ON THE EVOLUTION OF *WOLBACHIA*-INDUCED PARTHENOGENESIS IN *TRICHOGRAMMA* WASPS

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Contents

Abstract

Organisms display a great variety of sex ratios (ratios of females vs. males), ranging from 100% females to a male bias. These sex ratios are not always only determined by the genes of the organism itself but may actually often be manipulated or distorted by "sex ratio distorters". One sex ratio distorter, the bacterium *Wolbachia* that lives in the cytoplasm of the cells of its host organism, has received much attention by biologists all over the world. This interest mainly arises from the fact that it manipulates arthropod or nematode reproduction in several ways - feminization, induction of cytoplasmic incompatibility, male-killing and parthenogenesis-induction - to enhance its own inheritance from mother to daughter. Because sperm cells do not contain enough cytoplasm, they cannot transmit *Wolbachia* and males are a dead end for the bacterium. Recent estimates of *Wolbachia*'s prevalence range from 17 to even 76% of the insect species.

In many wasp, thrips and mite species *Wolbachia* has switched the mode of reproduction from sexuality to complete parthenogenesis (±100% females). However, in minute parasitoid wasps of the genus *Trichogramma*, which are used worldwide as natural enemies in biological control of lepidopteran pests, only a part of the females in a population is infected with *Wolbachia* and can therefore reproduce through parthenogenesis. My aim in this thesis is to gain more insight in the dynamics of parthenogenesis-inducing (PI) *Wolbachia* and to explain the coexistence of infected and uninfected forms in *Trichogramma* wasps. After first reviewing the literature on PI *Wolbachia* in **chapter 2**, I tried to further our understanding of the coexistence of the two reproductive forms in natural *Trichogramma kaykai* and *T. deion* populations by combining fieldwork, molecular techniques, behavioural- and crossing experiments with model studies.

Vertical transmission of *Wolbachia* from mother to daughter has been viewed as the main mode of transmission but in **chapter 3 & 4** we show an unexpectedly frequent natural inter- and intraspecific horizontal transmission between and within *Trichogramma kaykai* and *T. deion* larvae sharing a common food source, a butterfly egg. Originally uninfected immature wasps could acquire *Wolbachia* inside the host egg but not all newly infected females exhibit parthenogenesis. In *T. kaykai,* intraspecific

horizontal transfer was followed by complete parthenogenesis in future generations but when *T. kaykai* females received *Wolbachia* from *T. deion*, the infection tended to be lost several generations after interspecific horizontal transfer. Our results largely explain the discordance between *Wolbachia*- and (Trichogrammatid) host phylogenies. Frequent horizontal transfer might select for high virulence in these bacteria.

Because of a nuclear-cytoplasmic conflict between *Wolbachia* and the nuclear genes of *Trichogramma* and the previously described horizontal transfer of *Wolbachia*, the infection is most likely associated with fitness costs in populations where infected and uninfected individuals coexist. In **chapter 5** we show that infected *T. kaykai* suffer a reduced survival compared to uninfected conspecifics when they shared the same host. The survival rate of infected immatures was higher when they competed with other infected immatures from a different infected parent than in competition with uninfected immatures. This shows that PI *Wolbachia*-infected *Trichogramma* can suffer a substantial fitness cost. Because of this reduced competitive ability of infected larvae, horizontal transfer that occurs under the same superparasitism circumstances does not contribute much to an increase in the infection rate in the population.

Previous work showed that the presence of another sex ratio distorter in males, a B chromosome called *PSR* (Paternal Sex Ratio) that destroys the paternal chromosomes after fertilization thereby causing an all-male or a male-biased offspring sex ratio, contributes to a low infection frequency in *T. kaykai*. In **chapter 6** we determined if a *PSR* factor causes low infection frequencies in other species as well. Therefore, we studied natural populations of three *Trichogramma* species - *T. kaykai*, *T. deion* and *T. pratti* - from the Mojave Desert. Our data showed that all the male-biased and all-male *Trichogramma* broods collected from the butterfly *Apodemia mormo deserti* that contained males expressing the *PSR* phenotype, belonged to *T. kaykai*. In laboratory tests, 71.4% of the *T. kaykai PSR* males horizontally transmitted the *PSR* phenotype to *T. deion*. This percentage is comparable to the transmission rate of *PSR* to *T. kaykai* females, namely 81.6%. Consequently, the *PSR* can be transmitted to *T. deion* and we expect this to happen in the field because *T. kaykai* and *T. deion* sometimes emerge from the same butterfly egg. Despite this, we cannot find *PSR* in *T. deion*. Modeling shows that low *Wolbachia* infection frequencies can only be attained when the *PSR* rates are very high. Therefore, other factors should keep the PI *Wolbachia*-infection from spreading to fixation in this species, e.g. nuclear suppressor genes.

The mating structure in the host population plays a major role in the dynamics of PI *Wolbachia* and *PSR*. A *PSR* factor prevents the *Wolbachia* infection from spreading to all the females in *T. kaykai* because uninfected *T. kaykai* females show a high level of sib (brother-sister) mating. Sib mating is a barrier against the destructive effect of mating with a *PSR*-carrying male. Infected females do not have this advantage. Using a population genetic model with microsatellites as genetic markers in **chapter 7**, we estimated high levels of sib-mating of 70% and an off-patch mating of 15%. Thirty-five percent of the patches were estimated to be parasitized by two *T. kaykai* females. Incorporating such levels of sib mating in a previously developed model describing the dynamics of PI *Wolbachia* and *PSR* in a *Trichogramma* population, resulted in stable low frequencies of infection, i.e., a coexistence between infected and uninfected individuals, and of the *PSR* chromosome. Our results show how mating structure allows the two sex ratio distorters to coexist in the population.

The main conclusion from this thesis is that, despite the high vertical transmission and regular horizontal transfer of *Wolbachia*, a PI *Wolbachia*-infection can be attained at low frequencies in *Trichogramma*, due to the presence of a non-mendelian suppressor, like the male-biasing *PSR* factor in *T. kaykai*, but also due to other factors. In *T. deion*, for example, *PSR* does not keep the infection frequency at low levels but a nuclear mendelian suppressor against the PI *Wolbachia* might have evolved.

Next to their significance for the understanding of the evolutionary pathways of *Wolbachia*-host interactions, the results reported in this thesis may also have important implications for future use of natural enemies, and more specifically *Trichogramma* wasps, in inundative biological control. We may now have a good method to render wasps parthenogenetic, via super- or multiparasitism by infected and uninfected females, thereby increasing the efficacy of parasitoid releases against lepidopteran pests.

Dit moet titelpagina **Chapter 1** worden!!!!!!!!!!!

General introduction

1.1 SEX RATIOS

In nature, most organisms with separated sexes produce equal numbers of males and females. These 50% sex ratios (% females) are observed independent of the sex determination mechanism, either genetic, e.g. in mammals (male heterogamety with males being XY and females XX) (reviewed in Fredga 1994), or environmental, e.g. temperature dependency in crocodiles (Janzen & Paukstis 1991). A balanced sex ratio is supported by many theories of which Fisher's study (1930) is one of the most influencing. Certainly not all organisms display a balanced sex ratio: e.g in parasitoid wasps with a haplodiploid mode of reproduction, i.e. fertilized (diploid) eggs develop into females whereas unfertilized (haploid) eggs develop into males, female-biased sex ratios are found according to the Local Mate Competition theory by Hamilton (1967).
Most of the sex ratio theories are based on a sex ratio trait that is determined by nuclear genes that are transmitted equally to the offspring by the father and the mother, i.e. by genes with a mendelian inheritance. However, sex ratios may often be manipulated or distorted by selfish genetic elements that do not have such a mendelian inheritance. These selfish genetic elements are therefore also called sex ratio distorters (reviewed in Stouthamer *et al*. 2002).

1.2 SELFISH GENETIC ELEMENTS

In principle, all genetic elements in a genome have been selected to transmit as many copies of themselves as possible to future generations. Some however gain transmission relative to the rest of the genome. Such selfish genetic elements have been defined as elements that spread through populations despite the costs they may inflict on their hosts (Werren et al. 1988). Examples of such elements include cytoplasmically inherited microorganisms, meiotic drive chromosomes, homing endonucleases, transposable elements and B-chromosomes. They can cover relatively large parts of a genome, e.g. transposons comprise 45% of our human genome (International Human Genome Sequencing Consortium 2001). Selfish genetic elements are assumed to have an important role in several evolutionary processes, e.g. in speciation, extinction and the evolution of sex determination mechanisms (Hurst & Werren 2001). Cytoplasmically

inherited bacteria cause their hosts to produce a higher proportion of female offspring either by killing the male progeny or by converting eggs destined to be males into functional females. Such microbes exhibit this selfish behavior because they can only be vertically transmitted through eggs; sperm cells do not contain enough cytoplasm and being inside a male host therefore is a dead end for the bacteria. Infection with sex ratio distorting microbes causes the sex ratio produced by the hosts to differ from the optimal sex ratio for the nuclear genes. The conflicts between cytoplasmic elements with a maternal inheritance and nuclear genes are called nuclear cytoplasmic conflicts (Cosmides & Tooby 1981).

One cytoplasmic sex ratio distorter, the bacterium ***Wolbachia***, has received much attention by biologists all over the world, mainly because it has evolved several types of reproductive alterations in many different arthropod and nematode species to enhance its own maternal inheritance (O'Neill *et al.* 1997; Bandi *et al.* 2001). Parthenogenetic reproduction is one such alteration caused by *Wolbachia*, first discovered by Stouthamer *et al.* (1990), and now known from many hymenopteran, thrips and mite species (reviewed in Stouthamer 1997; Huigens & Stouthamer 2003 (=Chapter 2)). In this thesis I will focus on the evolutionary pathway of ***Wolbachia*-induced parthenogenesis** in *Trichogramma* wasps, minute parasitoids of lepidopteran eggs. Here, I will first introduce the selfish microbe *Wolbachia*.

1.3 *WOLBACHIA*

Intracellular α-proteobacteria of the genus *Wolbachia* infect a wide variety of insects, mites, isopods and filarial nematodes (O'Neill *et al.* 1997; Werren 1997; Stouthamer *et al.* 1999; Bandi *et al.* 2001). Infected adult host tissues are mainly reproductive tissues, but also haemolymph, glands and nervous tissues. These bacteria were first described in the mosquito *Culex pipiens* by Hertig & Wolbach (1924) and later on named *Wolbachia pipientis* (Hertig 1936). Some decades later, Yen & Barr (1971) discovered that a reproductive alteration in mosquitoes was induced by *Wolbachia*. Cytoplasmic incompatibility (CI) occurred when uninfected female mosquitoes mate with *Wolbachia* infected males: In such crosses the fertilized eggs die.

Studies on *Wolbachia* have expanded tremendously in the last decade after the application of various molecular (mainly PCR-based) methods. Recent surveys estimate *Wolbachia* to be present in 17 % or even up to 76% of the arthropods and nematodes

tested (Werren *et al.* 1995*a*; Bandi *et al.* 1998; Bouchon *et al.* 1998; Wenseleers *et al.* 1998; Jeyaprakash & Hoy 2000). Only one survey in molluscs did not reveal any infection (Schilthuizen & Gittenberger 1998). The interest amongst scientists for these bacteria largely arises from the fact that they are widespread and impose various reproductive alterations on their host. They are therefore seen as one of the evolutionary forces behind speciation and the bewildering variety of sex determination mechanisms in arthropods, and even other animals. Also, from an applied perspective, scientists are interested in their possible role in a) biological control, either as vector for the spread of genetic modifications (Sinkins *et al.* 1997) or to enhance the efficacy of parasitoids used as natural enemies (Stouthamer 1993), and b) (human) diseases caused by filarial nematodes (Bandi *et al.* 2001; André *et al.* 2002).

1.4 PHYLOGENY OF *WOLBACHIA*: EVIDENCE FOR HORIZONTAL TRANSFER

Wolbachia are closest related to rickettsia-like bacteria: they form a monophyletic cluster relative to other Rickettsiae (Stouthamer *et al.* 1993; O'Neill *et al.* 1992). In general, *Wolbachia* can be divided into five groups, A-E (Werren *et al.* 1995*b*; Bandi *et al.* 1998; Vandekerckhove *et al.* 1999). The A- and the B-group contain the arthropod-*Wolbachia*, whereas the nematode-*Wolbachia* form group C and D. Group E exists of only a single *Wolbachia* infection in the springtail *Folsomia candida* (Vandekerckhove *et al.* 1999). This *Wolbachia* is more closely related to group A and B than to C and D. A-B and C-D seem to have diverged approximately 100 million years ago, after which A and B diverged close to 60 Million years ago (Bandi *et al.* 1998).

