virions on lower foliage, where consumption by caterpillar hosts may occur.

Although this remarkable change in host behaviour has been known for a long time, the underlying mechanisms have remained largely obscure. A recent study (Hoover et al. 2011) demonstrated that in *Lymantria dispar* caterpillars infected with the baculovirus *L. dispar* multiple nucleopolyhedrovirus (LdMNPV) a viral gene, the ecdysteroid UDP-glucosyl transferase gene (*egt*), is responsible for tree-top disease. However, this function of *egt* is not conserved among baculoviruses, as *egt* does not play a role in tree-top disease induced by *Autographa californica* MNPV (AcMNPV) (Chapter 7), indicating that other baculovirus genes may be involved in this behavioural change. Regardless of the nature of the viral gene inducing tree-top disease, it is unknown which host response(s) is/are triggered by the virus. Theoretically, baculoviruses induce tree-top disease by triggering a response related to geotaxis or phototaxis, or a combination thereof.

In this chapter the hypothesis was tested that tree-top disease results from a phototactic response. This was done by investigating the effect of light on tree-top disease induced by the baculovirus *Spodoptera exigua* multiple nucleopolyhedrovirus (SeMNPV) in its single host, the beet armyworm *S. exigua*. To this aim behavioural assays were performed under different light/dark conditions. We conclude that tree-top disease in infected caterpillars is caused by a strong attraction to light prior to death.

Materials and methods

Insect larvae and virus

Spodoptera exigua larvae were reared on artificial diet at 27 ºC with 50% relative humidity as described before (Smits et al. 1986) and a 14 h light/10 h dark photoperiod (14:10 LD) (7 am lights on, 9 pm lights off). A stock of wild type (WT) SeMNPV viral occlusion bodies (OBs) of the G25 genotype (Murillo et al. 2006) was kindly provided by Amaya Serrano, University of Pamplona, Spain. OBs were amplified once in *S. exigua* 3rd instars to obtain more virus. OBs were purified from infected larvae by grinding deceased larvae in water and filtering through a double layer of cheese cloth. The suspension was first centrifuged at 500x*g*, after which the supernatant was centrifuged at 4000x*g* to pellet the OBs. Finally, OBs were resuspended in water and stored at 4 ºC.

Behavioural assays under normal light/dark conditions (14:10 LD)

Newly moulted 3rd instar *S. exigua* larvae (moulted and starved overnight for 16 h) were infected with an LC_{90} dose (10^5 OBs/ml) of virus using droplet feeding with a 10% sucrose solution containing 0.4% (w/v) Patent Blue V food colouring as described before (van Houte et al. 2012). Subsequently, virus-infected larvae were placed individually in glass jars (120 mm tall x 71 mm wide), which were closed with a metal lid containing small holes to allow ventilation. Jars were lined with sterile mesh wire to facilitate climbing and contained a piece of artificial diet (approximately 3.5 cm^3) at the bottom. The jars were then incubated in a climate chamber at 27 ºC with 50% relative humidity. A 14:10 LD (7 am lights on, 9 pm lights off, as during rearing) was applied using three luminescent tube lamps of 18 Watts each, with a 30 cm distance between the luminescent tubes and the top of the jars. The vertical position of the larvae was monitored twice per day, starting from one day post-infection (dpi) until all larvae were either dead or had pupated. Larvae that did not die due to virus infection (died of other causes or survived despite being droplet fed with virus) were excluded from analyses. The assay was performed twice.

Behavioural assays under dark conditions or using light from a single direction

For behavioural assays under dark conditions a 0 h light/24 h dark photoperiod (0:24 LD) was applied. In behavioural assays in which light was applied only from above, a 14:10 LD photoperiod was applied as described above, but jar walls were protected from light using aluminium foil. In this case jars were covered with a piece of transparent plastic Saran wrap, in which three holes were made to allow ventilation. The jars were

then placed in a black box to prevent any light from other directions than above. In behavioural assays in which light was applied only from below, a 14:10 LD photoperiod was applied as described above with a 30 cm distance between the luminescent tubes and the bottom of the jars. Jar walls were protected from light using aluminium foil, and jars were closed with metal lids containing small holes to allow ventilation. A black box was placed over the jars to prevent any light from other directions than below.

For those three behavioural assays (dark, light from above, light from below) the height at death was recorded at 4 dpi. Larvae that did not die due to virus infection (died of other causes or survived despite being droplet fed with virus) were excluded from the analyses. Each assay was performed twice.

Behavioural assays using uninfected larvae

Newly moulted 3rd instar *S. exigua* larvae (moulted and starved overnight for 16 h) were droplet fed with a virus-free 10% sucrose solution containing 0.4% (w/v) Patent Blue V food colouring dye as described in van Houte et al. (2012). Larvae were placed in glass jars as described above, and jars were incubated either under normal light/dark conditions (14:10 LD) or completely dark conditions (0:24 LD). The vertical position of the larvae was monitored twice per day until all larvae had pupated. To avoid the exposure of larvae to normal light during the measurements, the position of the larvae kept under dark conditions was measured using a 20 Watts red light bulb.

Statistical analysis

The height at death of virus-infected larvae kept under different light conditions was analysed for significant differences using a Kruskal-Wallis non-parametric test (SPSS). Bonferroni correction was applied to adjust for multiple comparisons.

Results

Behavioural assays were performed to investigate the effect of light on tree-top disease induced by SeMNPV infection in larvae of its single host *S. exigua* larvae. As it was unknown whether tree-top disease is observed at all during SeMNPV infection, we first performed a behavioural assay to investigate whether SeMNPV causes this behavioural change in its single host *S. exigua*. To this aim, 3rd instar *S. exigua* larvae were infected with an LC_{90} dose of the SeMNPV wild type (WT) baculovirus and behavioural assays were performed under normal light/dark conditions (14:10 LD). Virus-infected larvae did not show any significant vertical movement until 76 h after infection (Fig. 1). From

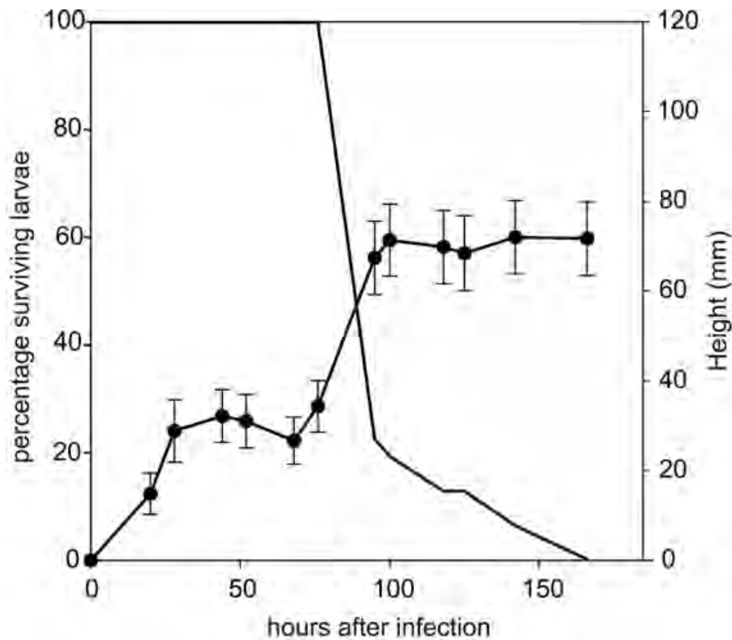


Figure 1. SeMNPV infection in *S. exigua* larvae induces tree-top disease under normal light/dark conditions (14:10 LD). The black line represents the survival rate (% survival, left y-axis) of infected larvae. The line with closed circles represents the average height (mm, right y-axis) of infected larvae (n=31) at different hours after infection. Error bars represent the standard error of the mean (SEM).

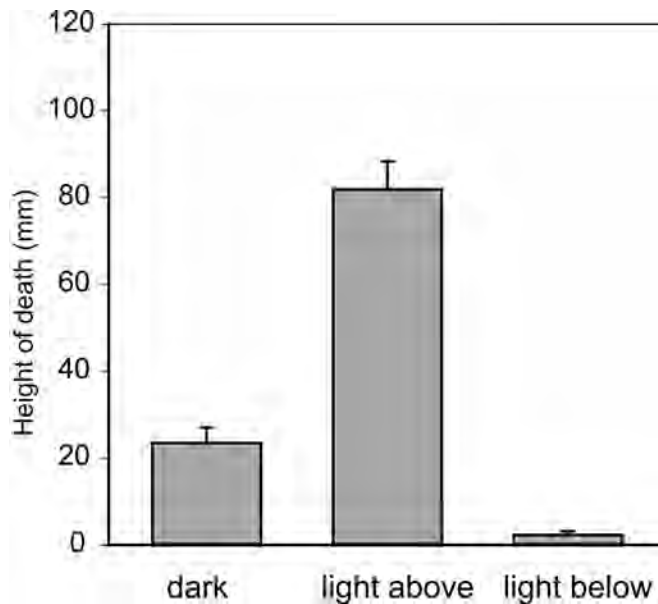


Figure 2. Tree-top disease in SeMNPV-infected larvae is the result of a positive phototactic response. Average height at death (mm) of larvae kept under complete dark conditions (dark; n=123); normal light conditions (14:10 LD) with light from above only (light above; n=62); normal light conditions (14:10 LD) with light from below only (light below; n=62). Error bars represent the SEM.

this time point onwards diseased larvae rapidly ascended (35 mm at 76 h after infection; 69 mm at 90 h after infection) (Fig. 1). Shortly after these larvae ascended, the majority succumbed to the virus infection (100% survival at 76 h after infection; 20% survival at 90 h after infection). Thus, these larvae showed a strong climbing response in the last

hours prior to death, resulting in tree-top disease.

We hypothesized that SeMNPV hijacks host signalling pathways leading to a response related to phototaxis. Hence we determined whether light was an important factor in inducing the strong climbing response prior to death. To this end, the average height at death of infected larvae that were exposed to different light conditions was measured. Interestingly, these experiments showed that infected larvae, which were exposed to complete darkness (0:24 LD), died at low positions (23 ± 3.6 mm) (Fig. 2), indicating that light was required for death of infected larvae at elevated positions.

Next we investigated whether the presence of light *sensu strictu* was sufficient to evoke climbing behaviour of the host or whether the infected larvae had become positive phototactic. To this end, infected larvae were exposed to a light source from a single direction. Infected larvae exposed to light only from above (14:10 LD) died at very high positions (82 ± 6.5 mm) (Fig. 2), which was significantly different from larvae that were kept under dark conditions ($P<0.0001$). In contrast, when exposed to light only from below (14:10 LD), virus-infected larvae died at extremely low positions (2.3 ± 0.9 mm) (Fig. 2), which was significantly lower than larvae kept in the dark ($P=0.019$) and larvae exposed to light from above ($P<0.0001$). These data showed that SeMNPV induces a strong positive phototactic response in infected caterpillars.

To understand whether this response was specifically evoked by baculovirus infection, or whether larvae were also positive phototactic in the absence of virus infection, behavioural assays were performed using uninfected 3rd instar larvae. These larvae showed several climbing peaks that coincided with larval moults during their development from 3rd instar to pupae (Fig. 3A). Climbing prior to moulting is known to occur in *S. exigua* larvae, although this response is much more prominent in other lepidopteran species, such as *Trichoplusia ni* (Chapters 6 and 7). A relatively low climbing peak at 28 h after droplet feeding coincided with the larval moult from the 3rd to the 4th instar (Fig. 3A). A higher climbing peak at 99 h after droplet feeding coincided with the moult from the 4th to the 5th instar. Prior to pupation, healthy larvae descended to pupate in the diet plug (Fig. 3A).