Phylogenies of *Wolbachia* have been based on several genes, including 16S rDNA, *ftsZ*, *groEl* and *wsp* (reviewed in Stouthamer *et al.* 1999). The latter, *wsp* (*Wolbachia* Specific Protein, a cell surface protein), is now commonly used for the classification of *Wolbachia*. Independent of the studied gene, all phylogenies show a discordance between *Wolbachia*- and host phylogenies in the A- and the B-group: closely related *Wolbachia* can be found, and cause different reproductive alterations, in very diverse hosts (Werren 1997; Stouthamer *et al.* 1999). This lack of congruence indicates that horizontal transfer of *Wolbachia* must have taken place on an evolutionary time scale. Such transfer is mainly considered a rare event (Stouthamer *et al.* 1999; Cook & Butcher 1999). Only in filarial nematodes there seems to have been a striking co-

evolution of *Wolbachia* and host (*Wolbachia*- and nematode phylogenies are completely congruent) (Casiraghi *et al.* 2001)

1.5 REPRODUCTIVE ALTERATIONS CAUSED BY *WOLBACHIA* OTHER THAN PARTHENOGENESIS

1.5.1 *Cytoplasmic incompatibility*

The most widely distributed reproductive alteration caused by *Wolbachia* is cytoplasmic incompatibility (CI): an incompatibility between sperm and egg induced by *Wolbachia* results in an elimination of the paternal chromosomes. In diploid species such fertilized eggs will die (Yen & Barr 1971) whereas in haplodiploid species, the fertilized eggs will also die (Female Mortality CI type) (Breeuwer 1997; Vavre *et al.* 2000) or develop into males (Male Development CI type) (Breeuwer & Werren 1990; Giordano *et al.* 1997). More recently, Vavre *et al.* (2001) even described a third type of CI in the parasitoid wasp *Leptopillina heterotoma* in which some fertilized eggs of a single female die and others develop into males (Intermediate CI type). CI is known from many insect orders (for a review, see O'Neill *et al.* 1997), from several mite (Breeuwer 1997) and one isopod species (Rigaud & Rousset 1996). Phylogenetic analysis shows that CI-*Wolbachia* fall both in the A and the B-group. Most CI is unidirectional: crosses between infected males and uninfected females are incompatible whereas the reciprocal cross between infected females and uninfected males is compatible (Werren 1997). Bidirectional CI occurs when males and females harbor a different *Wolbachia* strain that are mutually incompatible (O'Neill & Karr 1990; Mercot *et al.* 1995; Perrot-Minnot *et al.* 1996). The latter form of CI is especially interesting with respect to speciation since it facilitates reproductive isolation. In *Nasonia* this type of CI most likely preceded other post-zygotic isolation mechanisms and may have played a role in speciation in this genus (Bordenstein *et al.* 2001). *Wolbachia* infections expressing CI can spread rapidly through a population by reducing the uninfected part of the population: infected eggs are compatible with both infected and uninfected sperm whereas uninfected eggs are only compatible with uninfected sperm. Theoretical and experimental work has shown that infection expressing CI are selectively favoured and can spread to fixation, i.e., a situation where all females in the population are infected (Turelli & Hoffmann 1991; Turelli 1994).

1.5.2 *Feminization*

In amphipod and terrestrial isopod crustaceans that display female heterogamety (males are ZZ and females ZW), genetic males are being feminized into functional females when infected with *Wolbachia*. This form of feminization does not only occur in these crustaceans: Recently *Wolbachia* was also found to be associated with feminization in two butterfly species (Hiroki *et al.* 2002; Kageyama *et al.* 2002). Feminizing (F) *Wolbachia* may play an important role in the evolution of sex determination in crustaceans since feminization would lead to a loss of female heterogamety. This idea is supported by the occurrence of several populations of the isopod *Armadillidium vulgare* that do not harbor ZW females (Rigaud 1997). A survey estimated 35 % of the terrestrial isopod species to be infected with *Wolbachia* (Bouchon *et al.* 1998). All *Wolbachia* in isopods fall within the B-group (Bouchon *et al.* 1998). All but one of the *Wolbachia* in the Oniscoidae form a monophyletic group but in other isopod groups *Wolbachia* seem to be more distantly related. The infection rates in populations vary to quite some extent but are, with the exception of one species that was fixed for the infection, generally between 10-50%. *A. vulgare* is the best studied example and in this species, the presence of F *Wolbachia* seem to have led to the evolution of suppressor (in this case masculinizing) genes to counteract the effect of *Wolbachia* in some populations (Rigaud & Juchault 1993).

1.5.3 *Male-killing*

Already in 1947, Lus recorded maternally inherited factors to kill male progeny during embryogenesis in the ladybird beetle *Adalia bipunctata*. At present, six different bacteria have been described to be associated with male-killing (MK) suggesting this trait has more easily evolved in microbes than other reproductive alterations (Stouthamer *et al.* 1999). Beneficial effects of MK are assumed to be 1) a decrease in the rate of inbreeding, 2) a direct access to resources through cannibalism on the dead males, and 3) a decrease in the level of antagonistic interactions between siblings (Hurst *et al.* 1997). MK *Wolbachia* are currently known to infect *A. bipunctata* and the butterfly *Acraea encedon*. With the ladybirds being male heterogametic and the butterflies female heterogametic this suggests *Wolbachia* has also evolved different mechanisms to recognize the host sex. Infection rates vary tremendously, namely 20-30% of the females in Russian populations of *A. bipunctata* and 80% of the females of

A. encedon (Hurst *et al.* 1999). Details on male-killing induced by *Wolbachia* are still lacking as this field of research is now starting to emerge.

1.5.4 *Fecundity and fertility increase*

In the parasitoid wasp *Trichogramma bourarache* the transmission of *Wolbachia* is enhanced by an increase in the offspring production of infected females (Girin & Bouletreau 1995; Vavre *et al.* 1999). Infected females produce on average twice as many offspring as conspecifics cured from their infection through antibiotic treatment. This *Wolbachia* was shown to fall in the A-group (Vavre *et al.* 1999). Unfortunately, experimental and theoretical work on this fecundity effect has not been continued so far. Similar phenomena after antibiotic treatment were shown for CI-*Wolbachia* in *Drosphila simulans* and *Nasonia vitripennis* but results were rather inconclusive (Poinsot & Mercot 1997; Stolk & Stouthamer 1995). We can also find two cases where *Wolbachia* positively affects the fitness of its host. In a stalked eyed fly, *Sphyracephala beccarri*, and the flour beetle, *Tribolium confusum*, fertility of infected males was higher than that of cured males (Wade & Chang 1995; Hariri *et al.* 1998). Here, *Wolbachia* seem to have evolved a, somewhat, mutualistic behavior.

1.5.5 Wolbachia *essential for oogenesis*

Very recently, Dedeine *et al.* (2001) found a *Wolbachia* variant to be essential for oogenesis in the parasitoid wasp *Asobara tabida*. All female *A. tabida* in French populations are infected with three *Wolbachia* variants (based on the *wsp* gene), of which two induce CI and one is involved in oogenesis. Wasps cured from the latter variant by antibiotic treatment fail to produce mature oocytes. Similar phenomena have been described in nematode *Wolbachia*, where antibiotic treatment had detrimental effects on two species of filarial nematodes, *Brugia pahangi* and *Litomosoides sigmodontis* (Bandi *et al.* 2001). In this case, a long co-evolution between host and symbiont has lead to a situation where the host has become dependent on *Wolbachia*, i.e. an obligatory symbiosis.

1.6 *WOLBACHIA*-INDUCED PARTHENOGENESIS IN *TRICHOGRAMMA* SP.

Wolbachia is known to induce parthenogenesis in 14 of approximately 180 *Trichogramma* species known (Pinto 1999; Huigens & Stouthamer 2003 (=Chapter 2)).

Trichogramma displays a haplodiploid reproduction mode in which daughters (diploid) arise from fertilized eggs and sons (haploid) from unfertilized eggs. Females infected with parthenogenesis-inducing (PI) *Wolbachia* however produce daughters from both their fertilized and unfertilized eggs. Unfertilized infected eggs develop into females because of a doubling of the haploid set of maternal chromosomes in the first mitotic division, a process called gamete duplication (Stouthamer & Kazmer 1994). This form of parthenogenetic reproduction can be cured after antibiotic treatment (Stouthamer *et al.* 1990).

Wolbachia in *Trichogramma* sp. are unique compared to almost all other *Wolbachia*-host associations because these *Wolbachia* cluster together in the B-group in all phylogenetic trees based on several *Wolbachia* specific genes (Stouthamer *et al.* 1999). A detailed phylogenetic analysis of the *Wolbachia-Trichogramma* sp. association revealed an obvious discordance between *Trichogramma* phylogeny and *Wolbachia* phylogeny that is most likely explained by horizontal transfer of *Wolbachia.* These *Wolbachia* all seem to have a common ancestor and have only shifted from one species in this genus to another (Schilthuizen & Stouthamer 1997).

Parthenogenesis-inducing (PI) *Wolbachia* in *Trichogramma* differ from those in other host species because infections have not reached fixation in host populations, i.e. all females in the population are infected. In all *Trichogramma* species except two, we find low infection frequencies, what seems to be a unique situation for PI *Wolbachia* (Stouthamer 1997). Only *Trichogramma cordubensis* and *T. oleae* populations are fixed for the infection. Theoretical work has shown that stable low infection rates amongst females require some form of suppressing element of *Wolbachia* or its effect when infected females produce at least as many daughters as uninfected females and the transmission fidelity of *Wolbachia* from mother to daughter is high (Stouthamer 1997). In *Trichogramma kaykai*, where 6-26% of the females are infected, an extensive search for elements suppressing the spread of *Wolbachia* brought to light the presence of another selfish genetic element. About 10% of the males carry a *PSR* (Paternal Sex Ratio) chromosome (Stouthamer *et al.* 2001). Until then such a *PSR* chromosome was only found in the parasitoid wasp *Nasonia vitripennis* (Werren 1991). When a female mates with a *PSR*-carrying male, the paternal chromosomes in fertilized eggs are functionally destroyed and only the maternal chromosomes and the *PSR* factor itself remain. Such fertilized eggs develop into sons that carry a haploid set of chromosomes

from the mother and the *PSR* chromosome from their father. *PSR* therefore causes uninfected and infected females to produce sons from their fertilized eggs. In uninfected *T. kaykai* a large fraction of the uninfected females mate with their brothers on the natal patch and are thereby protected against the destroying effect of the *PSR* factor. In contrast almost all infected females emerge from a host with only sisters and mate with unrelated males from the population, and 10% of these are carriers of *PSR*. The consequence of this asymmetry is that the higher daughter production of the infected population is suppressed more than that of the uninfected population. Such a mating structure and presence of a *PSR* factor should allow a stable coexistence of both forms within the population (Stouthamer *et al.* 2001).

In populations where infected and uninfected individuals coexist, a nuclear cytoplasmic conflict between the *Wolbachia* and the nuclear genome of the host is expected. *Wolbachia* favors a 100% female bias whereas the nuclear genes favor a sex ratio involving at least some males. In these situations an arms race between the nuclear genes and those of the *Wolbachia* may result in nuclear genes trying to suppress the *Wolbachia* or its effect. Consequently, a much higher physiological cost of being infected is expected in mixed host populations. The reduced offspring production by infected *Trichogramma* females from mixed populations might result from the nuclear cytoplasmic conflict (Stouthamer & Luck 1993; Stouthamer *et al.* 1994).

In Chapter 2 I will expand the discussion about the work done on parthenogenetic reproduction associated with *Wolbachia* in approximately the past 12 years after its first discovery by Stouthamer *et al* (1990).

1.7 BIOLOGY OF *TRICHOGRAMMA* SP. IN THE MOJAVE DESERT

Trichogramma kaykai, *T. deion* and *T. pratti* occur sympatrically in the Mojave Desert of the American Southwest (Pinto 1999). *T. kaykai* (Figure 1.1a) and *T. deion* are both infected with PI *Wolbachia* and comprise of mixed populations (Stouthamer 1997; Stouthamer *et al.* 2001). All three species parasitize eggs of the mormon metalmark butterfly *Apodemia mormo deserti* (Lepidoptera, Lycaenidae) (Figure 1.1c).

According to literature, the subspecies *A. m. deserti* has two generations per year, one flies from March to April and the other from September to November (Emmel & Emmel 1973). We, however, also found eggs in the end of June suggesting a second generation in spring.

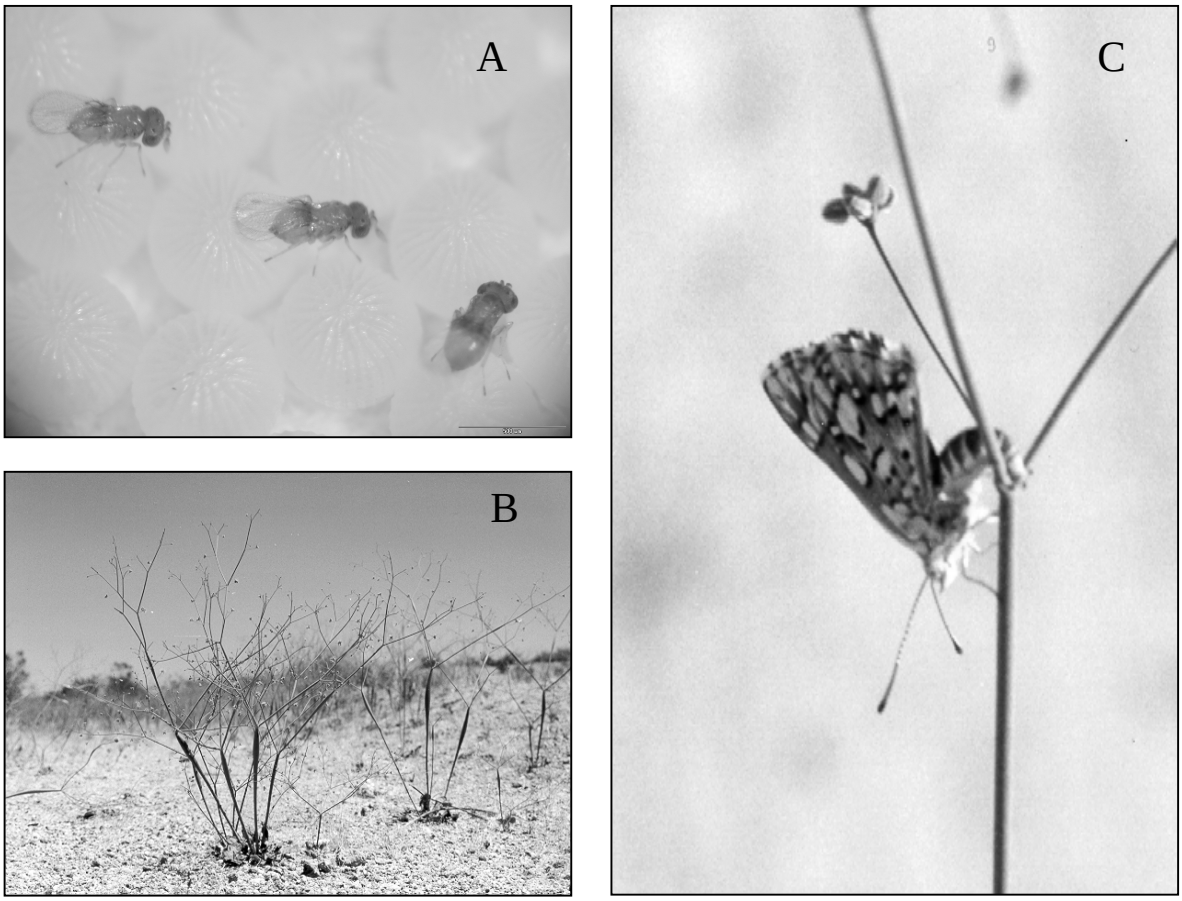

Figure 1.1 a) *Trichogramma kaykai* females parasitizing *Mamestra brassicae* eggs under laboratory conditions (Photo Nina Fatouros). b) *Eriogonum inflatum* (Photo Richard Stouthamer). c) *Apodemia mormo* female ovipositing on *E. inflatum* (Photo Marc Maas)

A. m. deserti uses *Eriogonum inflatum* (Polygonacae), known as desert trumpet, as its larval host plant (Figure 1.1b). This patchily distributed perennial is found throughout the Mojave Desert mainly growing near washes, roadsides and on foothills (Munz 1959). It has grey-green to green stems originating from a basal rosette. The upright stems have a large number of trichotomous and/or dichotomous branchings. Some parts of the 10 to 120 cm tall stems are inflated (Munz 1959). The flowers are yellow and the major flowering period is in spring (Stone & Mason 1979). It is on the branchings of the stems where *A. m. deserti* lays its eggs (Figure 1.1c).

From 1998-2001 we yearly monitored natural *Trichogramma* populations on *A. m. deserti* for PI *Wolbachia*-infection rate and the presence of other factors, like the *PSR* chromosome, that may help explaining a stable coexistence of infected and uninfected individuals. *T. kaykai* was by far more abundant on *A. m. deserti* than the two other

species. We sometimes found wasps of two different species emerging from a single host egg (Chapter 6).

1.8 AIM AND OULINE OF THIS THESIS

The main aim of this study is to expand our understanding of the evolutionary pathways of *Wolbachia*-induced parthenogenesis in *Trichogramma* wasps. Or more specifically: we want to explain the coexistence of infected and uninfected forms in these parasitoids. Stouthamer *et al.* (2001) showed in a more theoretical study the presence of a *PSR* factor in males to mainly contribute to stable low PI *Wolbachia*-infection rates in *Trichogramma kaykai*, thereby taking into account the egg-fertilization rate and the sib-mating frequency amongst uninfected individuals. Two other hypotheses of a) nuclear supressor genes and b) bacterial transmission could not explain the low levels of infection in this species. Modelling the latter hypothesis, Stouthamer *et al.* (2001) calculated the equilibrium infection rate as a function of the vertical transmission efficiency of *Wolbachia* and fitness costs associated with *Wolbachia* infection in terms of offspring production.

The evolutionary pathway of a PI *Wolbachia*-host interaction is, however, most likely determined by more elements that each play their own role in the trajectory leading to a loss of the infection, fixation or stable low infection frequencies. In this thesis I test and discuss several new elements that may have important implications for the evolution of *Wolbachia*-induced parthenogenesis (and also *Wolbachia*-host interactions in general) using a combination of extensive field work, behavioral experiments and the application of molecular methods.

Chapter 2: Here, most of the work done on *Wolbachia*-induced parthenogenesis in approximately the past twelve years is reviewed. The discovery of symbiotic microbes has changed the perspective on parthenogenetic reproduction. Research on this topic has revealed that many cases of parthenogenetic reproduction in arthropods are actually forced upon them by intracellular *Wolbachia* bacteria and not regulated by the genes of the host itself. I discuss PI *Wolbachia* research on different levels, from the cytogenetic mechanisms of PI to the evolution and dynamics of infections in host populations.

Chapter 3: The mode by which *Wolbachia* can be transmitted has important implications for the co-evolution of *Wolbachia* and their host. *Wolbachia* have evolved several host reproduction manipulations because of their vertical cytoplasmic inheritance. Such vertical transmission of *Wolbachia* has been viewed as the main mode of transmission. Here I test if horizontal transfer of PI *Wolbachia* from infected to uninfected *Trichogramma kaykai* larvae can occur when they share a common food source. Such horizontal transfer might select for higher virulence in these bacteria and result in relatively high fitness costs for the host compared to a situation of pure vertical transmission of *Wolbachia*.

Chapter 4: *Wolbachia*- and host phylogenies are not congruent, as is also the case with PI *Wolbachia* and their Trichogrammatid hosts. Horizontal transfer of PI *Wolbachia* when two species share the same food source might explain the discordance between the phylogenies. I investigate if inter- and intraspecific transfer is common in *Trichogramma* wasps under those conditions. In this chapter I discuss why some *Wolbachia* might be transmitted horizontally more easily than others and emphasize that, despite the fact that we might not detect horizontal transfer in certain cases, it might occur frequently enough on an evolutionary time scale to explain the discordance between *Wolbachia*- and host phylogenies.

Chapter 5: Much of the work done on *Wolbachia* has focussed on the evolution of the main reproductive alterations it is associated with but few studies have actually looked at the costs these bacteria may inflict on their host. In this chapter we focus on direct effects of PI *Wolbachia* on their host's fitness. I test if PI *Wolbachia* bacteria reduce competitive ability of *Trichogramma kaykai* under situations of low and high intraspecific competition for food sources. Moreover, I discuss why a reduction is to be expected when populations of infected and uninfected individuals co-occur and how the effect of PI *Wolbachia* on competitive ability influences the spread of the bacteria in the field.

Chapter 6: In this chapter I discuss the ecology and population dynamics of PI *Wolbachia* in different *Trichogramma* populations, thereby expanding our understanding of the coexistence between infected and uninfected forms that has so far

mostly been studied in *T. kaykai*. I first describe the PI *Wolbachia* infection rates in natural populations of *T. kaykai, T. deion* and *T. pratti* found during the field seasons of 1998, 1999, 2000 and 2001 and subsequently test if the *PSR* factor might explain low infection rates in *T. deion* as it does in *T. kaykai,* i.e. if a *PSR* factor suppressing the PI *Wolbachia* infection is a more common phenomenon.

Chapter 7: Sex ratio distorting elements are expected to have played a major role in the evolution of different sex ratio strategies. How strong their selection pressure on the host's sex ratio can be, largely depends on the mating structure of the host population. In this study I investigate the role of mating structure in the evolution of PI *Wolbachia* and the *PSR* factor in *Trichogramma kaykai*. More specifically, I estimate the level of sib (brother-sister) mating in uninfected *T. kaykai* using a population genetic model in which we use microsatellites as genetic markers. Such sib mating acts as a barrier against the destructive effect of mating with *PSR*-carrying males thereby indirectly influencing the suppressive effect of the *PSR* factor on the PI *Wolbachia* infection in a population.

Chapter 8: In the general discussion and summary, I first review the most important results from the studies described in the previous chapters. Thereafter inter- and intraspecific horizontal transmission of *Wolbachia* (Chapter 3 & 4), host fitness (Chapter 5), the presence of other selfish genetic elements, like the *PSR* factor (Chapter 6) and mating structure of the host population (Chapter 7) and, are synthesized with previous work on PI *Wolbachia* to understand more of the evolutionary pathways of *Wolbachia*-induced parthenogenesis and, more specifically, to explain the stable coexistence between PI *Wolbachia* infected and uninfected forms in *Trichogramma* wasps. I suggest directions for future research and also stress the importance of the results for future applications of parasitoids in biological control.

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Dit moet titelpagina **Chapter 2** worden!!!!!!!!!!!

Parthenogenesis associated with *Wolbachia*

2.1 PARTHENOGENESIS MEDIATED BY MICROBIAL INFECTIONS

Since the nineteenth century biologists have been puzzled by the fact that sexual reproduction is so common and only some, mostly lower, organisms have parthenogenetic reproduction. Parthenogenetic reproduction has advantages due to its simplicity, efficiency, effectiveness and it is inexpensive (Crow 1994). For an individual, many disadvantages are associated with sex, such as finding a mate, competition for mates and the chance of obtaining sexually transmitted diseases. In one of the many reviews on the advantages of sexual reproduction, Crow (1994) mentions two of the most plausible explanations. The “oldest” explanation is that greater genetic variability in a population through sex can keep up the population with changes in the environment Second, harmful mutations can be eliminated more easily through recombination and therefore do not accumulate according to the Muller’s ratchet (Felsenstein 1974), as happens in parthenogenetic populations (Muller 1964). Most studies on the advantages of sexual reproduction are theoretical and hypotheses remain very difficult to test experimentally.

Recent studies on different modes of reproduction have forced us to change our perspective, because the evolutionary forces behind parthenogenetic reproduction are often not the genes of the organism itself but those of their bacterial symbionts (Stouthamer *et al.* 1990a). In this chapter we will focus on microbe-induced parthenogenesis.

Studies on extraordinary sex ratios by Hamilton (1967, 1979) and Cosmides & Tooby (1981) suggested that the differences in inheritance of cytoplasmic factors and nuclear genes could result in a conflict over offspring sex ratios. While cytoplasmic factors that are maternally inherited benefit from an offspring sex ratio of 100% females, nuclear genes located on autosomal chromosomes favor an optimal sex ratio with at least some male offspring in populations that are able to reproduce sexually. Many cytoplasmically inherited factors cause female-biased sex ratios (Werren *et al.* 1988; Wrensch & Ebbert 1993), but the extreme bias of 100% females and the induction of parthenogenesis were shown for the first time by Stouthamer *et al.* (1990*a*). In many cases microbe-induced parthenogenesis may not be optimal for the organism (host), but it is forced upon them

by the fitness advantages their symbionts derive from their host's parthenogenetic reproduction.

These parthenogenesis-inducing (PI) symbionts inhabit the cytoplasm and can therefore be transmitted vertically only through egg cells. Males are considered to be a dead end for the symbionts. By inducing parthenogenesis the symbionts enhance their own transmission despite the cost they may inflict on their host. Two different microbes have been described to be associated with parthenogenesis in insects. A potential third case was found in nematodes.

At present the most common symbiotic microbe found to induce parthenogenesis is the bacterium *Wolbachia pipientis*, named by Hertig (1936). It was first identified as the microbe inducing parthenogenesis in parasitoid wasps of the genus *Trichogramma* (Rousset *et al.* 1992; Stouthamer *et al.* 1993). In general *Wolbachia* was thought to be unique in its ability to induce parthenogenesis, but recently Zchori-Fein *et al.* (2001) found an undescribed vertically transmitted bacterium also associated with parthenogenesis. The microbe was found in six parthenogenetic *Encarsia* species. *Encarsia* is a genus of parasitoids that uses whiteflies as their host. The bacterium belongs to the Cytophaga-Flavobacter-Bacteroides (CFB) group, thus being unrelated to *Wolbachia.* Bacteria belonging to the CFB group are also capable of inducing feminization (Weeks *et al*. 2001). Vandekerckhove *et al.* (2001) discovered a potential third case of microbial involvement in parthenogenesis. They detected a verrucomicrobial species that is maternally transmitted and seems to be associated with parthenogenesis in the nematode species *Xiphinema americanum*. These bacteria are the first endosymbionts found among the *Verrucomicrobia*. More experimental evidence is needed to show that these bacteria do indeed induce parthenogenesis.

This chapter describes current knowledge of PI *Wolbachia* in insects. Because studies on the involvement of the CFB bacterium and the verrucomicrobial species in parthenogenesis have just started, these endosymbionts will only be mentioned briefly.

2.2 *WOLBACHIA*-INDUCED PARTHENOGENESIS IN HAPLODIPLOIDS

The main requirement for parthenogenesis-induction (PI) by *Wolbachia* seems to be a haplodiploid mode of reproduction of the host species. Haplodiploidy is known from the arthropod groups Hymenoptera, Acari, Thysanoptera and a few genera in the Coleoptera (Wrensch & Ebbert 1993). However, there is no fundamental reason why species with a diplodiploid sex-determination system should not be vulnerable to microbial induction of parthenogenesis.