In contrast to the climbing prior to death observed in baculovirus-infected caterpillars, the climbing peaks related to moulting were light-independent. Under completely dark conditions (0:24 LD) these larvae showed a remarkably similar climbing pattern as under normal light/dark conditions (14:10 LD), with climbing peaks that coincide with moulting to the next instar (moult to 4th instar at 24 h after droplet feeding; moult to 5th instar at 73 h after droplet feeding) (Fig. 3B). This indicates that climbing related to moulting

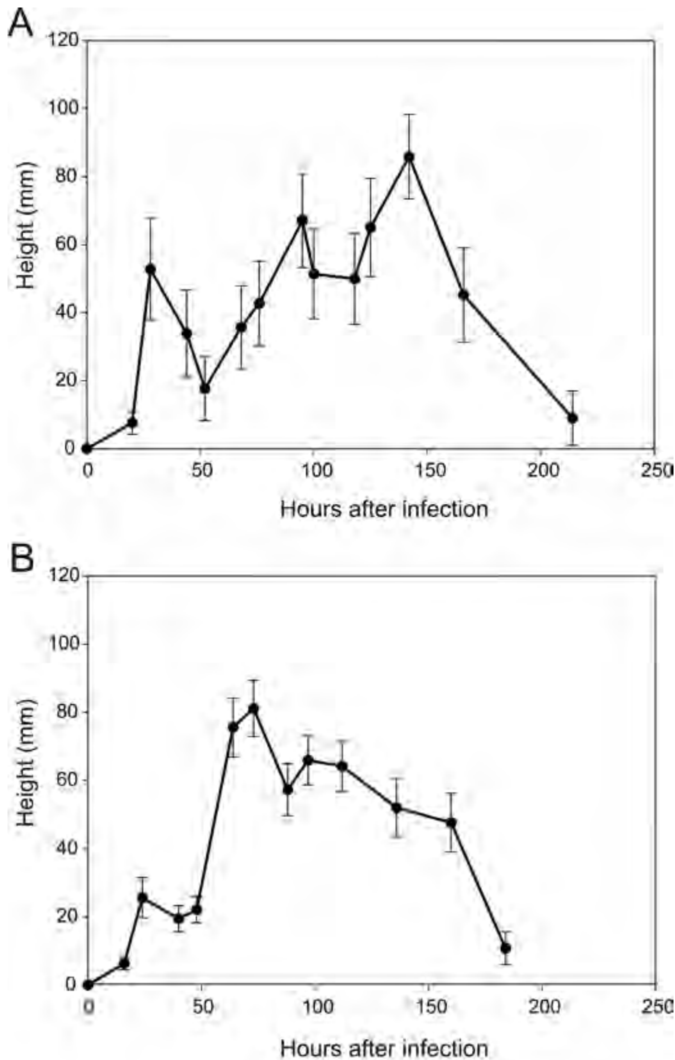


Figure 3. Climbing behaviour of uninfected control larvae is not affected in the absence of light. (A) Height (mm) of control larvae (n=23) at different hours after infection during normal light/dark conditions (14:10 LD). (B) Height (mm) of control larvae (n=29) at different hours after infection during completely dark conditions (14:10 LD). Error bars represent the SEM.

observed in uninfected caterpillars and the positive phototaxis prior to death in infected caterpillars are unrelated processes.

Discussion

Although several studies have investigated baculovirus-induced locomotion and tree-top disease of infected caterpillars (Goulson 1997; Hofmann 1891; Kamita et al. 2005; van Houte et al. 2012), the proximate mechanisms have remained largely elusive. In this paper we demonstrate that SeMNPV-induced tree-top disease in infected caterpillars is the result of an altered response to light. As the climbing behaviour of uninfected (3rd and 4th instar) larvae is light-independent, we conclude that this positive phototactic response is specifically triggered during virus infection. This observation is in accordance with a study on innate phototactic behaviour in *S. exigua* (Griswold & Trumble 1985), which reported that 3rd and 4th instar larvae respond neutrally to light. Interestingly, the latter study also showed that 1st and 2nd instar larvae were positively phototactic and 5th instars showed strong negative phototaxis. The observation that uninfected 3rd and 4th instar larvae climb even in the absence of light may suggest that this moulting-related climbing behaviour is

the result of a response related to geotaxis (movement in response to gravity). It would be of interest to study the effect of SeMNPV on the mobility of 1st, 2nd and 5th instar larvae under these conditions.

A recent study by Hoover et al. (2011) identified a gene from LdMNPV that induces tree-top disease in the caterpillar *L. dispar.* This gene (*egt*) is present in all sequenced baculovirus genomes, except one clade of granuloviruses (Chapter 7), and encodes ecdysteroid UDP-glucosyl transferase, an enzyme that inactivates host moulting hormones (O'Reilly & Miller 1989). However, this behavioural effect of *egt* from LdMNPV appears not to be conserved among all baculoviruses, as tree-top disease induced by the baculovirus *Autographa californica* MNPV (AcMNPV) in *S. exigua* and *T. ni* larvae was shown to be independent of *egt* (Chapter 7). Whether *egt* plays a role in tree-top disease in SeMNPV-infected larvae remains to be determined.

To understand more about the mechanism used by SeMNPV to induce positive phototaxis, we may learn from other parasite-host combinations. Manipulation of host climbing behaviour occurs in a variety of parasite-host systems and several parasites are known to induce phototactic responses in their hosts (reviewed in Adamo (2012; van Houte et al. (2013)). For example, positive phototaxis is seen in gammarids (freshwater crustaceans) infected with trophically transmitted parasitic worms (Bethel & Holmes 1977) and in crickets infected with Gordian worms (Ponton et al. 2011). Even though these two parasite-host systems are phylogenetically unrelated, similar proteins involved in light perception are differentially expressed during parasitic manipulation of the host (Biron et al. 2006; Ponton et al. 2006). This may suggest evolutionary convergence on both a physiological and molecular level.

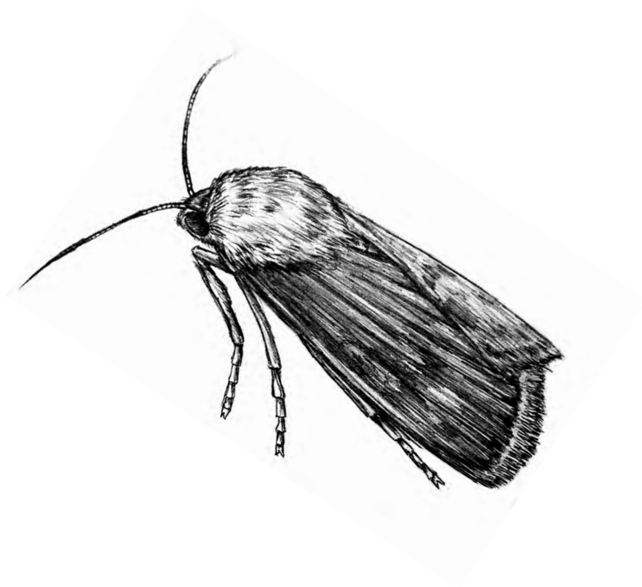
Likewise, such mechanistic parallels underlying positive phototaxis may exist between these systems and baculovirus-infected caterpillars. Presumably, host genes or proteins related to innate phototactic behaviour and/or light perception are targeted (Kain et al. (2012); Yamanaka et al. (2013) and reviewed in Keene & Sprecher (2012)). Several candidate genes are worth investigating in more detail for a possible role in SeMNPV-induced phototaxis. For example, two genes (*tim* and *per*), encoding components of the circadian clock, are important for phototactic behaviour in *D. melanogaster* larvae (Gong 2009; Keene & Sprecher 2012; Mazzoni et al. 2005). The neuropeptide pigment-dispersing factor (PDF), important in the maintenance of circadian behavioural rhythms (Gong et al. 2010; Keene & Sprecher 2012) and geotaxis (Toma et al. 2002), is also thought to be important for phototaxis in *D. melanogaster* larvae (Gong et al. 2010). Furthermore, prothoracicotropic hormone (PTTH), a brain hormone that controls the moulting process, was recently shown to be required for regulating phototactic beha-

viour in this species (Yamanaka et al. 2013). Finally, the neurotransmitter serotonin is implicated in phototactic behaviour in *D. melanogaster* (Rodriguez Moncalvo & Campos 2009), in the honey bee *Apis mellifera* (Thamm et al. 2010) and in gammarids infected with parasitic worms.

Overall, we conclude that tree-top disease in baculovirus-infected caterpillars is the result of a positive phototactic response prior to death, and that this response is specifically triggered during virus infection.

Acknowledgements

Han Yue is acknowledged for experimental contributions. Amaya Serrano, University of Pamplona, Spain, is acknowledged for kindly providing the SeMNPV G25 virus stock. SVH and VIDR are both supported by the Program Strategic Alliances of the Royal Dutch Academy of Sciences (project 08-PSA-BD-01) and VIDR is supported by a VENI grant of the Netherlands Organisation for Scientific Research (project 863.11.017).



Chapter

9

General discussion

Behavioural manipulation of organisms by parasites is a widespread phenomenon that has important ecological and evolutionary consequences for both the host and the parasite. The research described in this thesis aims to provide answers to several key questions on the underlying mechanism of behavioural manipulation of insects by baculoviruses. This chapter discusses the answers that this thesis provides to these questions and some directions for future research that, in my opinion, are worth exploring. As was stated in the introduction, this thesis studies mechanism(s) of behavioural manipulation of caterpillar hosts by baculoviruses and focuses on three main topics: i) the molecular mechanism that underlies hyperactive behaviour induced by baculovirus protein tyrosine phosphatase (PTP); ii) understanding whether inducing hyperactivity and inducing death at elevated positions (tree-top disease) are independent strategies that are regulated by distinct virus genes; and iii) the mechanisms that underlie tree-top disease in lepidopteran hosts leading to death at elevated positions. What do we now understand about each of these topics, and what are the remaining gaps in our knowledge?

The molecular mechanism of baculovirus *ptp*-induced hyperactive behaviour

As compared to the relatively detailed descriptions in literature on aberrant climbing behaviour of baculovirus-infected caterpillars, hyperactivity induced by baculoviruses is somewhat less well documented. This is probably related to the fact that hyperactivity is less visible in the field, while liquefied caterpillars hanging on the upper leaves or in the top of plants or trees ('Wipfelkrankheit') easily catch the observer's eye. Chapters 3 and 4 of this thesis describe investigations on the induction of hyperactive behaviour by *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) in *Spodoptera exigua* caterpillars, the role of the viral *ptp* gene therein, and the identity of potential (host) targets of the PTP enzyme.

AcMNPV induces hyperactive behaviour in S. exigua larvae, and presence of the viral *ptp* gene is required for this behavioural change (Chapter 3). Baculovirus PTP has now been implicated in hyperactive behaviour in two different baculovirus-host associations (*Bombyx mori* nucleopolyhedrovirus (BmNPV) – *B. mori* and AcMNPV – *S. exigua*), suggesting that it may play an evolutionarily conserved role in manipulation of locomotion behaviour by baculoviruses (Chapter 3). However, the exact mechanism by which *ptp* seems to exert its function in larval hyperactivity appears to be different between these two systems. In BmNPV-infected *B. mori* larvae the phosphatase activity of the encoded PTP enzyme is not required for behavioural manipulation (Katsuma et al. 2012). As deletion of the BmNPV *ptp* gene was shown to affect budded virus (BV) production

in many different tissues including the larval brain, it was hypothesized that BmNPV PTP may exert its behavioural function as a structural protein (see below) rather than as an enzyme. How this decrease in virus production in the brain could cause hyperactive behaviour in *B. mori* is unknown. In contrast, in AcMNPV-infected *S. exigua* larvae the PTP phosphatase activity is required for induction of hyperactivity providing strong evidence that a host or viral gene or protein is targeted by viral PTP to cause this behavioural change. In AcMNPV, PTP is also a virion protein that is known to associate with both BVs and occlusion-derived viruses (ODVs) (Li & Miller 1995a; Wang et al. 2010). However, no indications were found that deletion of *ptp* had an effect on virus production in AcMNPV-infected *S. exigua* larvae, although it cannot be excluded that this may be overlooked in our experiments. Therefore, it is questionable whether there is a single mechanism by which baculovirus *ptp* may induce hyperactivity.

The phosphatase activity of the viral PTP is important for this behavioural change of *S. exigua* larvae (Chapter 3). What are the intermediate steps between PTP enzymatic activity and the actual onset of hyperactivity during baculovirus infection? To address this question, two (related) factors are of importance: i) the location in the organism where PTP might exert its function in behaviour; ii) the host gene(s) and/or protein(s) that are targeted by viral PTP to induce hyperactivity. The latter issue is addressed in Chapter 4, and will be discussed below. The former issue has not been the main focus of this thesis, but will also be briefly discussed.

Taking into account that locomotion behaviour is primarily controlled by the central nervous system (CNS) (Strauss 2002), any alteration of this behaviour is likely to have its basis in the CNS. Several studies show that during baculovirus infection both budded viruses (BVs) and occlusion bodies (OBs) can be detected in nerve tissue, including the brain, of infected larvae (Herz et al. 2003; Katsuma et al. 2012; Knebel-Morsdorf et al. 1996; Torquato et al. 2006). As the PTP enzyme is a virion protein (see above), this may imply that PTP itself is present in the CNS. Connecting brain-specific PTP expression to behavioural manipulation is challenging, as it may require disentanglement of the role of PTP as a virion protein and as a behavioural determinant, since these two are not necessarily related. Many parasite taxa that invade host brain tissue are thought to specifically do so to efficiently manipulate behaviour (Biron & Loxdale 2013) (see Chapter 2 for some examples). Future research is needed to find out whether the presence of AcMNPV PTP in the brain is required for hyperactive behaviour in *S. exigua* larvae.

On the other hand, PTP may alter behaviour from a distance by inducing (a) pathway(s) outside the CNS. This phenomenon, manipulating behaviour via an indirect effect on the CNS, is thought to occur in many parasite-host associations where physical contact

between the parasite and the CNS appears to be absent (Adamo 2013; Biron & Loxdale 2013). For example, PTP may exert its effect via another viral protein which could subsequently target a host gene or protein to alter behaviour. In this context, the identification of six viral proteins that co-purify with PTP (Chapter 4) is of interest, although the exact nature and biological significance of their putative interaction with PTP requires further analysis. Alternatively, PTP could affect the production of a hormone in a tissue other than the CNS. This hormone could then travel to the CNS and induce downstream pathways there.

Tissues other than the CNS that are particularly interesting to study in this context are the prothoracic glands (PGs). These are endocrine glands that regulate insect moulting, and serve as the primary source of the moulting hormone ecdysone (E) during larval stages. This hormone is subsequently converted to its active form, 20-hydroxyecdysone (20E) (Marchal et al. 2010). PGs were shown to become strongly activated during baculovirus infection, resulting in high levels of ecdysteroids in the haemolymph (Park et al. 1996). Interestingly, bracoviruses also disturb PG functioning, presumably through the effect of bracovirus-encoded PTP enzymes (see Chapter 2) (Beckage & Gelman 2004; Falabella et al. 2006; Pennacchio et al. 2001). This PTP-mediated disruption of PGs is thought to act through dephosphorylation of key proteins upstream of a signalling cascade that regulates ecdysteroidogenesis (Falabella et al. 2006). Although baculovirus PTP and PTP2 are phylogenetically unrelated to bracovirus PTPs (Provost et al. 2004), they may have a similar effect on PG functioning. Whether baculovirus replication takes place in these glands is not entirely clear; virions were detected in the PGs of granulovirus-infected *Trichoplusia ni* larvae (Dougherty et al. 1987), but not in PGs of *Lymantria dispar* (Ld) MNPV-infected gypsy moth larvae (Park et al. 1996). Interesting in this context is that the baculoviruses EGT enzyme, encoding ecdysteroid UDP-glucosyl transferase (see below), blocks ecdysteroidogenesis by inhibiting host moulting hormones (O'Reilly & Miller 1989).

The second issue of importance in understanding the mechanism of PTP-induced hyperactivity is the identification of potential (host) target gene(s) and/or protein(s). A substrate analysis was performed to identify possible host targets of PTP in cell culture, which is described in Chapter 4. In total six viral and six host proteins were found to co-purify with PTP. Some of these viral proteins may associate with PTP, as they are also virion proteins (PCNA, sulfhydryl oxidase and VP91 (P95)). Viral protein kinase 1 (PK1) also co-purified with PTP. An interesting hypothesis that requires further testing is that PTP may form a complex with PK1. Such a complex may for example be important in regulating the phosphorylation status of certain target proteins. Furthermore, a viral inhibitor of apoptosis and a host caspase enzyme both co-purified with PTP, indicating a

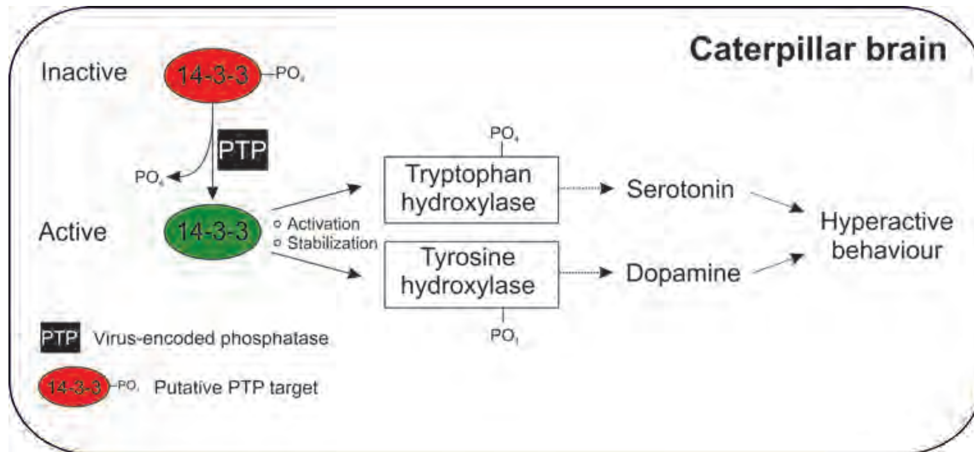


Figure 1. Model of the putative interaction of AcMNPV PTP protein with 14-3-3 ζ that finally leads to hyperactive behaviour PTP dephosphorylates 14-3-3 ζ in an infected caterpillar. Subsequently dephosphorylated 14-3-3 ζ binds to phosphorylated tyrosine hydroxylase and/or tryptophan hydroxylase, which activates and stabilizes these enzymes (Banik et al. 1997). This in turn causes an increase of dopamine and/or serotonin synthesis in neuronal cells. Higher levels of these neurotransmitters then lead to the induction of hyperactive behaviour in caterpillars.

possible connection of PTP with apoptosis, although PTP did not show a pro-apoptotic effect upon transient expression in insect cells (Chapter 5).

Amongst the other host proteins that co-purified with the AcMNPV PTP protein, several are expressed in invertebrate brain tissue. These include arginine kinase, subunits of two serine/threonine phosphatases (PP1 and PP2A) and the 14-3-3 ε and ζ isoforms. Additional RNA expression analysis demonstrated a relation between the levels of AcMNPV *ptp* and *14-3-3 ζ* expression in infected *S. exigua* larvae. 14-3-3 proteins are broadly involved in cell signalling, intracellular trafficking and regulation of cell cycle progression (Aitken 2006). They mediate interactions between a wide variety of target proteins, which are based on the phosphorylation status of target proteins and on phosphorylation of 14-3-3 itself (Aitken 2006). Phosphorylation of 14-3-3 is generally assumed to prevent interaction of 14-3-3 with its target proteins (Aitken 2006). A direct role for 14-3-3 in behaviour was shown in mice, where 14-3-3 deficiency caused hyperactivity and a reduced ability to learn and memorize (Cheah et al. 2012). Studies in *Drosophila* also demonstrated an important function for 14-3-3 ζ in learning and memory formation (Skoulakis & Davis 1996). Originally, 14-3-3 was described as activator protein of the enzyme tryptophan hydroxylase (also named tryptophan-5-monooxygenase) and tyrosine hydroxylase (also named tyrosine-3-monooxygenase) (Ichimura et al. 1988; Ichimura et al. 1987). These are rate-limiting enzymes in the synthesis of the neurotrans-

mitters serotonin and dopamine, respectively. These neurotransmitters both play a role in locomotion activity in invertebrates and vertebrates (Akasaka et al. 2010; Friggi-Grelin et al. 2003; Kamyshev et al. 1983; Mignon & Wolf 2002; Pendleton et al. 2002; Segalat et al. 1995) and are associated with parasitic manipulation of host behaviour in a variety of parasite-host systems, as outlined in Chapters 2 and 4. In vertebrates, tryptophan hydroxylase and tyrosine hydroxylase are both bound by 14-3-3 protein when they are phosphorylated (Banik et al. 1997). Binding of 14-3-3 to these phosphorylated enzymes has two effects: (i) it increases their hydroxylase activity, and (ii) it prevents their dephosphorylation by cellular phosphatases (Banik et al. 1997).

Based on the finding in this thesis that viral PTP and host 14-3-3 may physically interact, a model is proposed on the mechanism that baculoviruses use to induce hyperactive behaviour (Fig. 1). We hypothesize that PTP interacts with 14-3-3 ζ in the baculovirus-infected larva, possibly leading to dephosphorylation of 14-3-3 ζ. This in turn allows 14-3-3 to bind to and thereby activate tryptophan hydroxylase and/or tyrosine hydroxylase. These steps may occur in the brain, as 14-3-3 ζ and both hydroxylases are all known to be highly expressed in this tissue (Aitken 2006; Monastirioti 1999). Subsequently serotonin and/or dopamine synthesis and release is increased in interneuronal cells (Meneses & Perez-Garcia 2007; Monastirioti 1999; Ogren et al. 2008). Consequently, these neurotransmitters bind to and activate G-protein coupled receptors on the cell membranes of effector neurons that innervate different tissues, including organs, muscles and hormonal glands (Meneses & Perez-Garcia 2007). The *ptp*-dependent RNA expression levels of 14-3-3 ζ may indicate a positive feedback loop, whereby activation of 14-3-3 ζ by PTP leads to increased *14-3-3 ζ* expression levels. However, such a positive transcriptional feedback system has not been described for 14-3-3 and this hypothesis thus requires further analysis.

Alternatively, PTP may form a complex with 14-3-3 ζ and (some of) the other co-purified host or viral proteins. Of particular interest are the host phosphatases PP1 and PP2A. The latter enzyme was previously shown to form a complex with 14-3-3 and several other proteins during polyoma virus infection in vertebrate cells (Mullane et al. 1998). Both PP1 and PP2A have been described as important phosphatases regulating the activity of the transcription factor cAMP-responsive element binding protein (CREB) (Benito & Barco 2010; Lonze & Ginty 2002). In vertebrates, CREB is known to regulate transcription of certain isoforms of 14-3-3 (Lonze & Ginty 2002). Whether the same effect may occur in insects is unknown to date. If this would be the case, it may imply that the observed effect of PTP on 14-3-3 ζ expression is mediated by PP1 and/or PP2A with CREB as an intermediate step. However, CREB itself was not identified in this study as a potentially interacting protein of PTP.

Direct evidence that the interaction between PTP and 14-3-3 is a relevant step for inducing hyperactivity is lacking. This would be a highly challenging but crucial step towards drawing a more detailed picture of how PTP alters behaviour. For example, RNAi-mediated gene silencing on *14-3-3* (or one of the other candidate proteins identified in Chapter 4) in whole larvae could provide valuable information on whether down-regulated expression of a candidate gene alters locomotion behaviour. However, successful RNAi knockdown in lepidopteran insects is often difficult to achieve (Terenius et al. 2011), and efficient knockdown of *14-3-3* is likely lethal due to its functional plethora. The role of neurotransmitters such as serotonin or dopamine in PTP-induced hyperactivity could be investigated by injecting these molecules, or their inhibitors or activators, in baculovirus-infected caterpillars. This method has been successfully applied in several studies on the behavioural consequences of neurotransmitters in insects (Akasaka et al. 2010; Fussnecker et al. 2006; Tain et al. 2006). In addition, levels of serotonin and dopamine in the brain could be measured using techniques such as immunohistochemistry or high-performance liquid chromatography (HPLC) (Akasaka et al. 2010; Blenau & Thamm 2011). In this way neurotransmitter levels of larvae infected with wild type AcMNPV or a *ptp* deletion mutant can be compared. Alternatively, a genome-wide transcriptomic and/or proteomic approach could provide information on host genes and/or proteins that are differentially expressed in larvae during infection with either wild type AcMNPV or an AcMNPV *ptp* deletion mutant, respectively. In addition, profiling and comparing the phosphoproteome of brains of larvae infected with wild type AcMNPV or *ptp*-deleted AcMNPV likely aids in the identification of the host and viral proteins dephosphorylated by PTP.

Besides the brain as tissue of interest, the aforementioned PGs are also essential in the functioning of several (neuro-)hormones and neurotransmitters, which often play important roles in behaviour as outlined in Chapter 2. For example, the neuronal signalling molecule nitric oxide (NO) is produced in the PGs (Caceres et al. 2011), where it interacts with several receptor molecules. Disruption of these interactions in the PGs causes aberrant locomotion behaviour. NO production is dependent on the activity of the aforementioned arginine kinase, which was shown to interact with baculovirus PTP in cell culture (Chapter 4). This suggests a possible link between viral PTP and NO signalling. Furthermore, adipokinetic hormone (AKH), a protein potentially involved in behavioural manipulation (see Chapter 2), is produced in neuronal cells in the CNS. These neurons innervate the PGs of larvae (Lee & Park 2004), which may be important in the role of AKH in locomotion behaviour.