Haplodiploidy, or arrhenotoky, is a mixture of parthenogenetic and sexual reproduction; males develop from unfertilized (haploid) eggs whereas females develop from fertilized (diploid) eggs (Hartl & Brown 1970; White 1973). A completely parthenogenetic mode of reproduction is thelytoky. In thelytoky all eggs, fertilized or not, develop into females. In arrhenotoky only a single barrier has to be overcome for the induction of parthenogenesis, namely diploidization of the unfertilized egg. An additional barrier to complete parthenogenesis exists in diplodiploid species: egg development also must be induced, which is normally initiated by sperm penetration in sexual species. The terminology surrounding parthenogenesis in haplodiploids is complicated and is clearly in need of revision (Luck *et al*. 1992, Stouthamer 1997), in this thesis I will call arrhenotoky and thelytoky respectively sexual and parthenogenetic reproduction.

The first indications for microbial involvement in parthenogenesis were found through exposure of the parthenogenetic parasitoid wasp *Habrolepis rouxi* to elevated rearing temperatures (Flanders 1945). Females reared at 26,6^{o}C or less produced only daughters whereas females reared at 32,2^{o}C produced sons and daughters or only sons. Several other studies confirmed male production by thelytokous wasps at higher temperatures (Flanders 1965; Bowen & Stern 1966; Orphanides & Gonzalez 1970; Jardak *et al.* 1979; Wilson & Woolcock 1960*a*,*b*; Cabello & Vargas 1985; Laraichi 1978).

To determine the genetic basis of parthenogenesis, Stouthamer *et al.* (1990*b*) backcrossed the nuclear genome of a temperature-treated parthenogenetic line of *Trichogramma pretiosum* into a sexual line of the same species. If the parthenogenesis trait had been inherited through genes on the chromosomes backcrossed females, when kept at low rearing temperatures, should reproduce by parthenogenesis. After nine generations of backcrossing, unmated *T. pretiosum* females still produced only male offspring. Therefore they concluded that parthenogenesis was not caused by a simple Mendelian trait and might be caused by a cytoplasmic factor. Strong evidence for

microbial involvement came from feeding parthenogenetic *Trichogramma* wasps antibiotics (Stouthamer *et al.* 1990*a*). Antibiotic treatment (Tetracycline hydrochloride, sulfamethoxazole and rifampicin) caused male offspring production and reverted females from parthenogenetic to sexual reproduction. Temperature treatment had the same effect. Three years later, Stouthamer & Werren (1993) showed the presence of microbes in eggs of parthenogenetic *Trichogramma* females. They were absent in eggs from lines cured by antibiotic treatment and in field-collected sexual lines. The microbes were identified as *Wolbachia* (Rousset *et al.* 1992; Stouthamer *et al.* 1993). Grenier *et al.* (1998) provided the definite proof of *Wolbachia* as the causal agent of parthenogenesis. They infected eggs of the sexual species *Trichogramma dendrolimi* with PI *Wolbachia* from a *T. pretiosum* line through microinjection. After several generations the *Wolbachia* was still present in *T. dendrolimi* and a low level of parthenogenesis was induced. Pintureau *et al*. (2000*b*) tested the dynamics of infection in two transfected *T. dendrolimi* lines and found the frequency of infected females to decrease dramatically from 52,9% in generation 44 to 3,7% in generation 60 in one line and from 75,5% in generation 32 to 4,5% in generation 48 in the other line.

Recently we found evidence for horizontal transfer of PI *Wolbachia* under natural conditions, followed by complete expression of the parthenogenesis (Huigens *et al.* 2000 (=Chapter 3)). When infected and originally uninfected *T. kaykai* larvae share the same food source, a butterfly egg, approximately 40% of the female offspring of the uninfected line acquire the infection and produce some daughters from unfertilized eggs. In subsequent generations perfect (100%) transmission of, and PI by, *Wolbachia* was observed. This study, together with the work of Grenier *et al.* (1998), showed that *Wolbachia* was the causal agent of parthenogenesis in *Trichogramma*.

It still remains unclear how common *Wolbachia*-induced parthenogenesis is. So far, it has been detected mainly in Hymenoptera because the reproduction in these wasps has been studied intensively for their application in biological control (Stouthamer, 1997). Sixty six hymenopteran species have been reported as being most likely infected with a PI microbe. *Wolbachia* was detected in 56 of these species (Table 2.1A), 14 cases are unknown (Table 2.1B) and 6 *Encarsia* species are infected with the CFB bacterium.

In some parthenogenetic species, there are strong indications of PI by microbes other than *Wolbachia*, as is the case in *Galeopsomyia fausta*, where evidence for microbial

involvement was found through antibiotic treatment, but *Wolbachia* could not be detected (Argov *et al.* 2000).

Outside the Hymenoptera, Werren *et al* (1995) detected *Wolbachia* in a parthenogenetic beetle *Naupactus tesselatus* but it remains uncertain if *Wolbachia* causes parthenogenesis in this species. Pintureau *et al.* (1999) found *Wolbachia* in two parthenogenetic thrips species, *Heliothrips haemorrhoidalis* and *Hercinothrips femoralis*. Antibiotic or heat treatment should show *Wolbachia*‘s involvement in their parthenogenesis. Arakaki *et al.* (2001*a*) showed the first strong evidence of PI by *Wolbachia* in the predatory thrips *Franklinothrips vespiformis*. In this case, a population fixed for the infection and completely parthenogenetic is found on a Japanese island. Sexual populations occur in Central and South America.

In Acari, the only “non insect” order with haplodiploidy, parthenogenesis is widely distributed. In oribatid mites whole families reproduce parthenogenetically (Norton *et al.* 1993). Perrot & Norton (1997) tested eight oribatid species for the presence of *Wolbachia* but could not find the symbiont. Weeks & Breeuwer (2001) found *Wolbachia* infection associated with parthenogenesis in six species within the phytophagous mite genus *Bryobia*. Through antibiotic treatment they showed that in two of those species, *Bryobia praetiosa* and an unidentified species, the *Wolbachia* infection was strictly associated with parthenogenesis.

Table 2.1 A) Cases of parthenogenetic reproduction in which evidence exists of *Wolbachia* involvement. B) Cases of parthenogenetic reproduction in which evidence exists of microbial involvement.
The evidence is classified as males following heat treatment (h), males following antibiotic treatment (a), molecular evidence for *Wolbachia* presence (w). In addition information is given if the parthenogenetic forms are found in populations where parthenogenesis is fixed in the population or if it occurs mixed with sexual reproduction (p), and if the males and females are capable of successful copulations (c).

A) **Taxon**	**h**[1]	**a**[1]	**w**[1]	**p**[2]	**c**[3]	**reference**
			Insecta **Hymenoptera**			
Pteromalidae						
Muscidifurax uniraptor	+	+	+	f	-	Legner 1985*a,b* Stouthamer *et al*. 1993 Stouthamer *et al.* 1994
Spalangia fuscipes	?	?	+	?	?	Werren *et al.* 1995 van Meer *et al.* 1999
Aphelinidae						
Aphytis chilensis	?	?	+	?	?	Gottlieb *et al*. 1998
A. chrysomphali	?	?	+	?	?	Gottlieb *et al*. 1998
A. diaspidis	?	?	+	?	?	Zchori-Fein *et al*. 1994 Zchori-Fein *et al.* 1995
A. lignanensis	?	+	+	?	+[4]	Zchori-Fein *et al*. 1994 Zchori-Fein *et al.* 1995
A. yanonensis	?	+	+	?	?	H. Nadel pers. com. Werren *et al.* 1995
Encarsia formosa	?	+	+	f	-	Zchori-Fein *et al*. 1992 van Meer *et al*. 1995 Werren *et al*. 1995
Eretmocerus staufferi	?	?	+	?	?	van Meer *et al*. 1999
E. mundus	?	+	+	f	+[4]	De Barro & Hart 2001
Platygasteridae						
Amitus fuscipennis	?	?	+	?	?	van Meer *et al*. 1999 Manzano *et al*. 2000
Encyrtidae						
Aponanagyrus diversicornis	+	+	+	f	+[5]	Pijls *et al*. 1996 van Meer, 1999
Coxxidoxenoides peregrinus	+	?	+	f	+	Flanders 1965 van Meer *et al*. 1999
Scelionidae						
Telonomus nawai	+	+	+	f	+[4]	Arakaki *et al*. 2000
Trichogrammatidae						
Trichogramma brevicapillum	+	+	+	m	+	Stouthamer *et al*. 1990*a,b* Werren *et al*. 1995
T. chilonis	+	+	+	m	+	Stouthamer *et al*. 1990*a,b* Chen *et al*. 1992 Schilthuizen & Stouthamer 1997
T. cordubensis	+	+	+	f	+	Cabello & Vargas 1985 Stouthamer *et al*. 1990*b* Stouthamer *et al.* 1993 Silva & Stouthamer 1996
T. deion	+	+	+	m	+	Stouthamer *et al*.

						1990*a,b* Stouthamer *et al*. 1993
T. embryophagum	+	+	+	?	+	Birova 1970 Stouthamer *et al*. 1990*b* Almeida pers. com.
T. evanescens (rhenana)	+	+	?	?	+	Stouthamer *et al*. 1990*b* H. van Oosten pers. com.
T. kaykai	?	+	+	m	+	Stouthamer & Kazmer 1994 Schilthuizen & Stouthamer 1997 Schilthuizen *et al.* 1998
T. nubilalae	?	?	+	?	?	Schilthuizen & Stouthamer 1997 van Meer *et al*. 1999
T. oleae	+	+	+	?	+	Stouthamer *et al*. 1990*b* Rousset *et al*. 1992
T. platneri	+	+	+	m	+	Stouthamer *et al*. 1990*a* Schilthuizen & Stouthamer 1997
T. pretiosum	+	+	+	m	+	Orphanides & Gonzalez 1970 Stouthamer *et al*. 1990*a,b*
T. sibericum	+	?	+	?	?	Schilthuizen & Stouthamer 1997 van Meer *et al*. 1999
T. atopovirilla	?	+	+	?	?	Ciociola *et al.* 2001 R.P. Almeida pers. com.
T. semblidis	?	?	+	?	?	Pintureau *et al.* 2000*a*
Eucoilidae						
Gronotoma micromorpha	?	+	+	f	+[4]	Arakaki *et al*. 2001*b*
Figitidae						
Leptopilina australis	?	?	+	?	?	Werren *et al*. 1995
L. clavipes	?	?	+	?	?	Werren *et al*. 1995
Cynipidae						
Diplolepis rosae	?	?	+	f,m	-	Stille & Dävrig 1980 van Meer *et al*. 1995 Plantard *et al*. 1999
D. spinosissima	?	?	+	f,m	?	Plantard *et al*. 1998 Plantard *et al*. 1999
D. mayri	?	?	+	f	?	Plantard *et al*. 1999
D. fructuum	?	?	+	m	?	Plantard *et al*. 1999
D. eglanteriae	?	?	+	f	?	Plantard *et al*. 1999
D. bicolor	?	?	+	f	?	Plantard *et al*. 1999
D. californica	?	?	+	f	?	Plantard *et al*. 1999
D. nodulosa	?	?	+	f	?	Plantard *et al*. 1999
D. polita	?	?	+	f	?	Plantard *et al*. 1999
D. radicum	?	?	+	f	?	Plantard *et al*. 1999
D. spinosa	?	?	+	f	?	Plantard *et al*. 1999
Liposthenes glechomae	?	?	+	f	?	Plantard *et al*. 1999
Timaspis lampsanae	?	?	+	f	?	Plantard *et al*. 1999
Phanacis hypochaeridis	?	?	+	f	?	Plantard *et al*. 1999
P. centaureae	?	?	+	f	?	Plantard *et al*. 1999

			Coleoptera			
Curcilionidae						
Naupactus tesselatus	?	?	+	?	?	Werren *et al.* 1995
			Thysanoptera			
Thripidae						
Heliothrips haemorrhoidalis	?	?	+	?	?	Pintureau *et al*. 1999
Hercinothrips femoralis	?	?	+	?	?	Pintureau *et al*. 1999
Aeolothripidae						
Franklinothrips vespiformis	+	+	+	f	+[4]	Arakaki *et al*. 2001*a*
			Arachnida			
			Acari			
Tetranychidae						
Bryobia kissophila	?	?	+	f	?	Weeks & Breeuwer 2001
B. praetiosa	?	+	+	f	+	Weeks & Breeuwer 2001
B. graminum	?	?	+	f	?	Weeks & Breeuwer 2001
B. rubrioculus	?	?	+	f	?	Weeks & Breeuwer 2001
B. neopraetisosa	?	?	+	f	?	Weeks & Breeuwer 2001
B. sp.*x*	?	+	+	f	+	Weeks & Breeuwer 2001
B)						
Taxon	**h[1]**	**a[1]**	**w[1]**	**p[2]**	**c[3]**	**reference**
			Insecta			
			Hymenoptera			
Tenthredinoidae						
Pristiphora erichsonii	+	?	?	?	?	Smith 1955
Aphelinidae						
Aphelinus asynchus	+	?	?	?	?	Schlinger & Hall 1960
Apytis mytilaspidis	?	?	?	m	+	Rössler & DeBach 1973
Signiforidae						
Signiphora borinquensis	+	?	?	?	+	Quezada *et al*. 1973
Encyrtidae						
Pauridia peregrina	+	?	?	f	+	Flanders 1965
Ooencyrtus submetallicus	+	?	?	?	-	Wilson & Woolcock 1960*a*,*b* Wilson 1962
O. fecundus	+	?	?	?	?	Laraichi 1978
Plagiomerus diaspidis	+	?	?	?	-	Gordh & Lacey 1976
Trechnites psyllae	?	+	?	?	?	T.R. Unruh pers. com.
Habrolepis rouxi	+	?	?	?	?	Flanders 1965
Trichogrammatidae						
T. sp	+	?	?	?	?	Bowen & Stern 1966
T. telengai	+	?	?	?	?	Sorakina 1987
Eulophidae						
Galeopsomyia fausta	?	+	?	?	+[4]	Argov *et al.* 2000
Cynipidea						
Hexicola sp. near websteri	+	?	?	?	?	Eskafi & Legner 1974

[1] + evidence exists, ? information not available
[2] (f) Parthenogenesis fixed in population, (m) parthenogenesis and sexual reproduction occur in populations, ? information not available
[3] copulations are successful (+) or not (-)
[4] Mating and sperm transfer take place, but no successful fertilization of eggs
[5] Mating of males of parthenogenetic lines is successful with closely related sexual females, but not with parthenogenetic females.

We expect that the incidence of PI *Wolbachia* among species to be much higher than reported so far. In particular low infection frequencies with PI *Wolbachia* have most likely been underestimated. Low frequencies of infection with PI *Wolbachia* in field populations have only been found in *Trichogramma* sp. (Stouthamer 1997). This may simply be a consequence of the way populations are sampled. In general, laboratory colonies of species are initiated by pooling large number of field-collected individuals. In this way, low levels of parthenogenesis are easily overlooked because partially parthenogenetic populations cannot be distinguished from sexual populations; in both cases, the colony consists of both males and females. *Trichogramma* sp. stands out because the field populations have been sampled by establishing isofemale lines from wasps emerging from field-collected host eggs. All-female isofemale lines (= infected lines) are easily detected using this protocol (Pinto *et al.* 1991).

2.3 CYTOGENETICS: PARTHENOGENESIS IS NOT ONLY THROUGH GAMETE DUPLICATION

Cytogenetic processes involving diploidization can be divided in meiotic and postmeiotic modifications (Stouthamer *et al.* 1997). A review of parthenogenesis in insects shows that most belong to the first group (Suomalainen *et al.* 1987). In most species, the meiosis is entirely suppressed and the division has a mitotic character. The process of diploidization in parasitoid wasps due to *Wolbachia* infection, however, seems to be mostly a postmeiotic modification. Stouthamer & Kazmer (1994) described in detail the restoration of diploidy for several infected *Trichogramma* species. They showed that the meiosis was the same for infected and uninfected eggs. At first, in both infected and uninfected eggs, the haploid number of chromosomes is doubled in the prophase of the first mitotic division. In the normal anaphase, each haploid set of chromosomes is pulled to a different pole. In infected eggs, however, the two haploid sets of chromosomes do not separate during the anaphase and result in a single nucleus containing the two identical sets of haploid chromosomes. The following mitotic

divisions are the same in infected and uninfected eggs. An unfertilized infected egg therefore develops into a diploid female, homozygous at all loci, and an unfertilized uninfected egg into a haploid male. This diploidization process, which results in a fusion of two identical sets of chromosomes, is called gamete duplication. Allozyme analysis showed that infected females from the three *Trichogramma* species studied -*T. pretiosum, T. deion* and *T. kaykai*- still fertilized their eggs with sperm from conspecific males. The heterozygous F1 virgin females produced only homozygous offspring. This confirmed the gamete duplication. When infected eggs are fertilized, sperm prevents the diploidization during the first mitotic division caused by *Wolbachia* infection and heterozygous infected females develop.

Besides *Trichogramma* sp., two additional cases of gamete duplication due to *Wolbachia* infection have been reported. Stille & Därving (1980) and Gottlieb *et al*. (2002) observed a slightly different cytogenetic process in the parthenogenetic gall wasp *Diplolepis rosae* and the pteromalid wasp *Muscidifurax uniraptor* respectively. Diploidy restoration is not achieved because of an aberrant anaphase but following the completion of the first mitotic division. At that time, the products of the two mitotic nuclei fuse. These studies show that either different forms of *Wolbachia* induced gamete duplication exist or the observations of Stouthamer & Kazmer (1994) are incorrect.

The gamete duplication in some *Trichogramma* species appears to be far from perfect, Tagami *et al* (2001), found in lines of two infected *Trichogramma* sp., a higher pre-pupal mortality of infected eggs compared to uninfected eggs. Cytological analysis of the developmental stage of 6- to 48-h-old eggs and larvae showed that in up to 35% of unfertilized infected eggs the embryonic development was arrested in the early mitotic stages. This arrestment was not found in eggs of the sexual forms or in a *T. cacoeciae* line where parthenogenesis was not associated with *Wolbachia*. The researchers concluded that the high pre-pupal mortality of infected eggs was a result of these failures in gamete duplication.

Several studies on the cytogenetic process of cytoplasmic incompatibility (CI) induced by *Wolbachia* have also been carried out. There is some similarity in this process to gamete duplication. Incompatibility in the mosquito *Culex pipiens* showed that the paternal chromosome set does not faithfully fuse with the female chromosomes after fertilization (Jost 1970). Reed & Werren (1995) observed the same phenomenon in the

parasitoid wasp *Nasonia vitripennis*. Both cytogenetic processes of gamete duplication and incompatibility due to *Wolbachia* show defects in the early mitotic divisions. Lassay & Karr (1996) state that the cytogenetic process of incompatibility is pleiotropic and can be classified into several categories. One defect, in *Drosophila simulans* for example, seems to occur in the anaphase of the first mitotic division (Callaini *et al.* 1996; Callaini *et al.* 1997) as with gamete duplication. In this fruit fly, the maternal and paternal chromosomes do not condense synchronically when the father was infected and the mother uninfected. The maternal chromosomes normally enter the anaphase of the first mitotic division and migrate to the two poles. The paternal chromosomes are delayed and stay in the mid-zone of the spindle. This results in embryos with aneuploid or haploid nuclei that eventually die (Callaini *et al.* 1997). In general, we can say that *Wolbachia* acts in the early mitotic divisions. The main difference between the cytogenetic processes of diploidization and incompatibility induced by *Wolbachia* is of course that gamete duplication is prevented by sperm whereas incompatibility occurs after fertilization.

From the cytogenetic studies done in Hymenoptera, one might conclude that the most common diploidization process in PI *Wolbachia* infected eggs is gamete duplication. However, recent work by Weeks & Breeuwer (2001) shows that another mechanism of *Wolbachia* induced parthenogenesis is found in some mites, i.e. a meiotic modification in eggs infected with PI *Wolbachia*. Microsatellite loci in six infected mite species of the genus *Bryobia* indicate the mechanism of parthenogenesis to be most likely a meiotic modification, with progeny being identical to their infected heterozygous mother. It is clear that *Wolbachia* has evolved different mechanisms to induce parthenogenetic development.

2.4 DISTRIBUTION AND DENSITY OF *WOLBACHIA* IN PARTHENOGENETIC WASPS

Transmission of *Wolbachia* from mother to daughter is through the cytoplasm of the eggs. A study on two *Aphytis* species suggested that, inside the ovaries, the microorganisms multiply inside the nurse cells and then move, together with all other maternal substances, into the developing oocytes through cytoplasmic bridges (Zchori-Fein *et al.* 1998). Subsequently, they move to the posterior pole where they are found in freshly laid eggs, as was also shown in *Trichogramma* species (Figure 2.1; Stouthamer & Werren 1993). In later stages they migrate to the center of the eggs surrounding nuclei throughout the embryo, although this does not appear to be the case in *Trichogramma sp.* eggs (Stouthamer & Werren 1993). Nothing is known about the distribution of PI *Wolbachia* throughout adult host tissues, other than the reproductive tissue.

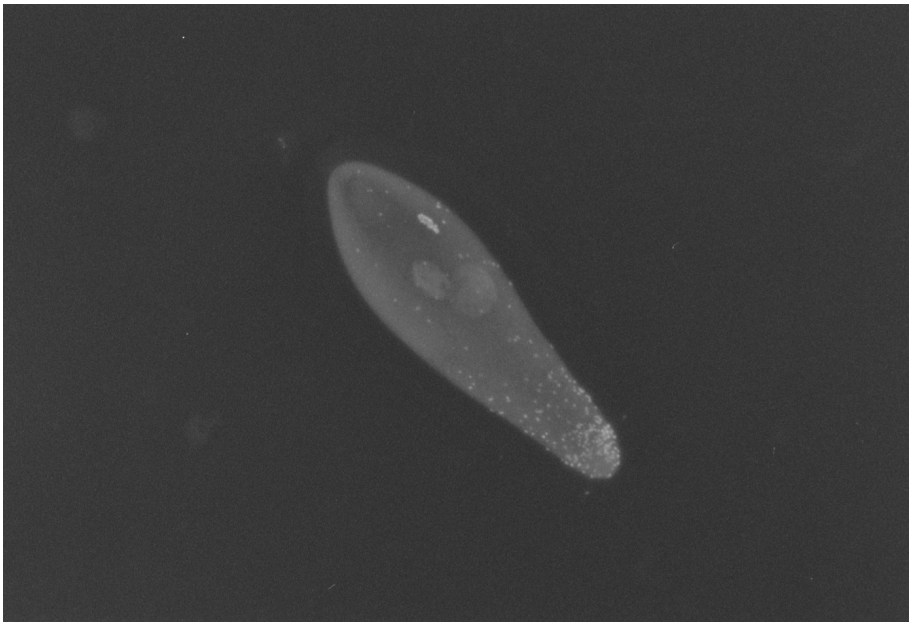

Figure 2.1 *Wolbachia* located at the posterior pole of a freshly laid *Trichogramma kaykai* egg (Photo Merijn Salverda).

PI is most likely related to the density of *Wolbachia.* As in CI it seems to be a titer effect. In *Muscidifurax uniraptor*, male production increased with increasing antibiotic dose (Zchori-Fein *et al.* 2000). In *Trichogramma* sp., infected females start producing more and more males after a 5 or 6 days of being able to parasitize a non-limiting

number of host eggs (Stouthamer unpublished results). The titer of *Wolbachia* in the ovaries, most likely, becomes too low to infect all the oocytes after few days of oviposition. The uninfected oocytes then develop into males. This might also be the case in *T. kaykai* females that acquired *Wolbachia* through horizontal transfer (Huigens *et al.* 2000 (=Chapter 3)). Newly infected virgin females always produced both sons and daughters.

2.5 PHYLOGENETICS: NO CLUSTERING OF PI *WOLBACHIA*

PI *Wolbachia* do not form a monophyletic group within the phylogeny of *Wolbachia* based on genes used thus far for reconstructing its phylogeny, i.e., *ftsZ* (Holden *et al.* 1993; Werren *et al.* 1995; von der Schulenburg *et al.* 1999), *wsp* (Braig *et al.* 1998; Zhou *et al.* 1998; Schulenburg *et al.* 1999), 16S rDNA (O' Neill *et al.* 1992; Stouthamer *et al.* 1993), 23S rDNA (Rousset *et al.* 1992), SR2- and 5S rDNA-regions (Fialho *et al.* 1997; van Meer *et al.* 1999).

Wolbachia phenotypes are intermixed in the phylogeny of *Wolbachia* (reviewed in Stouthamer *et al.* 1999). Several hypotheses exist to explain the scattered distribution of the *Wolbachia* phenotype over the phylogeny (Stouthamer 1997). According to one, the induction of parthenogenesis undergoes multiple evolution. A second hypothesis is that specific host effects that allow a certain *Wolbachia* phenotypes to express themselves in a particular host. Curing a host that showed phenotype A and infecting it with a *Wolbachia* that induced phenotype B in its original host may prove this. Infecting cured parasitoid wasps that previously showed CI with *Wolbachia* associated with parthenogenesis could provide support for this hypothesis. van Meer & Stouthamer (1999) microinjected uninfected *Drosophila simulans* embryos with PI *Wolbachia* from *Muscidifurax uniraptor*. Infection could be detected in the new host, but no effect was expressed, and the infection only persisted for seven generations. A third hypothesis states that the genes used for phylogenies are not linked to the genes associated with the effect. Mobile genetic elements, within the genome of *Wolbachia*, such as the recently discovered bacteriophage (Masui *et al.* 2000), may induce the effect and not *Wolbachia* itself. Masui *et al.* (2000) show evidence for frequent horizontal transmission of this phage between different *Wolbachia*.