What about the induction of hyperactive behaviour by group II NPVs, none of which carries a homolog of the *ptp* gene? Hyperactivity was not consistently observed in larvae

of the gypsy moth *L. dispar* infected with the group II NPV LdMNPV (K. Hoover, personal communication). However, larvae of the cabbage moth *Mamestra brassicae* infected with the group II NPV *M. brassicae* (Mb) MNPV were found to disperse over a larger area than uninfected ones (Goulson 1997; Vasconcelos et al. 1996). This implies that at least some group II NPVs induce hyperactivity. Given the absence of the *ptp* gene in all group II NPVs, hyperactivity is likely induced by (a) different unknown viral gene (s). Possibly, the *ptp2* gene, which also encodes a protein tyrosine phosphatase but is phylogenetically unrelated to *ptp*, plays a role in hyperactive behaviour in some group II NPVs. The *ptp2* gene is present in several group II NPVs (Chapter 3), including MbMNPV and SeMNPV, but not LdMNPV. Several group I NPVs also carry *ptp2*, but these do not include AcMNPV and BmNPV. Furthermore, two granuloviruses (GVs) carry *ptp2*. Chapter 5 describes the functional characterization of the SeMNPV *ptp2* gene. These results indicate that *ptp2* induces mild apoptosis in cultured cells, which may be related to its putative activity as MAPK phosphatase. Furthermore, similar to AcMNPV PTP, the SeMNPV PTP2 protein interacts with 14-3-3 ε and ζ and with arginine kinase, which may indicate (partial) functional overlap between the two proteins. In this thesis the function of SeMNPV *ptp2* in behaviour was not assessed and remains to be determined, e.g. using a *ptp2* deletion mutant of SeMNPV. Whether *ptp2* plays a role in hyperactivity in *M. brassicae* larvae infected with MbMNPV, as described above, also requires further research.

Hyperactivity and climbing behaviour are independent strategies governed by distinct virus genes

Hyperactivity and aberrant climbing leading to death at elevated positions (tree-top disease) both occur during baculovirus infection in lepidopteran caterpillars, but whether these behavioural phenotypes have a common genetic basis was unclear. The combined results from the Chapters 3 and 6 of this thesis show that a single virus can induce both hyperactivity and aberrant climbing, but (at least for AcMNPV in *S. exigua*) these two strategies have a distinct mechanistic basis. Hyperactivity and tree-top disease occur in different phases of the infection process; while for AcMNPV infection in *S. exigua* larvae hyperactivity is observed at 3 days post-infection (corresponding to about 70 hpi), the climbing behaviour that leads to tree-top disease commonly occurs later, starting from about 75 hpi. Presumably, these two behaviours are induced at different time points during the infection. In the case of AcMNPV, *ptp* is responsible for hyperactivity, while the viral gene underlying tree-top disease is currently unknown for AcMNPV. As discussed above, hyperactivity is hypothesized to be induced through the possible effect of PTP on neurotransmitter production and/or release through several intermediate steps. However,

tree-top disease may also be induced via interference with the host serotonergic or dopaminergic system, as will be discussed in more detail below. This would suggest that PTP and this unknown gene involved in tree-top disease target the same pathway in the host, although the behavioural outcome is different.

The question is whether hyperactivity and tree-top disease are also independently induced in other baculovirus-host systems. Apart from AcMNPV, the only group I NPV for which behavioural studies have been performed is BmNPV, but this study was limited to the induction of hyperactivity (Kamita et al. 2005; Katsuma et al. 2012). As discussed above, two studies on MbMNPV (a group II NPV) infection in *M. brassicae* larvae demonstrated that infected larvae had higher dispersal rates than uninfected larvae, and that they died more often on exposed parts of the plants than on lower or more concealed parts of the plant (Goulson 1997; Vasconcelos et al. 1996). This indicates that also this virus affects both hyperactivity and climbing behaviour. However, the genetic basis of this induction has not been studied for this virus.

Mechanisms underlying virus-induced climbing behaviour in lepidopteran hosts

'Tree-top disease' or 'Wipfelkrankheit', climbing behaviour induced by baculovirus infection leading to death at elevated positions, has been described in literature for the first time in 1891 in larvae of the nun moth *Lymantria monacha* (Hofmann 1891). AcMNPV infection had an effect on tree-top disease in the two lepidopteran hosts *T. ni* and *S. exigua*, (Chapter 7), but these effects were clearly host-dependent. In 3rd instar *T. ni*, virus infection always caused the larvae to climb up and die at elevated positions, regardless of the larval stage at death. For *S. exigua* however, only larvae that had moulted during the infection (from 3rd to 4th instar) climbed up and died at elevated positions. Those that did not undergo moulting moved downwards and died at low positions. Furthermore, even though the moulted *S. exigua* larvae died at elevated positions as compared to the unmoulted ones, they still died at much lower positions than *T. ni* larvae. While AcMNPV induces tree-top disease in both host species, its net effect on the height at death is clearly different. This difference might be related to the normal climbing behaviour that these two species display even in the absence of virus infection. In our setup, uninfected *T. ni* larvae showed a very strong climbing behaviour related to moulting, while in *S. exigua* this behaviour is less prominent. It may be expected that a manipulative parasite would make use of an already existing behavioural pathway in its host, rather than creating a completely new one. A possible way for AcMNPV to induce tree-top disease could be by interfering with the existing host pathway underlying moulting-related climbing. For

example, it might affect the same genes or proteins that are downstream targets of host moulting hormones in uninfected larvae. However, the absence of an effect of AcMNPV ecdysteroid UDP-glucosyl transferase (EGT) (discussed in more detail below) in tree-top disease makes this hypothesis less likely, given that EGT can efficiently inactivate host moulting hormones. It is possible that AcMNPV has evolved alternative ways of interfering with host moulting-related climbing behaviour independent of EGT. Interference with the functioning of the aforementioned prothoracic glands (PGs) could be one way of achieving this.

For *S. exigua*, AcMNPV-infected larvae that moulted died at approximately 2.5 times higher positions than larvae that did not moult. A similar difference in height at death was also observed when infecting 2nd instar *S. exigua* larvae instead of 3rd instars; larvae that died as 2nd instar died at low positions, while larvae that died as 3rd instar died at elevated positions (S. van Houte, unpublished). This may suggest that the behavioural difference is related to the moulting process itself rather than to physiological differences between the 3rd and 4th instar. Further research is needed to understand the nature of this difference.

What do we know about AcMNPV gene(s) involved in tree-top disease? The requirement of *egt* to induce death at elevated positions in *L. dispar* infected with LdMNPV (Hoover et al. 2011) rendered this gene a strong candidate to test in manipulation of climbing behaviour by other baculoviruses. In AcMNPV-infected *T. ni* and *S. exigua* hosts no effect of *eg*t was found on the height at death (Chapter 7). In addition, an opposite effect of *egt* was found on the time to death in the two host species; in *T. ni* larvae *egt* shortened time to death, while for *S. exigua egt* had a prolonging effect on the time to death. The latter finding contrasts with previous studies that reported a shorter life span for *T. ni* and *S. exigua* larvae infected with *egt* deletion mutant strains of AcMNPV (although this effect was highly stage-dependent) (Cory et al. 2001; Knebel-Morsdorf et al. 1996; Wilson et al. 2000), but is in accordance with another study that did not find an effect on time to death for *egt* during AcMNPV infection in *S. exigua* larvae (Bianchi et al. 2000). It appears that the effect of *egt* on time to death is dependent on many factors, including the developmental stage of the larva at the time of infection, the time between last moult and infection, and the ingested viral dose (Cory et al. 2004; Cory et al. 2001).

The role of light in tree-top disease

In Chapter 8 the effect of SeMNPV infection on tree-top disease of *S. exigua* caterpillars was investigated. While infected larvae did not display any significant climbing

response in the first two days after infection, they climbed upwards in the last 16 hours before they died, causing the larvae to die at elevated positions. These data indicate that tree-top disease is induced by both AcMNPV and SeMNPV, and thus appears to be a conserved strategy among baculoviruses. The question is whether this behavioural change is conserved on the mechanistic level as well. If different baculoviruses utilize distinct mechanisms, this may imply that inducing tree-top disease has evolved within the family *Baculoviridae* multiple times. As such it may be regarded as a case of convergent evolution, although strictly speaking this term is only applicable to phylogenetically unrelated taxa (Poulin 1998).

During SeMNPV infection in *S. exigua* infected larvae became strongly attracted to light in the last 16 hours before they died resulting in death at elevated positions (Chapter 8). In the absence of light, all infected larvae died at very low positions in the jar. This strong attraction to light was likely a consequence of virus infection, as uninfected larvae were not only found at low positions in the absence of light. Thus, SeMNPV presumably manipulates climbing behaviour by (somehow) altering the host's response to light. Preliminary data from AcMNPV infection in *T. ni* demonstrated that in this case light was not important for death at elevated positions (V. Ros, unpublished data). This difference may be due to distinct mechanisms that AcMNPV and SeMNPV use to induce tree-top disease, or may reflect host-specific differences. Future research aimed at finding out whether AcMNPV-induced tree-top disease in *S. exigua* is also dependent on the presence of light should provide more insight in this observation.

Whether the *egt* gene has any effect on tree-top disease in SeMNPV-infected *S. exigua* larvae remains to be determined. If it has an effect, it is possible that *egt* directly interferes with the host response to light. Another viral gene worth to be investigated for its putative involvement in tree-top disease is the *ptp2* gene, as discussed in Chapter 5.

Which host pathway(s) might possibly be hijacked by SeMNPV to induce phototaxis? Studies in *Drosophila* larvae demonstrated that visual behaviour, i.e. positive and negative phototaxis, is tightly linked to regulation of the circadian clock (Keene & Sprecher 2012). Flies carrying mutations in the clock genes *tim* or *per* display a strong reduction in light avoidance (Gong 2009; Mazzoni et al. 2005). A recent study identified two brain neurons that regulate the behavioural switch from a negative phototactic to a strong positive phototactic response in *Drosophila* larvae (Gong et al. 2010). Activity of these neurons is possibly regulated by pacemaker neurons that express pigment-dispersing factor (PDF), which is important in the maintenance of circadian behavioural rhythms (Gong et al. 2010; Keene & Sprecher 2012) (see Chapter 2). A recent study demonstrated that these two neurons are prothoracicotropic hormone (PTTH)-producing

cells (Yamanaka et al. 2013). Downregulation of the *ptth* gene or the gene encoding the PTTH receptor (*torso*) both impair light avoidance, demonstrating that the PTTH/Torso signalling pathway is required for regulating light preference in *D. melanogaster* larvae (Yamanaka et al. 2013). In addition, the serotonergic system appears to play an important role in the behavioural response to light (Rodriguez Moncalvo & Campos 2009; Thamm et al. 2010), which is in agreement with the finding that serotonin is involved in the positive phototaxis of gammarids infected with acanthocephalan worms, as described in Chapters 2 and 4.

Some baculoviruses may interfere with the host circadian rhythm through the expression of a photoreactivation (*phr*) gene. Although these genes are commonly involved in DNA repair, a *phr* gene present in the tomato looper *Chrysodeixis chalcites* (Chch) NPV was recently shown to interact with the murine molecular clock (Biernat et al. 2012). Whether this interaction also occurs in the insect host and has any behavioural effect remains to be established, but baculoviruses that carry a copy of this gene might interfere with host behaviour through manipulation of the host circadian rhythm (Biernat et al. 2011). So far, *phr* genes have been identified in ChchNPV (van Oers et al. 2005), in *T. ni* SNPV (TnSNPV) (Willis et al. 2005) and in five additional group II NPVs infecting insects from the lepidopteran subfamily Plusiinae (Xu et al. 2008), and in the granulovirus *Spodoptera litura* GV (SpliGV) (Wang et al. 2008).

Although the abovementioned host and viral genes are certainly worth being investigated, a genome-wide approach would give valuable additional insight in host genes and/or proteins involved in SeMNPV-induced positive phototaxis. This could be achieved by comparing host transcriptomes and/or proteomes of baculovirus-infected *S. exigua* larvae kept under either dark or light conditions.

Ecological significance of behavioural manipulation by baculoviruses

Research on parasitic manipulation of host behaviour has only in the past few decades shifted its focus towards understanding the mechanisms by which a parasite manipulates host behaviour. Before that, host behavioural modification during parasite infection was studied mainly in terms of its adaptive significance. Although for various examples of behavioural manipulation the adaptive value for the parasite seems evident (e.g. Gordian worms that cause their hosts to jump into the water where the worms can find a mate), for other examples the advantage for the parasite is not so clear. In many cases, understanding the adaptive significance of behavioural modification by a parasite

requires knowledge on the underlying mechanism. For example, the identification of a specific parasite gene necessary for behavioural manipulation allows one to compare parasite survival and transmission rates in the presence and absence of that specific gene, which provides valuable insights in the adaptive consequences of parasitic manipulation of a behavioural trait. With the identification of baculovirus *ptp*, a parasite gene required for hyperactive behaviour in caterpillars, an obvious next step to take is to understand the adaptive significance of this manipulative strategy. Would the presence of *ptp* indeed increase virion distribution on plants over a larger area? And would that distribution lead to higher infection rates for future larval generations? Now that we have the possibility to compare baculovirus transmission levels in the presence or absence of *ptp* such research questions can be investigated. Below the possible adaptive value of hyperactive behaviour during baculovirus infection is discussed.

Horizontal dispersal of baculovirus-infected cadavers increased virus transmission rates in the Douglas-fir tussock moth *Orgyia pseudotsugata* and is thought to be an important factor in cycles of virus outbreaks in lepidopteran populations (Dwyer 1991, 1994). Furthermore, horizontal larval dispersal likely contributes to the conspicuousness of infected caterpillars (Goulson 1997), and the shedding of virions through regurgitation and defecation during this hyperactive period has been regularly observed (Ali et al. 1987; Jaques 1962; Vasconcelos 1996; Zelazny 1976). It is also thought that infected larvae may spread virus by leaking liquid from the anus that possibly contains OBs (Kamita et al. (2005) and personal observations). Alternatively, increased movement of infected larvae could reflect an adaptive strategy of the host to prevent virus transmission to conspecifics, as it could result in removal of viral inoculum from the insect population (Goulson 1997). However, the identification of a specific viral gene necessary to induce hyperactivity points towards an adaptive value for the parasite rather than for the host.

Manipulation of host climbing behaviour, discussed in Chapters 5, 6 and 7 of this thesis, is generally assumed to contribute to a more efficient spread of virions on the upper part of plants. Distribution of baculovirus-containing cadavers on higher and more exposed plant parts led to higher transmission rates than distribution on inner, less exposed plant structures (Vasconcelos et al. 1996). This implies that climbing behaviour of infected caterpillars prior to death, leading to death at elevated positions, contributes to virus transmission in the field. The finding that the presence of light from above is crucial for death at elevated positions of SeMNPV-infected *S. exigua* larvae provides a suitable experimental system to study the adaptive value of virus-induced climbing. For example, one could compare virus transmission rates of SeMNPV-infected caterpillars that are kept under either light or dark conditions.

Dying of the larvae at elevated positions also has the advantage that these larvae become pale and are easily and selectively recognized by birds (Vasconcelos et al. 1996). These birds are then responsible for the long-distance transport and dispersal of OBs, as has been demonstrated for *Panolis flammea* MNPV infecting the pine beauty moth *P. flammea* in pine trees in Scotland (Entwistle et al. 1993).

The adaptive significance of the strong attraction to light that underlies death at elevated position in SeMNPV-infected larvae is less clear if one takes into account that UV inactivation of baculoviruses frequently occurs in the field (Cory & Myers 2003; Rothman & Roland 1998). Presumably, a trade-off exists between virus spread over a large and exposed surface on the one hand, and protection of virions against high UV levels on the other. Consequently, induction of climbing behaviour leading to death at elevated positions may not be beneficial for the virus under all conditions. For example, baculoviruses occurring in tropical or subtropical regions, where levels of UV exposure are generally high, may have evolved different strategies to manipulate host behaviour than baculoviruses that occur in more temperate regions. Alternatively, viruses may have evolved mechanisms to reduce UV-induced damage to their genome, in line with the finding that some baculoviruses carry the abovementioned *phr* genes, which encode DNA repair enzymes (van Oers et al. 2008; Willis et al. 2005).

Environmental factors influencing behavioural manipulation by baculoviruses

All behavioural experiments described in this thesis have been performed in laboratory setups. Such setups are easy to handle, and they provide the possibility to perform the experiments under tightly controlled conditions. Now that we begin to understand more about baculovirus-induced host behaviour, it is important to extend these behavioural studies to ecologically more relevant systems. Many different environmental factors are likely to influence behavioural manipulation of insects in the field, several of which will be discussed here.

Probably one of the most important factors is the species of host plant on which the insect and virus occur. Plants are known to have an effect on insect pathogens in multiple ways (Cory & Hoover 2006). Plant phytochemicals, including tannins, phenols and peroxidases, can inactivate baculovirus OBs present on plant leaves, which may significantly decrease the persistence of a virus in the environment (Hoover et al. 1998a; Hoover et al. 1998b; Hoover et al. 1998c; Keating et al. 1988). On the other hand, plant defensive chemicals may also increase host insect susceptibility to virus infections, virus speed of

kill and virus yields, for example by affecting host development (Cory & Hoover 2006; Elliot et al. 2000). A recent study showed that herbivore-induced plant defences can increase baculovirus infection rates among populations of *L. dispar* larvae in red oak forests (Elderd et al. 2013). Furthermore, plant species may differ in the extent to which they can provide protection to the virus against UV inactivation (Duffey 1995), as was also discussed above. Host plants may influence endogenous insect behaviour (Cory & Hoover 2006) and this in turn could affect pathogen-induced insect behaviour. Both host species studied in this thesis (*S. exigua* and *T. ni*) are polyphagous pest species and *S. exigua* larvae are known to have distinct patterns of feeding and dispersal depending on the plant species they occur on (Smits et al. 1987). It is therefore conceivable that the outcome of a behavioural manipulation strategy by baculoviruses is highly dependent on the plant species on which the insect and virus reside. As plants clearly are important factors in the co-evolutionary dynamics of insect and parasite, future research on baculovirus-induced behaviour should take tritrophic interactions between plant, insect and pathogen into account.

A second important factor to influence behavioural manipulation in the field may be the frequency of occurrence of susceptible larval populations in a crop field. While some lepidopteran caterpillar populations have multiple generations per year (multivoltine), others have only a single generation per year (univoltine) (Schowalter 2011). This difference in population frequency likely requires different modes of persistence for pathogens like baculoviruses. Baculoviruses that infect univoltine host species may have to persist in the environment for a long time before a new generation of susceptible hosts will appear. On the other hand, baculoviruses infecting multivoltine hosts likely encounter a new population of susceptible hosts more frequently and consequently have to invest less in virus persistence in the environment. This difference in host ecology may also have consequences for the strategies of behavioural manipulation employed by baculoviruses, as is illustrated by a study using larvae of the winter moth *Operophtera brumata* (Raymond et al. 2005). When these larvae are infected with *O. brumata* MNPV (OpMNPV), they descend to the bottom and finally die at the lower part of the plant stems, near the soil. *Operophtera brumata* is a univoltine species with only a single generation per year, and it is hypothesized that the virus persists better in the soil where it is well-protected from UV damage and environmental decay (Raymond et al. 2005).

A third factor that needs consideration is the virus dose that is needed to successfully infect larvae and how this dose relates to what is commonly found in the natural situation. For AcMNPV a viral concentration of 10^8 OBs/ml was used to infect 3rd instar *T. ni* and *S. exigua* larvae, which corresponds to about 10^5 OBs per caterpillar. It is questionable how likely it is to find such high concentrations of OBs in nature. Consequently,

natural infection of 3rd instar caterpillars with AcMNPV may not occur very frequently in the field. However, the infectivity of natural AcMNPV strains may be much higher than the laboratory strains used in this research. An important factor in this may be the occurrence of endogenous baculoviruses ('covert' or 'latent' infections) in insect populations (Burden et al. 2003; Cory & Myers 2003; Kukan 1999). While these viruses are commonly present in the host in a non-replicating state, stress conditions (e.g. crowding, high temperatures or infection with a second pathogen) may lead to activation of the virus (Cory & Myers 2003; Fuxa 1999). As such, they are thought to be an important cause of virus outbreaks in the field, and they may increase the final viral load upon horizontal transmission of baculoviruses (Burden et al. 2003, Cory & Myers 2003).

The developmental stage of host caterpillars may also influence the outcome of behavioural manipulation by baculoviruses. In all experiments described in this thesis almost exclusively 3rd instar larvae of *T. ni* and *S. exigua* were used. However, larvae of different developmental stages are known to display distinct behavioural patterns in the field (Griswold & Trumble 1985; Smits et al. 1987). Given the host-dependent effects that were observed for tree-top disease induced by AcMNPV, the host developmental stage is likely to affect the final outcome of a manipulative strategy that the virus employs.

The molecular mechanisms behind parasitic manipulation of host behaviour: where are we now?

What does the research described in this thesis contribute to the field of parasitic manipulation? Do these findings on baculovirus-induced caterpillar behaviour lead to a better understanding of the concept and mechanisms of behavioural manipulation?

A clear outcome of the research described in this thesis is that there is no general mechanism by which parasites alter the behaviour of their host. Behavioural manipulation during parasite infections is often highly fine-tuned in space and time, and likely requires a tailor-made mechanism of the parasite to manipulate its host. The finding that infection of two different host species with a single virus gives distinct behavioural outcomes (Chapter 7), nicely illustrates the concept of such a tailor-made (and consequently host-dependent) mechanism. Likewise, different virus species can also cause distinct behavioural phenotypes in a single host (Chapters 6, 7 and 8), demonstrating that these viruses use distinct mechanisms to induce a behavioural change. Even though the role of *ptp* in hyperactivity is conserved in BmNPV (Kamita et al. 2005) and AcMNPV (van Houte et al. 2012) (Chapter 3), differences seem to exist in the exact mechanism by which *ptp* exerts its behavioural function (Katsuma et al. 2012; van Houte et al. 2012).

This illustrates a very important issue when studying mechanisms of behavioural manipulation. The two parasite 'behavioural' genes that have been identified to date, i.e. the baculovirus *ptp* and *egt* genes, both appear to have multiple functions within the viral life cycle. Besides an inducer of hyperactive behaviour, the baculovirus PTP enzyme is also a structural protein that, at least in some baculovirus-host systems, appears to contribute to virus production (Katsuma et al. 2012). Likewise, EGT is not only crucial for death at elevated positions of infected larvae, but may also cause a longer life span and a higher virus yield by inhibiting host development (Cory & Myers 2003; Hoover et al. 2011). In more general terms, observing that a parasite gene underlies a specific behavioural phenotype does not necessarily mean that this gene is directly involved in inducing this behaviour, nor that it is the sole determinant. Subsequent examination of host genes and proteins affected by this parasite gene should give more insights in whether such a behavioural effect is direct or indirect.

Despite the apparent mechanistic differences that underlie behavioural manipulation even between closely related parasites, do we also see parallels between mechanisms of behavioural manipulation? Manipulative strategies in distinct parasite-host systems likely have very different initial steps of induction. For example, baculovirus EGT has been identified as a manipulative parasite gene in LdMNPV-infected *L. dispar* larvae, but AcMNPV-induced climbing in *T. ni* and *S. exigua* larvae has a distinct genetic basis. However, similarities may exist in some steps within the behavioural cascades induced by a parasite gene. An example is the host serotonergic system, which seems to be important in behavioural manipulation in multiple parasite-host associations that are phylogenetically unrelated (Hughes et al. 2012). The serotonergic system is evolutionarily old and extremely conserved among different taxa, and consequently represents a suitable signalling route to be abused by parasites. Thus, although the initial step will be very different between distinct parasite-host systems, many of these pathways may finally converge and affect the host serotonergic system to alter host behaviour.

At this moment in time we still understand relatively little about how and why parasites manipulate host behaviour. Suitable model systems to study mechanisms of behavioural manipulation are still scarce. The model system studied in this thesis, baculoviruses and their lepidopteran hosts, has demonstrated its usefulness in studying parasitic manipulation and will certainly continue to do so in the future.

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Summary

Parasites often have severe effects on their hosts by causing developmental and physiological alterations in their hosts. These alterations may contribute to parasite growth, reproduction and survival. For example, host development may be inhibited so that more nutrients become available for the parasite. Parasites can also interfere with host behaviour as a strategy to increase parasite survival or transmission. This phenomenon is known as 'parasitic manipulation' or 'behavioural manipulation'. Although many examples of parasitic manipulation are known, the genetic basis underlying such manipulations is largely unexplored. A thorough understanding of how parasites manipulate their hosts' behaviour is therefore lacking, but it can be hypothesized that parasites carry specific genes that induce these behavioural alterations. Such 'behavioural' parasite genes likely affect one or more host proteins directly or via the expression of specific target genes in the host, which subsequently leads to altered behaviour. Understanding the details of such interactions between parasite and host is important as parasitic manipulation is thought to be wide spread in nature and to be a strong driver of the co-evolutionary arms race between parasite and host. Furthermore, the strategies employed by parasites to alter behaviour likely provide important insights in the molecular mechanism of many behavioural processes. Chapter 2 of this thesis reviews our current understanding of the mechanisms of behavioural manipulation in invertebrates. It discusses known examples of behavioural manipulation and the present knowledge on the mechanistic basis of these manipulations. Furthermore, an overview of host genes and proteins that play a conserved role in behavioural traits in different invertebrate species is given. These genes and proteins are worthwhile to be studied in more detail in the context of parasitic manipulation, as they might be suitable targets for parasites to induce behavioural changes.