Many PI *Wolbachia* are distributed in the B-group but a few also occur in the A-group (Werren *et al.* 1995; Gottlieb *et al.* 1998; Zhou *et al.* 1998; Plantard *et al.* 1999; Vavre

et al. 1999; van Meer *et al.* 1999; von der Schulenburg *et al.* 1999). When we review these studies and calculate the percentage of PI *Wolbachia* in both groups, using only those *Wolbachia* cases where the phenotype has been established, we find PI *Wolbachia* in 28% of the *Wolbachia* in group A (10 of 36) and 54% in group B (20 in 37)

Only in *Trichogramma* sp. do PI *Wolbachia* show a clear phylogenetic pattern. Independent of the studied gene, *wsp*, *ftsZ* or 16S rDNA, these *Wolbachia* always form a separate cluster. Schilthuizen & Stouthamer (1997) studied the phylogenetic relationships between PI *Wolbachia* and their *Trichogramma* hosts in detail. A comparison of the phylogenetic tree of the host species with the tree of *Wolbachia* in these species showed them to be incongruent. The major reason for this incongruence is most likely horizontal transfer of the *Wolbachia* among different *Trichogramma* species.

Do PI *Wolbachia* differ in this respect from *Wolbachia* inducing other effects? At present we cannot make strong statements concerning this pattern because our sample size is too small. For parasitoids of the genus *Aphytis* several PI *Wolbachia*-infected lines have been sequenced using the *ftsZ* gene (Gottlieb *et al*. 1998). In this genus the *ftsZ* sequence of the *Wolbachia* is identical, suggesting that here too we find a high similarity of PI *Wolbachia* within the genus. Similar patterns are also found to some extend in CI-*Wolbachia*, for example the sibling species of *Nasonia* sp. carry similar *Wolbachia*. The same applies to *Culex* sp. (Zhou *et al.* 1998). *Wolbachia* in *Diplolepis*, most likely associated with PI, show an extraordinary pattern: The *Wolbachia* in five *Diplolepis* species from France cluster together in the B-group whereas five other PI *Wolbachia* from North American species are distributed over the A- and the B-group (Plantard *et al*. 1999). This distribution would be consistent with the hypothesis that the peculiarities of the host induce the PI effect. The *Leptopilina* species are very interesting because here we CI- and PI *Wolbachia* exist within the same genus. The pattern we observe here is that all CI *Wolbachia* in *Leptopilina* are distributed over the A-group, mostly associated with the *Wolbachia* of their host species (Vavre *et al*. 1999), but the PI *Wolbachia* in *L. clavipes* and *L. australis* occur in a separate cluster in the B-group.

Besides Schilthuizen & Stouthamer (1997), many other phylogenetic studies detected indirect evidence of for horizontal transfer (O'Neill *et al.* 1992; Stouthamer *et al*. 1993; Rousset & Solignac, 1995; Werren *et al.* 1995; Vavre *et al.* 1999). Host-to-parasitoid and parasitoid-to-parasitoid transfer of *Wolbachia* have been considered mainly as

potential transmission routes. Horizontal transmission was shown for PI *Wolbachia* by Huigens *et al.* (2000) (=Chapter 3): Horizontal transfer of PI *Wolbachia* between *Trichogramma kaykai* parasitoids occurs while sharing the same food source.

2.6 FITNESS COSTS: HIGHER COSTS OF CARRYING *WOLBACHIA* IN MIXED POPULATIONS

The effects of *Wolbachia* on the fitness of its host are influenced by the following factors: transmission route, presence of a genomic conflict between *Wolbachia* and host, and the physiological cost of carrying bacteria. The relationship between *Wolbachia* and its host can range from an adversarial relationship in populations where only a fraction of the females is infected with the PI *Wolbachia* (under these circumstances we expect a genomic conflict in which the nuclear genes of the host may try to suppress the *Wolbachia* or its effect) to a completely mutualistic relationship when the infection with *Wolbachia* in a host population has gone to fixation and all females of a population are infected with *Wolbachia*.

Transmission route: Since the early part of the 20th century, the evolution of parasite virulence has received much attention. Although PI *Wolbachia* are not true parasites but non obligatory symbionts, theories on the evolution of virulence are applicable to PI *Wolbachia*. In general, the mode of parasite transmission, vertical and/or horizontal, is considered to be the key selective force behind the evolution of virulence (Ewald 1994). The two modes of transmission are expected to select for opposing virulence levels.

Vertical transmission should select for lower parasite 'virulence' to the host. Parasites depend on the host and benefit from a high reproductive success of its host. Hosts with less virulent parasites should produce more offspring than hosts infected with more virulent forms (Lipsitch *et al.* 1996). On the other hand horizontal transmission is expected to select for higher virulence because parasites are not dependent on the reproductive capacity of the host. When only horizontally transmitted, parasites obtain the highest fitness through a trade off between the negative effect of the parasites multiplication (virulence) on the longevity of the infected host. Too many parasites could make the host less capable of transmitting parasites (Messenger *et al.* 1999).

Some parasites have two modes of transmission. They provide ideal models for studying the evolution of virulence. Bull *et al.* (1991) studied phage virulence evolution in *Escherichia coli*. These phages can be both vertically and horizontally transmitted.

Two forms of the phage occur in natural populations - a mild form and a more virulent form that decreases the growth speed of the host. In situations where only vertical transmission was possible, the mild form prevailed. This was not the case when horizontal transmission was also allowed.

In *T. kaykai,* PI *Wolbachia* are also transmitted in two ways. Vertical transmission is the main mode but at high parasitoid densities horizontal transfer is expected to be frequent in the field (Huigens *et al.* 2000 (=Chapter 3)). The presence of both transmission forms in the PI *Wolbachia* of *T. kaykai,* will allow us to study the tradeoffs between virulence and transmission of *Wolbachia*.

Nuclear-cytoplasmic conflict: The theory of parasite virulence evolution can not be wholly applied to PI *Wolbachia* because they are not parasites in the true meaning of the word. They are reproductive parasites that drive through a host population by manipulating the sex ratio of the host. Therefore, when *Wolbachia* are only vertically transmitted, they can induce relatively high fitness costs and still spread through the host population. For PI *Wolbachia*, several aspects of a host population have implications for the fitness costs we can expect (Table 2.2).

Table 2.2 Fitness costs for hosts carrying PI *Wolbachia*, ranked from lowest (1) to highest (4), expected in several situations. Costs for hosts from populations fixed or mixed for the infection and from populations where horizontal transmission of *Wolbachia* does or does not occur are mentioned.

Infection status	Horizontal transmission	Fitness cost
Fixed	-	1
Fixed	+	2
Mixed	-	3
Mixed	+	4

First of all, fitness costs of carrying *Wolbachia* are expected to be different among host populations fixed for the infection and populations where infected and uninfected individuals coexist (Stouthamer 1997).

A) Initial infections that spread fastest through a population are the ones with little or no effect on the host fitness. Once an infection has spread throughout the entire host population the selective pressures that act on both host and symbionts are assumed to minimize the costs to the host of carrying these symbionts.

B) In cases where the infection has not reached fixation in populations, a nuclear cytoplasmic conflict between the *Wolbachia* and the nuclear genome of the host is expected. *Wolbachia* favors a 100% female bias whereas the nuclear genes favor a sex ratio involving at least some males. In these situations an arms race between the

nuclear genes and those of the *Wolbachia* may result in nuclear genes trying to suppress the *Wolbachia* or its effect. Consequently, a much higher physiological cost of being infected is expected in mixed host populations where infected and uninfected individuals co-occur.

In addition to the contribution of the genomic conflict to the potential fitness cost of the PI *Wolbachia* in mixed populations, the opportunities for horizontal transmission in such populations may also select for additional costs of being infected. In fixed populations all individuals are already infected, and we expect the horizontal transmission to be selected against.

Physiological cost: It is generally assumed that the physiological costs are minimized in those populations where the infection has gone to fixation. But this does not imply there is no cost at all because there are still energy-consuming bacteria present inside the host.

The best way to determine the effect of *Wolbachia* infection on the offspring production is by "curing" a line of its infection. Such cured lines should be maintained for several generations; then the offspring production of two genetically identical lines that differ in their infection status can be compared. Unfortunately this has been impossible to do for those cases where sexual lines cannot be established because males and females are no longer capable of sexual reproduction. In this case it is only possible to compare the offspring production of infected females with cured (antibiotic-treated) females. When such comparisons are made, infected females fed antibiotics are compared with those fed honey. It is important in these comparisons to avoid interference of toxic effects that the antibiotics may have on the females. Therefore, the lowest possible effective dose should be applied. Stouthamer & Mak (2002) showed that at a concentration of 50 mg/ml honey, the antibiotic tetracycline was toxic to *Encarsia formosa* females, while at 5mg/ml the treated females produced significantly fewer offspring than controls. At a concentration of 1mg/ml no difference in offspring production was found between treated and control females. Both the 5mg/ml and the 1mg/ml concentrations cured the treated females of their infection.

Fitness cost for infected hosts from mixed populations -a rare situation for *Wolbachia*-induced parthenogenesis- has been studied only in *Trichogramma* sp. In *T. pretiosum*, *T. deion* and *T. kaykai*, *Wolbachia* has a negative influence on lifetime offspring production under laboratory conditions (Stouthamer & Luck 1993; van Meer 1999;

Silva 1999). Infected wasps produce fewer offspring than genetically identical wasps cured from their infection when given an abundance of host eggs every day during their entire lifespan (Stouthamer & Luck 1993). Sometimes the number of daughters produced was even lower for infected mothers (Stouthamer *et al.* 1990*b*; Stouthamer & Luck 1993). These results show that the host can suffer severe fitness costs from being infected, but life-time offspring production cannot be extrapolated very well from the lab to the field because females are expected to be host-limited in the field (Stouthamer & Luck 1993). Some parameters that may be important for host fitness in the field were also studied. Van Meer (1999) determined pre-adult survival and showed it was reduced for infected *T. kaykai* and *T. deion* females. We compared several infected lines with several naturally uninfected lines of *T. kaykai* and found that offspring of infected females had a lower pre-adult survival and survived even dramatically less when both forms compete for the same food source (Chapter 5). Under high parasitoid densities, sharing of a food source may occur frequently in the field. The high mortality rates of unfertilized eggs in infected *T. kaykai* and *T. deion* lines found by Tagami *et al.* (2001), also confirms the general pattern of high fitness costs in mixed populations.

When the *Wolbachia* infections have gone to fixation in populations the infections do not seem to have a negative impact on the offspring production. In two *Trichogramma* species with fixed populations, *T. cordubensis* and *T. oleae*, sexual lines could be established after curing (van Meer 1999; Silva 1999). Infected females and sexual females from cured lines had the same offspring production in both species. In all other studies the effects on host fitness of PI *Wolbachia* have only been studied by comparing untreated infected females with infected females treated with antibiotics of heat. Infected wasps of *Muscidifurax uniraptor* produced as many offspring as antibiotic-fed wasps (Horjus & Stouthamer 1995). Another study on the same species, confirmed the absence of a negative effect of *Wolbachia* on fecundity, longevity and offspring survival (Zchori-Fein *et al.* 2000).

In *Eretmocerus mundus*, fecundity was unaffected by *Wolbachia* but offspring of infected females had much higher survival than offspring from antibiotic treated females (De Barro & Hart 2001). This low survival may be caused by the very high antibiotic dose of 29,8mg/ml Rifampicin that was used. Two cases of *Wolbachia*-induced parthenogenesis outside the Hymenoptera, with the population fixed for the

infection, also do not show a negative effect of *Wolbachia* on host fitness. In the parthenogenetic mite species *Bryobia praetiosa* and *B.* sp. *x*, antibiotic treated females (0,15% Tetracycline) produced fewer offspring than infected females (Weeks & Breeuwer 2001). Arakaki *et al.* (2001*a*) studied the predatory thrips species *Franklinothrips vespiformis* and found the same longevity and offspring production for infected and treated females. Pijls *et al.* (1996) compared parthenogenetic *Apoanagyrus diversicornis* females with sexual uninfected conspecific females, which were allopatric and studied the effect of antibiotic treatment in both forms. They found a lower offspring survival for treated originally parthenogenetic females. For *A. diversicornis* the results are however slightly ambiguous since allopatric females were compared. At the time of their study *Wolbachia* had not been detected yet in *A. diversicornis*, but this was later on confirmed by van Meer *et al.* (1999) In general, infection induces a fitness cost in mixed populations but not in populations fixed for the infection. For some aspects of host fitness, infection might have evolved to neutrality or even benevolence in fixed populations.

2.7 DYNAMICS OF PI *WOLBACHIA* AND THE EVOLUTION OF VIRGINITY MUTANTS

The dynamics of PI *Wolbachia* infections are in general very simple. A PI *Wolbachia* infects a female in a population, and as long as the infected female produces more infected daughters than an uninfected female produces uninfected daughters the infection will spread in the population, barring stochastic events. The infection frequency among females that such an infection can reach is a function of a number of variables: 1) the fitness cost of the infection, in terms of offspring production, 2) the transmission fidelity of the PI *Wolbachia* infection and 3) the egg fertilization frequency (Stouthamer 1997). Models show that the transmission fidelity of the infection is one of the most important variables that determine the equilibrium infection frequency. Once the infection has reached the equilibrium it may stay at that level for a long time, unless suppressor alleles, i.e., traits that either kill off the *Wolbachia* or suppress the *Wolbachia* induced phenotype, evolve and spread (Stouthamer *et al*. 2001). Alternatively *Wolbachia* may also evolve to a higher level of transmission and eventually reach fixation. However another option is very likely to be the most common. In this model the infection reaches fixation in the population because a

mutation that causes females not to fertilize their eggs any longer has an enormous fitness advantage when a substantial part of the population has become infected with the PI *Wolbachia*.