This thesis focuses on behavioural manipulation in insect hosts by baculoviruses. Baculoviruses are DNA viruses that infect the larval stages of mainly lepidopteran insects. These viruses alter host behaviour in multiple ways. They induce hyperactivity in the larvae, which likely contributes to virus dispersal over a large area. In addition, baculoviruses alter host climbing behaviour leading to death at elevated positions, a phenomenon known as 'tree-top disease' or 'Wipfelkrankheit'. This latter manipulation is thought to contribute to optimal virus dispersal on plant foliage. In the research described in this thesis baculoviruses and their lepidopteran insect hosts are used as a model system to study molecular mechanisms of behavioural manipulation. In Chapter 3 of this thesis the involvement of the protein tyrosine phosphatase (*ptp*) gene from the baculovirus *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) in the induction of hyperactivity of the beet armyworm *Spodoptera exigua* was studied. A homolog of this gene in *Bombyx mori*

nucleopolyhedrovirus (BmNPV) was previously shown to be important in hyperactivity in the silkworm *B. mori*. The results in Chapter 3 showed that the AcMNPV *ptp* gene induces hyperactive behaviour in *S. exigua* larvae and that the phosphatase activity of the encoded PTP enzyme is crucial for this behavioural change. Phylogenetic inference revealed that the baculovirus *ptp* is presumably transferred from a (ancestral) lepidopteran host to a baculovirus. Within the family Baculoviridae, *ptp* is only present in group I NPVs, which are a group of phylogenetically related baculoviruses. It is hypothesized that *ptp*-induced hyperactivity is an evolutionarily conserved strategy of group I NPVs to manipulate host behaviour.

To obtain insights into the target proteins of the baculovirus PTP enzyme to achieve hyperactive behaviour in infected caterpillars, a PTP substrate analysis was performed. Chapter 4 describes host and viral proteins that were found to co-purify with AcMNPV PTP. Many of these host proteins are known to be important in signalling pathways and behavioural traits. For one of these proteins, 14-3-3 ζ, mRNA transcript levels were found to be significantly higher in wild type AcMNPV-infected *S. exigua* larvae as compared to larvae infected with a mutant virus from which the *ptp* gene has been deleted (AcMNPV Δ*ptp*). The 14-3-3 protein is a known activator of the enzymes tryptophan hydroxylase and tyrosine hydroxylase, which in turn are required for synthesis of the neurotransmitters serotonin and dopamine. These signalling molecules are both important determinants in hyperactive behaviour in various organisms, and are associated with behavioural manipulation in several parasite-host systems. In Chapter 9 a model is proposed of how the putative interaction between baculovirus PTP and host 14-3-3 ζ may lead to hyperactive behaviour.

Within the baculoviruses two different genes that encode protein tyrosine phosphatases, *ptp* and *ptp2*, are found. While the *ptp* gene induces hyperactivity (described in Chapter 3), no function has yet been assigned to the *ptp2* gene. Chapter 5 describes the functional characterization of the baculovirus *ptp2* gene. PTP2 protein carries a conserved consensus sequence that is characteristic for mitogen-activated protein kinase (MAPK) phosphatases. SeMNPV *ptp2* induced a mild apoptosis and caspase activation in insect cells upon transient expression, which may be related to its putative function as MAPK phosphatase. Several host proteins that co-purified with SeMNPV PTP2 have known functions in apoptosis and/or MAPK signalling, rendering them promising candidate proteins to be involved in SeMNPV PTP2-induced apoptosis and possibly MAPK signalling. Whether PTP2 also has any behavioural effect is unknown, but the data from this chapter indicate that PTP2 likely has a cellular function during virus infection.

Baculoviruses are known to alter host climbing behaviour, commonly leading to death at elevated positions (tree-top disease). In Chapter 6 the hypothesis was tested that baculovirus-induced hyperactive behaviour and tree-top disease are induced by a single baculovirus gene. To this aim the effect of the hyperactivity-inducing *ptp* gene (Chapter 3) on tree-top disease was investigated. The results demonstrated that AcMNPV *ptp*, known to cause hyperactive behaviour in *S. exigua*, is not involved in tree-top disease in this host. This indicates that hyperactivity and tree-top disease induced by baculoviruses are governed by independent mechanisms. Furthermore, a moulting-dependent effect on tree-top disease in *S. exigua* was found, which may relate to physiological and/or ecological differences between moulted and unmoulted larvae.

In the next chapter (Chapter 7) the effect of AcMNPV infection on tree-top disease was investigated for two different host species, *Trichoplusia ni* and *S. exigua*. Data show that in *T. ni* larvae AcMNPV induces tree-top disease, causing death at elevated positions. In contrast, in *S. exigua* a moulting-dependent effect on the height at death was observed, as was also described in Chapter 6. Furthermore, in this chapter the role of the AcMNPV *egt* gene, encoding ecdysteroid UDP glucosyl transferase, on tree-top disease in *T. ni* and *S. exigua* larvae was analysed. A homolog of this gene causes tree-top disease in *Lymantria dispar* larvae infected with *L. dispar* (Ld) MNPV. The results (Chapter 7) show that AcMNPV *egt* does not play a role in the observed death at elevated positions in the two host systems studied. This indicates that the role of *egt* in tree-top disease may not be conserved among members of the family *Baculoviridae*.

In addition to the mechanisms employed by the generalist baculovirus AcMNPV to alter climbing behaviour, the effect of the specialist baculovirus *S. exigua* (Se) MNPV on tree-top disease in its only known host *S. exigua* was studied. In Chapter 8 it is shown that SeMNPV induces tree-top disease by triggering an aberrant response to light, and this positive phototaxis leads to death at elevated positions. A hypothesis is put forward that SeMNPV hijacks a host behavioural pathway that is involved in light perception to induce this positive phototactic response.

Overall, the results of this thesis show that hyperactivity and tree-top disease are induced by baculoviruses through independent mechanisms and that distinct baculovirus species presumably use different genes and proximate mechanisms to induce tree-top disease. While the baculovirus *ptp* gene induces hyperactivity, possibly by targeting host 14-3-3 ζ, the baculovirus *ptp2* gene may function as a pro-apoptotic gene. The baculovirus *egt* gene does not have a conserved function in tree-top disease, indicating that other viral genes may underlie this host manipulative strategy. This thesis also demonstrates that tree-top disease in SeMNPV-infected caterpillars is the result of a strong attraction to light.

Parasitic manipulation is a fascinating biological phenomenon that can provide crucial information on how behavioural traits are controlled at the molecular level. The research described in this thesis provides several new insights in the mechanisms by which parasites manipulate the behaviour of their hosts.

Samenvatting

Veel parasieten manipuleren hun gastheer op allerlei manieren om zo hun transmissie en overlevingskans te maximaliseren. Zo veranderen ze bijvoorbeeld de groei, ontwikkeling en voortplanting van hun gastheer. Sommige parasieten zijn ook in staat om het gedrag van hun gastheer te manipuleren. Dit fenomeen heet 'gedragsmanipulatie' of 'parasitaire manipulatie'. Er zijn ondertussen vrij veel gevallen bekend van gedragsmanipulatie door parasieten, maar in bijna geen enkel geval is het achterliggende mechanisme bekend waarmee de parasiet een gedragsverandering in de gastheer veroorzaakt. Waarschijnlijk hebben veel parasieten speciale genen die deze manipulaties induceren. Het is aannemelijk dat de producten van deze 'gedragsgenen' aangrijpen op bepaalde genen of eiwitten van de gastheer, en dat deze interacties vervolgens leiden tot de gedragsverandering. Kennis over deze interacties en hun uitwerkingen is belangrijk, omdat gedragsmanipulatie een wijdverbreid biologisch fenomeen is en een belangrijke factor is in de co-evolutionaire wapenwedloop tussen gastheer en parasiet. Daarnaast kunnen de strategieën die parasieten gebruiken om gastheergedrag te veranderen ook inzicht geven in de genetische en moleculaire basis van gedrag in het algemeen. Hoofdstuk 2 van dit proefschrift geeft een overzicht van hetgeen er bekend is over gedragsmanipulatie in ongewervelde dieren en van onze huidige kennis over de mechanismen die hieraan ten grondslag liggen. Verder wordt er in dit hoofdstuk een overzicht gegeven van gastheergenen en –eiwitten die een evolutionair geconserveerde rol spelen in gedrag bij verschillende ongewervelde diersoorten. Deze genen en/of eiwitten zijn interessant om te bestuderen in de context van parasitaire manipulatie, omdat zij een geschikt doelwit zouden kunnen zijn voor parasieten om een gedragsverandering te bewerkstelligen.

Dit proefschrift gaat over gedragsmanipulatie geïnduceerd door baculovirussen in insecten. Baculovirussen zijn grote DNA-virussen die rupsen infecteren van voornamelijk vlinders en motten (Lepidoptera). Deze virussen veranderen gedrag op diverse manieren. Ze induceren hyperactiviteit bij rupsen, wat waarschijnlijk bijdraagt aan efficiënte virusverspreiding over een groot oppervlak. Baculovirussen veranderen ook het klimgedrag van hun gastheerrupsen, waardoor de rups hoog in de plant of het gewas doodgaan. Dit fenomeen staat bekend als 'Wipfelkrankheit' oftewel 'tree-top disease', en draagt vermoedelijk bij aan een goede verspreiding van nieuwe virussen over het gebladerte. In het hier beschreven onderzoek worden baculovirussen en hun rupsengastheren gebruikt als modelsysteem voor het bestuderen van de moleculaire mechanismen die ten grondslag liggen aan gedragsmanipulatie. Zo wordt in Hoofdstuk 3 de rol van het 'protein tyrosine phosphatase' (*ptp*)-gen van het baculovirus *Autographa californica* kernpolyedervirus (multiple nucleopolyhedrovirus, AcMNPV) bestudeerd bij de inductie van

hyperactief gedrag van de geïnfecteerde rups van de florida-mot, *Spodoptera exigua*. De resultaten laten zien dat het AcMNPV *ptp*-gen hyperactiviteit induceert in deze rupsen, en dat de fosfatase activiteit van het door dit gen gecodeerde PTP-enzym cruciaal is voor deze gedragsverandering. Fylogenetische analyse laat zien dat in de evolutie van baculovirussen het *ptp*-gen vermoedelijk is overgesprongen van een voorouderlijke lepidoptera-gastheer naar een baculovirus. Binnen de familie *Baculoviridae* is het *ptp*-gen alleen aanwezig in groep I NPV's, een groep evolutionair verwante baculovirussen. De hypothese is dat *ptp*-geïnduceerde hyperactiviteit een evolutionair geconserveerde strategie van groep I NPV's is om gedrag te manipuleren.

Een PTP-substraat analyse werd uitgevoerd om inzicht te verkrijgen in de eiwitten waarop het baculovirus PTP eiwit aangrijpt om hyperactiviteit te induceren. In Hoofdstuk 4 worden de virale en gastheereiwitten besproken die op een affiniteitskolom meezuiverden met AcMNPV PTP, en dus mogelijk een interactie aangaan met PTP. Van veel van deze gastheereiwitten is bekend dat ze een belangrijke rol spelen in signaalroutes in de cel en bepalend zijn voor gedragskenmerken. Voor één van deze eiwitten, 14-3-3 ζ, bleek het transcriptieniveau significant hoger te zijn in wild-type AcMNPV-geïnfecteerde rupsen in vergelijking met rupsen geïnfecteerd met een mutant virus waaruit het *ptp*-gen was verwijderd. De 14-3-3 eiwitten functioneren als activatoren van de enzymen tryptofaan hydroxylase en tyrosine hydroxylase, die beide nodig zijn voor de aanmaak van de neurotransmitters serotonine en dopamine. Deze signaalstoffen spelen een rol in hyperactief gedrag bij verscheidene organismen, en zijn betrokken bij gedragsmanipulatie in een aantal parasiet-gastheer interacties. In Hoofdstuk 9 wordt een model gepresenteerd van hoe de interactie tussen baculovirus PTP en gastheer 14-3-3 zou kunnen leiden tot hyperactiviteit.

Binnen de baculovirussen kunnen twee verschillende genen worden gevonden die beide een eiwit-tyrosine-fosfatase coderen. Terwijl het *ptp*-gen hyperactiviteit induceert (beschreven in Hoofdstuk 3), is er nog geen functie bekend van het *ptp2*-gen. Hoofdstuk 5 beschrijft de karakterisering van dit *ptp2*-gen. Het PTP2 eiwit bevat een consensussequentie die karakteristiek is voor MAPK-fosfatases. Expressie van SeMNPV *ptp2* induceert een milde apoptose en activeert caspases in insectencellen, hetgeen gerelateerd zou kunnen zijn aan een mogelijke functie als MAPK-fosfatase. Een aantal gastheereiwitten dat meezuivert met SeMNPV PTP2 vormt geschikte kandidaateiwitten om betrokken te zijn bij SeMNPV PTP2-geïnduceerde apoptose en/of MAPK-signaalroutes. Het is onbekend of PTP2 ook gedragseffecten heeft, maar de resultaten van dit hoofdstuk geven aan dat PTP2 waarschijnlijk een cellulaire functie heeft tijdens virusinfectie.

Baculovirussen veranderen klimgedrag van hun gastheer, wat er vaak voor zorgt dat de rups hoog in de plant doodgaat ('tree-top disease'). In Hoofdstuk 6 is de hypothese getest dat baculovirus-geïnduceerde hyperactiviteit en 'tree-top disease' worden geïnduceerd door een enkel baculovirus gen. Hiervoor is het effect van het *ptp*-gen (Hoofdstuk 3) op 'tree-top disease' onderzocht. De resultaten laten echter zien dat AcMNPV *ptp*, dat hyperactiviteit induceert in *S. exigua*-rupsen, niet betrokken is bij 'tree-top disease' in deze gastheer. Dit alles toont aan dat baculovirus-geïnduceerde hyperactiviteit en 'tree-top disease' worden aangestuurd via onafhankelijke mechanismen. Daarnaast werd een vervellings-afhankelijk effect op 'tree-top disease' gevonden in *S. exigua*, hetgeen gerelateerd zou kunnen zijn aan fysiologische en/of ecologische verschillen tussen vervelde en niet-vervelde rupsen tijdens de infectie.

In het volgende hoofdstuk (Hoofdstuk 7) is het effect van AcMNPV-infectie op 'tree-top disease' vergelijkenderwijs in de twee gastheren *Trichoplusia ni* en *S. exigua* bekeken. De resultaten laten zien dat AcMNPV 'tree-top disease' ook in *T. ni*-rupsen induceert. In *S. exigua* is een vervellings-afhankelijk effect op 'tree-top disease' te zien, zoals ook beschreven in Hoofdstuk 6. Verder wordt in dit hoofdstuk de rol van het AcMNPV *egt*-gen bij 'tree-top disease' onderzocht. Een homoloog van dit gen is verantwoordelijk voor 'tree-top disease' in *Lymantria dispar*-rupsen, geïnfecteerd met het baculovirus *L. dispar* MNPV. Het blijkt echter dat in de twee bestudeerde gastheersystemen het AcMNPV *egt*-gen niet betrokken is bij 'tree-top disease', hetgeen aangeeft dat de rol van *egt* in 'tree-top disease' waarschijnlijk niet geconserveerd is binnen de familie *Baculoviridae*.

Naast de mechanismen die AcMNPV, een generalist onder de baculovirussen, gebruikt om klimgedrag te veranderen, is er ook gekeken naar het effect van het specialistische baculovirus SeMNPV op 'tree-top disease' in zijn enige gastheer *S. exigua*. Hoofdstuk 8 laat zien dat SeMNPV-infectie een afwijkende reactie op licht veroorzaakt, en dat deze positieve fototaxis leidt tot 'tree-top disease' in de rups. De hypothese is dat SeMNPV een gedragsroute van de gastheer, die betrokken is bij licht/donker-perceptie, misbruikt om deze gedragsverandering te induceren.

Alles bij elkaar genomen laten de resultaten van dit proefschrift zien dat baculovirussen hyperactiviteit en 'tree-top disease' induceren door middel van onafhankelijke mechanismen, en dat verschillende baculovirussen vermoedelijk verschillende genen en mechanismen gebruiken om 'tree-top disease' te induceren. Terwijl het baculovirus *ptp*-gen hyperactiviteit induceert, vermoedelijk door aan te grijpen op het gastheereiwit 14-3-3 ζ, heeft het *ptp2*-gen waarschijnlijk een pro-apoptotische functie. Het baculovirus *egt*-gen heeft geen geconserveerde rol in 'tree-top disease', hetgeen doet vermoeden dat ook andere virale genen verantwoordelijk kunnen zijn voor deze gedragsmanipulatie. Dit

proefschrift laat verder zien dat 'tree-top disease' in SeMNPV-geïnfecteerde rupsen het gevolg is van een sterke aantrekking tot licht.

Parasitaire manipulatie is een fascinerend fenomeen dat zeer waardevolle informatie kan geven over hoe gedragskenmerken van gastheerorganismen worden aangestuurd op moleculair niveau. Het onderzoek beschreven in dit proefschrift, geeft nieuwe inzichten in de mechanismen die parasieten gebruiken om het gedrag van hun gastheer, meer in het bijzonder insecten, te veranderen.

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About the author

Christina Geertruida Johanna (Stineke) van Houte was born on the 28th of May 1982 in Waarde, a small village on the Westerschelde shore in the province of Zeeland, the Netherlands. She followed secondary education at the Buys Ballot College in Goes and in 2001 decided to move to Wageningen to study Molecular Sciences. After finishing the Bachelor study, she spent seven months in Córdoba, Spain, for an Erasmus exchange. In September 2005 she started the major thesis for her Master degree in the Laboratory of Virology, Wageningen, under supervision of Monique van Oers. In this project she worked on the functional characterization of baculovirus photolyases, which are DNA repair enzymes. This work was awarded best thesis of Wageningen University '06/'07 in Life Sciences. After her major thesis, she went for a six-months internship to the Jacob Blaustein Institute for Desert Research in Sde Boqer, Israel, where she worked on the characterization of glutamine synthetase enzymes from microalgae. The Master study was completed with a four-months minor thesis on CRISPR/Cas-mediated antiviral defense in prokaryotes at the Laboratory of Microbiology, Wageningen. In November 2007 she obtained her MSc degree (*cum laude*). After finishing her studies she moved to Bologna, Italy, where she worked for a year as junior researcher at the Department of Evolutionary Biology on the neuronal function of the Cystatin B protein in Unverricht-Lundborg disease. In 2009 she moved back to the Netherlands to start her Ph.D. on baculovirus-induced behaviour of insects at the Laboratory of Virology in Wageningen under supervision of Monique van Oers, of which the results are described in this thesis. In 2012 she received the Mauro Martignoni award for best Ph.D. research from the Society of Invertebrate Pathology. In September 2013 she moved to the Biosciences Campus of the University of Exeter, which is located in Penryn, Cornwall. She currently works as a visiting scientist on virus infections and endosymbionts in moths under supervision of Nina Wedell and Mike Boots.

Acknowledgements

...en dan, na bijna vijf jaar, ben ik bij het dankwoord van mijn proefschrift aanbeland! Dat voelt natuurlijk heel goed, maar ook wel een beetje definitief, als de afsluiting van een tijdperk. Zonder twijfel één waar ik met enorm veel plezier op kan terugkijken. Och, bij tijd en wijle was ik wel eens moe van het eindeloos rupsen kweken (waardoor ik 's nachts alsmaar krioelende rupsen op mijn netvlies zag), rupsenfilmpjes analyseren en klimgaasjes afborstelen, maar het doel is nooit ver uit zicht geweest en er was altijd in elk geval een beetje licht aan het eind van de tunnel. Al die mensen die me tijdens mijn aio-tijd op het juiste (wetenschappelijke) pad hebben weten te houden (of dat in elk geval naarstig getracht hebben....), met me mee hebben gedacht, me op allerlei manieren geholpen hebben, of gewoon heel prettig volk waren om bij in de buurt te zijn, ik ben iedereen erg dankbaar omdat ze elk op eigen wijze mijn aio-tijd hebben veraangenaamd.

Monique, ik ben nog altijd blij dat ik in 2008 vanuit Italië een mail naar je stuurde, om te informeren of er misschien nog een leuk aio-project aan zat te komen in Wageningen. 'Wat betreft banen', schreef je me terug, 'zie ik een mooie mogelijkheid als je tenminste in Wageningen en in onze groep wil werken'. Het betekende voor mij de start van een compleet nieuw project aan gedragsveranderingen in rupsen door baculovirus-infecties, en ik heb geen seconde spijt gehad van de beslissing om hieraan te gaan werken. Het was een spannend project, waarbij we veel dingen vanaf de basis moesten uitzoeken. Het begin was moeizaam, met een verhuizing van de Binnenhaven naar het nieuwe Radix een half jaar na het begin van mijn project, een ingestorte rupsenkweek, en een nieuwe generatie rupsen die zich anders bleken te gedragen dan de vorige, waardoor ik mijn gedragsproeven weer van voor af aan kon beginnen... Mede door jouw grote betrokkenheid en actieve houding is dit project tot een goed einde gebracht. Hoe druk je het ook had, je maakte meteen tijd vrij als ik iets wilde bespreken. Ronduit fenomenaal is de snelheid waarmee je mijn manuscripten en proefschrifthoofdstukken becommentarieerde, wat zeker in de laatste fase van proefschrift schrijven erg handig was! Het is ook erg leuk dat jij je inaugurele rede een paar dagen voor mijn verdediging gaat geven; ik ben er van overtuigd dat je hoogleraarschap van Virologie goed gaat verlopen!

Just, in 2012 ben je officieel met emeritaat gegaan. Toch ben je nooit je interesse verloren in mijn project, en ik heb grote waardering voor je fascinatie en zeer aanstekelijk enthousiasme voor wetenschap. Ze zijn voor mij een bron van inspiratie geweest, en ik weet zeker dat ze dat zullen blijven voor menig aio. Ik herinner me jouw uitroep toen je even in het lab kwam koekeloeren tijdens mijn licht-donker proefjes heel goed: 'Dat moeten we naar Science sturen!'. Daarnaast toon je altijd veel vertrouwen in en waar-

dering voor je mensen. Imponerend is ook je vermogen om op vrij grote afstand koekjes te detecteren; toen ik de laatste weken op Virologie op een andere plek zat, wist je mijn verdekt opgestelde koektrommel feilloos te vinden. Ik verwacht je dan ook elk moment op onze stoep in Cornwall voor een stukje shortbread!

Dan mijn co-promotor, Vera! Toen je in 2009 aan de slag ging als postdoc bij Virologie, was aanvankelijk nog niet duidelijk dat wij zoveel zouden gaan samenwerken. Dat je, ergens in 2010, hebt besloten om ook aan gedragsmanipulatie te gaan werken is wat mij betreft echt een meesterzet geweest! Daardoor is dit echt 'ons' project geworden. Wat hebben we samen een hoop dingen beleefd in de afgelopen jaren, experimenten uitgedokterd en uitgevoerd, 'weekend-' en 'avond'diensten gedraaid voor de klim- en loopproeven (met *Stand by me* als favoriete hit), en vooral een hoop plezier gehad! Fantastisch waren ook de vele congressen die we samen bezocht hebben. Het ESEB congres in 2011 vond ik in dat opzicht wel echt een hoogtepunt, het congres was fantastisch, maar ook onze heenreis op de fiets door Duitsland was geweldig, ondanks dat ik op zeker moment met migraine in de tent lag en jij met mijn fietswiel (met maar liefst 8 kapotte spaken!) de bus in moest op zoek naar een fietsenmaker. De China-reis was ook grandioos, van het rondtoeren op onze fietsjes tussen de rijstvelden en karstbergen tot het aanschaffen (en weer terugverkopen) van malafide Chinese bustickets ('Two people take the bus to Ping An', ja ja!). Mede door jouw enthousiaste verhalen over de bacterie *Wolbachia* ben ik nu in Cornwall begonnen aan een onderzoeksproject over virus-infecties en *Wolbachia* in rupsen en motten. Heel veel succes in je verdere carrière, ik twijfel er niet aan dat die succesvol wordt! Ik ga 'ons' project natuurlijk enorm missen, maar verwacht dat we in de toekomst zullen blijven samenwerken, er is nog zoveel interessants te ontdekken!

En dan natuurlijk alle andere mensen van de baculogroep met wie ik de afgelopen jaren het lab, kantoor en de koffietafel gedeeld heb. Els, enorm bedankt voor al je hulp! In mijn eerste jaar hielp je me vaak met mijn bioassays, maar ook later heb je me geholpen bij het maken van kloons, de celkweek en niet te vergeten het opgroeien van gigantische hoeveelheden rupsen. Jouw hulp was voor mij heel handig, maar het was vooral ook altijd erg gezellig met jou erbij! Dear Ke, thanks for all the pleasant talks and interesting discussions we had. You were a much appreciated colleague and I wish you and your family all the best. Fang, you left Virology already quite a while ago, but we overlapped in my first year, when you were finishing your thesis (that felt like ages ahead for me back then, I remember well). All the best in the US! Magda, we share good memories of the SIP congress 2010 in Turkey (no, not the one where we were ill in our beds...), thanks a lot for the nice time! Good luck in Switzerland! Hoa, although you were a 'shrimp-lady', you were also part of the baculogroup for quite some time. You were very pleasant company in the lab and baculocorner and I hope you and your family will have a bright future in

Vietnam. Amaya, good luck with finishing your thesis! I'm sure one day you'll find out what this crazy ORF of you is doing. Stick to your great habit of cursing in Spanish to your computer when it's making you insane, I loved it! Dear Desrina, it was wonderful having you around in our lab, you always being cheery (even after a 1000 PCRs you were smiling) and generous. All the best for writing your thesis, I'll try my best to join your defense! Agah, wish you all the best in the future! Henry, good luck in Yale! Paulina, Joyce, Jingfang, I have very good memories of your stay in the baculogroup, all the best!