Imagine a population where half of the females are infected and produce mainly infected daughters as offspring, and let us assume 10% of the offspring of infected females is uninfected because of inefficient transmission of the *Wolbachia*. Let us also assume that all infected females mate. The uninfected females produce male and female offspring with a sex ratio that is optimal for the species. The male offspring of these uninfected females have a high fitness compared to both uninfected and infected females, because they can mate with several females. Assume that a mutation takes place in an uninfected female and that this mutation stops her either from mating or from fertilizing her eggs so that now she produces exclusively male offspring. This "virginity" mutation has a large fitness advantage, because of the female biased sex ratio in the population. When the mutant male mates with an infected female part of her offspring will be heterozygous for the mutant, and some of the offspring (50% of the unfertilized eggs) of these infected females will become homozygote for the "virginity" mutation and no longer mate with males. These infected mutant females will produce some uninfected eggs because of the inefficient transmission. These eggs will be unfertilized, and thus become males that are carriers of the mutation. These males also experience the large fitness benefit from being male in a population largely consisting of females. If these males mate with uninfected females they will increase the frequency of the mutant allele in the uninfected population, thus reducing the number of uninfected females produced. In the infected population, an increasingly larger number of females will carry the mutation and will become unavailable for mating with males, while the infected females not yet homozygous for the virgin mutation are still willing to mate. Simulations (Stouthamer & Vavre unpublished) have shown that even with a large negative impact on the fitness of their carriers in terms of offspring production this "virginity" mutation will spread through the population and results in a rapid fixation of the *Wolbachia* infection. In addition, the resulting all-female population will now consist entirely of mutant females that are no longer able of willing to mate. However, if males are induced by feeding the infected females antibiotics, these males are still capable of mating successfully with non-mutant females. This is indeed the pattern that is observed in a number of populations where both completely infected and uninfected

populations exist. Pijls *et al.* (1996) and Arakaki *et al.* (2000) studied species where both completely sexual and completely infected populations existed allopatrically. When males were produced by antibiotic treatment from the infected population, they did not mate successfully with females of the infected line, while successful matings took place with females from the sexual line. The females from the infected line did not mate successfully with either males from the infected or from the uninfected line. The lack of successful matings between cured males and infected females is a common phenomenon in populations where the infection has gone to fixation.

For example, in parthenogenetic parasitoid species such as *Encarsia formosa* (Zchori-Fein *et al.* 1992), *Muscidifurax uniraptor* ((Stouthamer *et al*. 1993; Stouthamer *et al*. 1994; Zchori *et al*. 2000), *Apoanagyrus diversicornis* (Pijls *et al*. 1996), *Aphytis* sp. (Zchori-Fein *et al*. 1995), *Telenomus nawai* (Arakaki *et al*. 2000) and *Eretmocerus mundus* (De Barro & Hart 2001), females do not produce offspring from fertilized eggs, when allowed to mate with conspecific males derived by antibiotic treatment. In addition, in the parthenogenetic mite species *Bryobia praetiosa* copulation took place between cured males and females but sperm was not used by the females (Weeks & Breeuwer 2001). In cured females of the thrips *Franklinothrips vespiformis*, sperm was found after they mated with "parthenogenetic" males (Arakaki *et al.* 2001*a*) but also not used.

In mixed populations, mating behavior of infected females does not seem to be different from uninfected females. Within several species -*Trichogramma kaykai*, *T. pretiosum* and *T. deion*- infected females successfully mate with males from cured or uninfected forms (Stouthamer *et al.* 1990b; Stouthamer & Luck 1993; van Meer 1999; Stouthamer *et al.* 2001). In all mixed populations, the virginity mutation could evolve and should lead to fixation of the infection in the population. The cases where mixed populations persist are an indication of the presence of some suppressing factor. Suppressor genes as such have not yet been found for PI *Wolbachia*. In *Trichogramma kaykai* detailed work on the prolonged persistence of infected and uninfected individuals within a population did not find any evidence for the presence of suppressors but brought to light the presence of a second sex ratio distorter (Paternal Sex Ratio, *PSR*) in the population. About 10% of the males carry this chromosome (Stouthamer *et al.* 2001). Such a *PSR* chromosome was until then found only in the parasitoid wasp *Nasonia vitripennis* (Werren 1991). When a female mates with a *PSR*-carrying male, the paternal

chromosomes in fertilized eggs are functionally destroyed and only the maternal chromosomes and the *PSR* factor itself remain. Such fertilized eggs develop into sons that carry a haploid set of chromosomes from the mother and the *PSR* chromosome from their father. *PSR* therefore causes uninfected and infected females to produce sons from their fertilized eggs. The mating structure of the uninfected *T. kaykai* is such that a large fraction of the uninfected females mate with their brothers (54-65%, Stouthamer & Kazmer 1994). In contrast, the infected females mate with males from the population, and 10% of these are carriers of *PSR*. As a consequence, approximately 10% of the infected females mate with *PSR* males and only 5.5-6.4% of the uninfected females. The consequence of this asymmetry is that the higher daughter production of the infected population is suppressed more than that of the uninfected population, allowing for a stable coexistence of both forms within the population (Stouthamer *et al.* 2001).

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Dit moet titelpagina **Chapter 3** worden!!!!!!!!!!!

Infectious parthenogenesis

ABSTRACT Parthenogenesis-inducing (PI) *Wolbachia* bacteria are reproductive parasites causing infected female wasps to produce daughters without mating (Stouthamer *et al*. 1990; Stouthamer *et al*. 1993). This manipulation of the host's reproduction enhances *Wolbachia*'s transmission to future generations because they are passed on vertically only from mothers to daughters. Males are dead ends for cytoplasmically inherited bacteria; they do not pass them on to their offspring. Vertical transmission of *Wolbachia* has been viewed as the main mode of transmission, but here we report frequent horizontal transmission from infected to uninfected wasp larvae sharing a common food source. The transferred *Wolbachia* are then vertically transmitted to the new host's offspring. This is the first known case of a natural horizontal transfer of PI *Wolbachia* intraspecifically. The unexpectedly frequent horizontal transfer of these bacteria has important implications for the co-evolution of *Wolbachia* and their host.

3.1 INTRODUCTION

Trichogramma kaykai (Hymenoptera; Trichogrammatidae) is a minute egg parasitoid of a butterfly species, *Apodemia mormo deserti* (Lepidoptera; Lycaenidae), that inhabits the Mojave Desert of Southern California (Pinto *et al*. 1997). *T. kaykai* normally lays a clutch of 3 to 5 offspring in an *A. mormo deserti* egg. This parasitoid wasp manifests haplo-diploid sex determination in which unfertilized eggs develop into sons and fertilized eggs develop into daughters. Between 6-26% of the *T. kaykai* females in these populations are infected with PI *Wolbachia* and produce daughters from their unfertilized eggs (Stouthamer & Kazmer 1994). These daughters are produced when PI *Wolbachia* suppresses spindle formation during anaphase of the first mitotic division, thus restoring diploidy by fusion of the two mitotic nuclei (Stouthamer 1997). In the field, the offspring of two *T. kaykai* females can share and mature in the same butterfly egg (Kazmer 1992). Thus, we tested whether *Wolbachia* transfers horizontally between the offspring of a *Wolbachia*-infected and an uninfected *T. kaykai* female when they share the same egg and, if so, whether this infection persists in its expression in subsequent generations.

3.2 MATERIAL AND METHODS

T. kaykai females collected at Last Chance Canyon, El Paso Mountains, Kern County, California were used to initiate infected and uninfected lines. We cultured these lines in the laboratory on eggs of the moth, *Trichoplusia ni* (Pak & Oatman 1982), that do not harbour *Wolbachia* (Stouthamer unpublished). Each of these lines differed in the size of a microsatellite DNA repeat. In our experiments, we offered a moth egg to a female *T. kaykai* from one line, allowing her to lay a normal clutch of 2 to 4 eggs. Two hours later, we exposed the same egg to a female from the second line. In half the cases, a *Wolbachia* infected *T. kaykai* was offered the egg first and in the other half the order was reversed. We continuously observed and recorded the number of eggs oviposited in a moth egg by each *T. kaykai* (Suzuki *et al*. 1984). The resulting offspring were linked unambiguously to their parental female using a microsatellite marker. If F1 offspring from a doubly parasitized egg consisted of only females, we exposed these virgin F1 females individually to host eggs and recorded the sex of their progeny. Because only infected virgins produce daughters, their presence in offspring of an F1 virgin originating from an uninfected line indicated horizontal transfer of PI *Wolbachia*.

3.3 RESULTS AND DISCUSSION

We found clear evidence for the horizontal transmission of PI *Wolbachia* and the expression of parthenogenesis. Twenty-one instances of horizontal transfer occurred in 56 all-female broods from moth eggs that contained infected and uninfected larvae (Table 3.1). Horizontal transfer to uninfected offspring occurred independent of the order in which the infected and uninfected females were exposed to the host egg ($\chi^2_{0.05,1} = 2.75$; n = 56). The bacteria's presence was confirmed in all newly infected females by means of PCR using *Wolbachia* specific primers (Braig *et al*. 1998). Both sons and daughters were produced by these newly infected, virgin F1 females, indicating inefficient vertical transmission from mother to offspring. We suspect that these females had insufficient *Wolbachia* titer to express parthenogenesis in all of their offspring. To test whether a new infection was maintained over several generations, we established three lines, each of which consisted of offspring from a different, newly infected female. Vertical transmission was inefficient in the first generation, but it was highly effective in the subsequent eight generations. The transmission efficiency was 100% for 10 virgin females of each line tested in generation 3 and 8.

Table 3.1 Twenty-one instances of horizontal transfer of parthenogenesis-inducing *Wolbachia* between *T. kaykai* wasps parasitizing the same host egg.

	P				**F2**			
Host egg	**N° eggs oviposited** ♀**1**	♀**2**		**F1**	♀**1**	♀**2**	♀**3**	♀**4**
1	3 197 (U)	**2** **203 (I)**	→	♀♀♀	**1♀: 2♂** **197 (U)**	**1♀: 0♂** **203 (I)**	**1♀: 0♂** **203 (I)**	
2	**2** **216 (I)**	2 194 (U)	→	♀♀♀♀	**17♀: 0♂** **216 (I)**	**15♀: 0♂** **216 (I)**	**7♀: 3♂** **194 (U)**	**4♀: 10♂** **194 (U)**
3	2 194 (U)	**1** **216 (I)**	→	♀♀	**18♀: 0♂** **216 (I)**	**9♀: 14♂** **194 (U)**		
4	**2** **222 (I)**	2 197 (U)	→	♀♀	**9♀: 5♂** **197 (U)**	**10♀: 0♂** **222 (I)**		
5	**2** **222 (I)**	2 197 (U)	→	♀♀	**1♀: 8♂** **197 (U)**	**9♀: 8♂** **197 (U)**		
6	2 194 (U)	**1** **222 (I)**	→	♀♀	**9♀: 7♂** **194 (U)**	**14♀: 19♂** **194 (U)**		
7	2 200 (U)	**1** **216 (I)**	→	♀♀	0♀: 14♂ 200 (U)	**4♀: 3♂** **200 (U)**		
8	2 200 (U)	**1** **216 (I)**	→	♀♀	0♀: 12♂ 200 (U)	**1♀: 14♂** **200 (U)**		
9	2 200 (U)	**2** **216 (I)**	→	♀♀	**1♀: 11♂** **200 (U)**	0♀: 20♂ 200 (U)		
10	2 197 (U)	**2** **203 (I)**	→	♀♀	**3♀: 14♂** **197 (U)**	**3♀: 0♂** **203 (I)**		
11	2 197 (U)	**2** **203 (I)**	→	♀♀	**7♀: 2♂** **197 (U)**	**1♀: 6♂** **197 (U)**		
12	2 197 (U)	**2** **203 (I)**	→	♀♀	**4♀: 5♂** **197 (U)**	**3♀: 6♂** **197 (U)**		
13	**2** **216 (I)**	1 194 (U)	→	♀♀	**6♀: 2♂** **194 (U)**	**7♀: 0♂** **216 (I)**		
14	**2** **216 (I)**	1 194 (U)	→	♀♀	**5♀: 21♂** **194 (U)**	DNE		
15	2 194 (U)	**2** **216 (I)**	→	♀♀	**11♀: 2♂** **194 (U)**	**1♀: 18♂** **194 (U)**		
16	2 194 (U)	**2** **216 (I)**	→	♀♀	**3♀: 25♂** **194 (U)**	**5♀: 14♂** **194 (U)**		
17	2 194 (U)	**1** **216 (I)**	→	♀♀	**6♀: 1♂** **194 (U)**	DNE		
18	2 194 (U)	**1** **219 (I)**	→	♀♀	**1♀: 37♂** **194 (U)**	DNE		
19	2 194 (U)	**1** **219 (I)**	→	♀♀	**9♀: 1♂** **194 (U)**	**1♀: 29♂** **194 (U)**		
20	2 194 (U)	**2** **219 (I)**	→	♀♀	**4♀: 3♂** **194 (U)**	**3♀: 5♂** **194 (U)**		
21	**2** **219 (I)**	1 194 (U)	→	♀♀	**5♀: 5♂** **194 (U)**	DNE		

Bold entries indicate *Wolbachia*-infection. The origin of the wasps is mentioned: U, Uninfected; I, Infected. DNE, Did Not Emerge. Size of the microsatellite fragment is also mentioned.