En dan al die andere mensen binnen Virologie die me hebben vergezeld deze jaren! Wat hebben we het leuk gehad, met alle borrels, diners, dansavondjes en natuurlijk de befaamde koffiepauze's, die nergens zo goed en gezellig zijn als bij Virologie. Corinne, jij bent in al die jaren de constante factor en toch ook wel een beetje de drijvende kracht achter veel van de gezelligheden geweest! Zonder jou was Viro nergens. Enorm bedankt voor alle gezelligheid deze jaren, voor alle heerlijke taart die je maakte toen ik 30 werd (je hebt wat van me tegoed in mei 2014 ;-), en zeker ook voor alle hulp in het lab. Ook Simon erg bedankt voor de gezelligheid en succes in Delft. Stefan, je bent al even weg, maar het is altijd weer een groot genoegen je te zien! Onze gezamenlijke tijd bij Viro was fantastisch, enorm bedankt daarvoor. Ik heb in Cornwall trouwens nog niemand kunnen vinden om mijn kruimeltjes aan te kunnen geven, typisch hè? Femke ook heel veel succes met je project in Lelystad! Jelke, heel veel succes met je muggenproject, dat gaat vast goedkomen! Mia, ik vind het superleuk dat je mijn paranimf wil zijn. Je werd aio bij ons toen ik al twee jaar bezig was, maar je bent in die tijd heel belangrijk geweest voor de sfeer binnen de groep, met je vrolijkheid en behulpzaamheid en vooral ook je positieve inslag. Heel veel succes met je zalmen! Paulus, al ben je iets later begonnen dan ik, ik heb toch het gevoel dat we samen op zijn gegaan in het aio-traject. Hartelijk dank voor alle gezelligheid afgelopen jaren en het bakjes 'lekkere' koffie halen (de Hosternokke-stelling heeft het helaas niet gered). Ik waardeer het erg dat je mijn paranimf wil zijn, zeker omdat je momenteel zelf tot over je oren in het werk zit. Dank ook dat je mijn repertoire geestelijke liederen altijd zo enorm kon waarderen, maar je was dan ook wel heel dankbaar publiek ;-). Veel succes met de afronding van je proefschrift! Karlijn, veel succes op je school vol brave kindertjes! Dryas, op dezelfde dag begonnen we als groentjes op de Binnenhaven! Onze tijd samen bij Virologie was superleuk. Erg bedankt voor de gezelligheid in Cambridge en in ons 'keethok' met Vera erbij. Heel veel succes met je nieuwe baan in Warmenhuizen! Ook Hannelie, succes in Amsterdam! Patrick, erg bedankt voor je positieve bijdrage aan de sfeer bij Virologie de afgelopen jaren, heel veel succes voor de toekomst! Marcio, take care and good luck with finishing your PhD! Athos, it was very nice sharing the office space with you in the last month of my stay in Wageningen. Wish you all the best for the future!

En dan is er natuurlijk nog een hoop vast personeel bij Virologie met wie ik vaak te maken had. Dick L, heel erg bedankt voor alle hulp in deze jaren maar vooral ook voor alle gezelligheid die je bracht. Ik vind het echt jammer dat ik het nu zonder je grapjes over het rupsen-vlooientheater en de jam moet stellen! Dick P, sinds de verhuizing naar Radix moesten we een 'hok' delen voor onze proefjes, waardoor er menigmaal een trips door het beeld vloog als ik rupsenfilmpjes maakte. Het was heel leuk om je verhalen te horen over je wilde proeven met rijstplanten en ganzen. Gorben, bedankt voor al je adviezen en hulp afgelopen jaren, je voortdurende interesse in het project en je bijdrage aan de goede sfeer! Ook Richard bedankt voor de leuke tijd, je interesse, en de (lange) discussies aan de koffietafel. Beste Jan, hartelijk dank voor alle gezelligheid die je meebracht (ondanks je beruchte verbolgenheid over mijn lawaaimakerij: 'Kan het hier wat stiller, ik probeer te WERKEN!') en je hulp waar nodig. Marleen, erg bedankt voor je vlotte hulp bij administratieve ellende en uit- (elkaar) vallende apparatuur, en zeker ook voor de gezelligheid. Dat je de hele leesversie van mijn thesis hebt geprint en ingebonden voor mij toen ik al in Cornwall zat was ook geweldig, veel dank! Hanke en Janneke, dank voor al jullie hulp en gezelligheid deze jaren!

I was lucky to have quite a number of BSc and MSc students under my supervision that helped me to finish this project in a good way. Christina, Tom, Merve, Aart, Nisa, Susan and Han, thanks a lot for all your great efforts and nice company in the lab! Without your hard work I would have been nowhere. It's great to see that several of you appear or will appear as co-author on a publication! Han, the fact that you are my 'successor' PhD on behavioural manipulation in our lab, binds us in a special way. I'm eager to see how the project will develop. Wish you the very best in the project, I'm convinced you can do it!

For several research projects I worked together with people from other departments or other universities. Jeroen, heel erg bedankt voor al je tijd en moeite bij het introduceren van EthoVision aan Vera en mij. Dat we in jouw kantoor daarmee mochten werken toen we zelf nog geen licentie hadden was ideaal. Nadia, enorm bedankt voor al je snelle hulp bij de statistische analyse van de hyperactiviteitsdata! Kelli, thanks a lot for starting up initial mobility assays during your stay in Wageningen! Lia, jouw hulp bij het analyseren van het door ons vergaarde oerwoud aan klimdata was onontbeerlijk, bedankt daarvoor! Bram, veel dank je voor je snelle analyse van de PTP substraat-samples, erg handig dat je dat wilde doen. Carmen en Esther, bedankt voor het vergaren van de qPCR data van hoofdstuk 4!

I also would like to thank all the people that were part of the PSA project, the collaborative programme between China and the Netherlands. Our China-meeting in 2011

was absolutely fantastic, but also our regular meetings here in the Netherlands were very pleasant. Peter, Berend-Jan, Ineke, Quishi, Li Xin, thanks a lot for the nice time with inspiring talks and interesting discussions!

Joop, Ties en Vera, we moeten het 'bijklussen' aan onze spannende sluipwespenproeven zeker blijven voortzetten in de toekomst! De data zijn veelbelovend, en Trichogramma's zijn geweldig (klein)! Veel dank voor de prettige samenwerking.

For the last couple of years I was involved in organizing the Wageningen Evolution and Ecology Seminar (WEES) series together with a group of PhDs and postdocs. WEES people, thanks a lot for the efforts we made and the fun we had, I think together we did a great job in organizing interesting lectures!

Dank ook aan alle goede vrienden die in de afgelopen jaren voor veel vertier zorgden buiten werktijden. Ard en Thijs, onze culinaire Knopstraat-vrinden, dank voor alle plezier en gezelligheid en al het heeeeeeeeeeeeerlijke eten wat we bij jullie altijd krijgen. Job en Dina, wij hebben elkaar leren kennen door Virologie, is dat niet prachtig! Veel dank voor de gezellig avondjes keuvelen en spelletjes spelen. Bij deze een volgend boek om aan Elin voor te lezen! En dan al mijn lieve 6v-vrienden! Weet je wat, ik noem jullie ALLEMAAL bij naam: Anne (en Maarten), Corine, Els, Froukje (en Hendrik en Maaike), Karin (en *batwoman* Marlotte), Lauke (en Femke), Marijn-Willem, Nelleke, Paulisca (en Sjoerd), Willeke (en Ko). Iedereen dank voor onze ondertussen al 12 jaar durende en nog steeds hechte vriendschap! We zien elkaar weliswaar niet vaak, maar bij elk weerzien is het weer als vanouds. Dat 6v-bejaardentehuis zie ik er nog wel van komen! Mauricio and Fernanda, thanks a lot for your great friendship, it's always nice to see you again. Both of you good luck with finishing your thesis! Martin, dank voor je vriendschap, zet 'm op met het afronden van je proefschrift!

Mijn Friese skoanfamylje: Heit en mem, tige tank voor al jullie goede zorgen in de afgelopen jaren en voor de altijd gezellige weekenden als we bij jullie langs komen! Hessel en Jeannette, erg bedankt voor het verhuizen van onze inboedel naar Cornwall, dat liep allemaal op rolletjes en was ook nog eens heel leuk! Tjalke en Imke, dank voor jullie interesse deze jaren, hopelijk komen jullie eens in Cornwall langs! Hanneke en Remco, dank voor alle gezelligheid en jullie interesse in ons wel en wee!

En dan natuurlijk mijn Zeeuwse familieleden, de Van Houte's! Lieve pa en ma, erg veel dank voor al jullie goede zorgen en nooit aflatende interesse in mij en mijn onderzoek. Het moet niet altijd gemakkelijk zijn om een kind te hebben dat tot voor kort niet vaak tijd had om in de weekenden langs te komen vanwege 'die rupsen' en toen dat eenmaal

achter de rug was er snel tussenuit kneep naar Cornwall. Als echte Anglofielen komen jullie hopelijk snel eens bij ons langs! Maarten en Titia, bij jullie langsgaan in Utrecht is altijd beregezellig, dank voor jullie interesse en vriendschap. Titia, heel erg bedankt voor het maken (om half 3 'ʻs nachts...) van alle mooie rupsen- en mottentekeningetjes die bij elk hoofdstuk terecht zijn gekomen! Kees, ik vind het erg leuk dat we elkaar nu wat vaker zien (nou ja, da's relatief natuurlijk nu ik in Cornwall zit, maar toch!), veel dank voor je grote interesse in mijn onderzoek en voor je gezelligheid!

Als 'icing on the cake' is het laatste stukje voor Edze! Ons gezamenlijk door een aio-schap gaan was een heel drukke tijd (er was vrijwel geen weekend dat een van ons niet aan 't werk was, en meestal allebei), maar het was vooral ook enorm leuk en stimulerend. Zeker in de laatste fase van mijn proeven (vaak tot laat in de avond), kwam je me geregeld avondeten brengen, op een gegeven moment zo vaak dat de receptiedames stikjaloers op me waren! Dank ook voor al die keren dat je me hebt geholpen met figuren maken als ik weer eens gek werd van de computer. We wonen nu al even in Cornwall, waar we volgens mij samen een prima tijd zullen gaan hebben. Wie weet waar de toekomst ons hierna zal brengen!

PE&RC PhD Education Certificate

With the educational activities listed below the PhD candidate has complied with the educational requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities).

Review of literature (4.5 ECTS)
- Walking with insects: molecular mechanisms behind parasitic manipulation of host behaviour

Writing of project proposal (4.5 ECTS)
- Genetic basis of baculovirus-induced insect behaviour

Post-graduate courses (5.4 ECTS)
- A dip into EBI resources; EMBL (2009)
- Molecular phylogenies; EPS (2010)
- Survival analysis; PE&RC (2011)
- Proteomics; VLAG (2011)
- Autumn school host-microbe interactions; EPS (2011)

Invited review of (unpublished) journal manuscript (1 ECTS)
- Insect Molecular Biology: the role of protein kinase C (PKC) in baculovirus infection (2012)

Deficiency, refresh, brush-up courses (1.4 ECTS)
- Basic statistics (2009)

Competence strengthening / skills courses (2.1 ECTS)
- PhD Competence assessment; WGS (2009)
- Techniques for writing and presenting a scientific paper; WGS (2010)
- Presentation skills; WGS (2011)

PE&RC Annual meetings, seminars and the PE&RC weekend (2.1 ECTS)
- PhD Weekend (2009)
- PE&RC Annual Meeting (2009-2012)

Discussion groups / local seminars / other scientific meetings (6.8 ECTS)

- WEES Seminars (2009-2013)
- Experimental Evolution Discussion Group (2010-2013)

International symposia, workshops and conferences (9 ECTS)

- Nederlandse Entomologendag (2009)
- Annual Meeting of the Society for Invertebrate Pathology (2010)
- Meeting of the European Society of Evolutionary Biology (2011)
- 6th International Congress of Molecular Insect Science (2011)
- Annual meeting of the Society for Invertebrate Pathology (2012)
- Meeting of the Society for Molecular Biology and Evolution (2012)
- Dutch Annual Virology Symposium (2013)
- Netherlands Annual Ecology Meeting (2013)

Supervision of 3 MSc students

- The role of the SeMNPV *ptp2* and *egt* gene in baculovirus–induced behaviour
- Substrate identification of baculovirus PTP proteins and investigation of the putative role of SeMNPV PTP2 in the induction of apoptosis
- Localization of the AcMNPV PTP protein in *Spodoptera exigua* larvae

The research described in this thesis was financially supported by the Program Strategic Scientific Alliances of the Royal Dutch Academy of Sciences (project 08-PSA-BD-01).

Printing: Gildeprint, Enschede, the Netherlands.

Pencil drawings: Titia Hoogeveen, www.kaekuri-design.com.

Cover art: S. van Houte.