The process by which uninfected *Trichogramma* larvae acquire *Wolbachia* remains unclear. Several possible acquisition mechanisms exist. Infected larvae may die and be

consumed by uninfected larvae. Larvae may fight within the host egg and the uninfected acquire an infection through blood to blood contact as found in the woodlice *Armadillidium vulgare* (Rigaud & Juchault 1995). Finally, infected females may inject *Wolbachia* into the host egg when they oviposit in it and the larvae acquire the infection either orally or through wounds caused by larval conflict. The first possibility can be eliminated. In 3 of the 21 cases all the eggs laid by the infected female survived to become adults, yet some of the offspring from the uninfected female became infected. We cannot distinguish between the two other possibilities. Our results show that offspring of uninfected females can acquire sufficient PI *Wolbachia* to express parthenogenesis when they share a butterfly egg with offspring of an infected female. We know that such egg sharing occurs in the field. Maximum rates at which the offspring of *Wolbachia* infected and uninfected females share the same butterfly egg in the field can be estimated from observed parasitism rates (van Alphen *et al*. 1992). *T. kaykai* can parasitize up to 80% of the *Apodemia mormo deserti* eggs, suggesting that offspring of two or more females could share the same egg in as many as 48% of the cases. Up to 9 % of the latter cases would involve offspring of both infected and uninfected females (assuming a 10% infection rate among females). Although such high parasitism rates are infrequent, they illustrate the potential for horizontal transfer under field conditions.

Purely vertically inherited symbionts suffer from an accumulation of mutations similar to the Muller's ratchet in asexual organisms (Moran 1996). However, horizontal transfer of PI *Wolbachia* among offspring of infected females offers the potential for recombination between different *Wolbachia* variants and thus for circumventing Muller's ratchet.

Vertical transmission has been considered the only common mechanism by which PI *Wolbachia* is transmitted. Such transmission should select for accommodation between *Wolbachia* and its host wasps. However, when horizontal transfer and vertical transmission are frequent, they should select for conflicting adaptations because they are considered to require opposing adaptations (Ewald 1994). The reduced offspring production by PI *Wolbachia* females (Stouthamer & Luck 1993) may be a result of such opposing adaptations.

Intraspecific horizontal transfer of *Wolbachia* from infected to uninfected larvae within a host suggests the possibility of interspecific transfers. These latter transfers have been

predicted based on phylogenies of *Wolbachia* and its Trichogrammatid hosts (Schilthuizen & Stouthamer 1997). Host eggs that are shared by offspring of more than one *Trichogramma* species have been found in field populations in the Mojave Desert (Chapter 4 & 6). One of these species, *T. deion*, is known to be infected, but with a phylogenetically different *Wolbachia* isolate (Schilthuizen *et al*. 1998). Thus, horizontal transfer between species may either be more difficult or infection may not be maintained in the new species. Such interspecific horizontal transfers, however, may offer a means with which to investigate the co-evolution between *Wolbachia* and its host wasp.

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Dit moet titelpagina **Chapter 4** worden!!!!!!!!!!!

Natural inter- and intraspecific horizontal transfer of parthenogenesis-inducing *Wolbachia* in *Trichogramma* wasps

ABSTRACT The intracellular bacterium *Wolbachia* is one of the most common symbionts in arthropods and, because of its manipulative effects on host reproduction, assumed to be an important factor in several evolutionary processes. These bacteria are mainly vertically transmitted from mother to daughter through the egg cytoplasm and horizontal transmission is generally assumed to be rare. Here, we show natural inter and intraspecific horizontal transfer of parthenogenesis-inducing (PI) *Wolbachia* between parasitoid wasps of the genus *Trichogramma*. Horizontal transfer was observed when infected and uninfected larvae shared the same host egg. This is the first report on interspecific horizontal transfer of *Wolbachia* between closely related sympatric species. Originally uninfected immature wasps could acquire *Wolbachia* inside the host egg but not all newly infected females also exhibited the parthenogenesis phenotype. In general, intraspecific horizontal transfer was more successful than interspecific transfer. *Wolbachia* could undergo vertical transmission in a new species but infection tended to be lost several generations. Our results have important implications to understand the evolution of *Wolbachia*-host associations.

4.1 INTRODUCTION

Prevalence of *Wolbachia* is estimated at 17-76% of the insect species (Werren *et al.* 1995*a*; Jeyaprakash & Hoy 2000). This endosymbiont has received much attention mainly because it has evolved several alterations of it's host reproduction, thereby optimizing its vertical cytoplasmic inheritance. These alterations include cytoplasmic incompatibility (CI) (Yen & Barr 1971; Hoffmann, *et al.* 1986; Breeuwer & Werren 1990; O'Neill & Karr 1990), feminization (Rigaud *et al.* 1991), male-killing (Hurst *et al.* 1999) and parthenogenesis-induction (PI) (Stouthamer *et al.* 1990; Stouthamer *et al.* 1993).

It is generally assumed that vertically inherited symbionts cospeciate with their host but this is certainly not the case for *Wolbachia.* Phylogenetic evidence showed that horizontal transfer of these bacteria must have occurred in the course of evolution because closely related bacterial strains can be found in unrelated hosts (O 'Neill *et al.* 1992; Rousset *et al.* 1992; Stouthamer *et al.* 1993; Werren *et al.* 1995*b*). Similarly, micro-injection studies successfully transferred *Wolbachia* into naïve hosts both intra-

and interspecifically (Boyle *et al.* 1993; Braig *et al.* 1994; Grenier *et al.* 1998). Recently, Fujii *et al.* (2001) were able to show that, after transfection, a single *Wolbachia* strain can induce different phenotypes in different hosts. In general it is however very difficult to maintain a high infection rate over many generations in newly infected lines (Pintureau *et al.* 2000*b*; van Meer & Stouthamer 1999; McGraw *et al.* 2002).

In nature, horizontal transfer can only occur when a donor and a recipient host are in close confinement, since *Wolbachia* is assumed not to survive outside host tissues. *Wolbachia* were first semi-naturally transferred in woodlice via blood-blood contact (Rigaud & Juchault 1995). Such transfer might occur in nature when individuals become injured during crowding. Close confinement is certainly the case in host-parasitoid associations. Two phylogenetic studies revealed the possibility for more frequent horizontal transfers in such communities (Schilthuizen & Stouthamer 1997; Vavre *et al.* 1999). Heath *et al*. (1999) thereafter showed a natural host-parasitoid transfer from an infected host (*Drosophila simulans*, where *Wolbachia* induces CI) to a parasitoid wasp (*Leptopillina boulardi*). Frequent natural horizontal transfer between conspecifics was first shown in the parasitoid wasp *Trichogramma kaykai* (Huigens *et al.* 2000 (= Chapter 3)). When PI *Wolbachia*-infected and uninfected *T. kaykai* larvae share the same host, originally uninfected larvae can acquire the infection. Newly infected females thereafter produce daughters from their unfertilized eggs. It is however still unknown how common such horizontal transfer is. Here, we investigate intra- and interspecific natural horizontal transfer in several *Trichogramma* species.

Trichogramma wasps display a haplodiploid mode of reproduction in which daughters (diploid) arise from fertilized eggs and sons (haploid) from unfertilized eggs. *Wolbachia* is known to induce parthenogenesis in at least 14 *Trichogramma* species (Stouthamer 1997; Schilthuizen & Stouthamer 1997; Pinto & Stouthamer 1999; Pintureau *et al*. 2000*a*; Ciociola Jr. *et al.* 2001) of 180 nominal species known (Pinto 1999). Females infected with PI *Wolbachia* produce daughters from both their fertilized and unfertilized eggs. In unfertilized infected eggs, a modification of the anaphase in the first mitotic division causes a doubling of the haploid set of maternal chromosomes, a process called gamete duplication (Stouthamer & Kazmer 1994). Such parthenogenetic reproduction can be cured after antibiotic treatment (Stouthamer *et al.* 1990).

Wolbachia in *Trichogramma* are unique compared to almost all other *Wolbachia*-host associations because "*Trichogramma Wolbachia"* cluster together in all phylogenetic trees based on several *Wolbachia* specific genes (Stouthamer *et al.* 1999*a*). Phylogenetic analysis of the *Wolbachia*-*Trichogramma* association revealed an obvious discordance between *Trichogramma* and *Wolbachia* phylogenies that is most likely explained by horizontal transfer of *Wolbachia* (Schilthuizen & Stouthamer 1997). Such horizontal transfer might take place when different species use the same host egg (Huigens *et al.* 2000 (=Chapter 3). Horizontal transfer can even result in double or triple infections when wasps infected with different *Wolbachia* oviposit in the same host egg. Multiple infections have not yet been described in *Trichogramma* but are known from several other host species, mainly associated with CI-*Wolbachia* (Werren 1997). Multiple infection opens the way for recombination between different *Wolbachia.* Such genetic exchange has been shown in the *Wolbachia* surface protein (*wsp*) gene of several strains of *Wolbachia* (Jiggins *et al*. 2001; Werren & Bartos 2001).

Here, we study natural intra- and interspecific horizontal transfer of PI *Wolbachia* in three situations: 1) superparasitism, in which larvae of an infected and an uninfected mother of a single species share the same host egg, 2) multiparasitism, in which larvae of an infected and an uninfected mother of different species share the same host egg, and 3) multiparasitism, but now when larvae of two infected mothers of different species share the same host egg.

4.2 MATERIAL AND METHODS

4.2.1 *Trichogramma cultures*

Intra- and interspecific horizontal transfer were attempted using iso-female lines of four *Trichogramma* species: *T. kaykai, T. deion, T. pretiosum* and *T. atopovirilia*.

T. kaykai and *T. deion* lines were initiated with wasps collected on eggs of the butterfly *Apodemia mormo deserti* in the Mojave Desert, CA, USA. In both species infected and uninfected females coexist. A study on the *ftsZ* gene of *Wolbachia* in the two species revealed that *Wolbachia* in *T. kaykai* most likely originates from a single infection (Schilthuizen *et al.* 1998). The two infected (LC 19-1 and LC 10-1) and three uninfected *T. kaykai* lines (LC 105A, LC 19-1 cured and LC 110 cured) all originate from Last Chance Canyon, El Paso Mountains, Kern County, California. Of *T. deion* we used an infected (SW 436-1) and an uninfected line (SW 649) from Sidewinder Mnts, Kern

County, California, an uninfected line (LC 151) from Last Chance Canyon, El Paso Mountains, Kern County, California and an infected line (223) initiated with wasps collected at Sanderson, Texas. The infected *T. pretiosum* line (Tpre-13) was collected in Santa Catarina, Brasil (host species unknown). The infected (Tato-01) and uninfected (Tato-02) *T. atopovirilia* were collected in Minas Gerais State, Brazil and in Colombia respectively (host species unknown). The infection status of the natural *T. pretiosum* and *T. atopovirilia* populations is unknown. *Trichogramma* lines were cultured on eggs of the moth *Ephestia kuehniella* for many generations before the experiments were conducted.

4.2.2 *Intra- and interspecific horizontal transfer*

Intraspecific horizontal transfer of *Wolbachia* was attempted by giving an infected (donor) and an uninfected (recipient) female of a single species the opportunity to oviposit in the same host egg. This superparasitism was carried out with three species *T. kaykai, T. deion* and *T. atopovirilia.* Here, *T. kaykai* was used as a control because horizontal transfer should occur in this species (Huigens *et al.* 2000 (=Chapter 3)).

Interspecific horizontal transfer was determined by allowing (1) infected *T. kaykai* and uninfected *T. deion*; (2) infected *T. deion* and uninfected *T. kaykai:* and (3) infected *T. pretiosum* and infected *T. atopovirilia* females the opportunity to parasitize the same host egg. The latter multiparasitism might result in females carrying two different *Wolbachia* strains, i.e. a double infection.

4.2.3 *Test for horizontal transfer of* Wolbachia

A moth egg, either *Trichoplusia ni* or *Mamestra brassicae*, both uninfected hosts, was offered to a female (‘line A’) and two hours later, we exposed the same egg to a second female (‘line B’). The latter female was either a conspecific (superparasitism) or a congener (multiparasitism). In eggs of both lepidopteran species, a *Trichogramma* female usually lays a clutch of 2-4 eggs. In half the cases, a female from ‘line A’ was offered the egg first and in the other half the order was reversed. We observed and recorded the number of eggs oviposited in a moth egg by each female using behavioral criteria described by Suzuki *et al.* (1984). If only F1 females emerged from a super- or multiparasitized egg, we exposed these virgin F1 females individually to host eggs and recorded the sex of their progeny.

The F1 females were linked to their parental female using a molecular marker and, in the multiparasitism experiments, female body color. To determine the origin of the F1 females in the superparasitism combinations we used a microsatellite DNA repeat TTG 49 for *T. kaykai,* a TAC 47 microsatellite repeat for *T. deion* and specific primer for DNA amplification of the ITS2 region for *T. atopovirilia.* In infected *T. atopovirilia* one DNA fragment is amplified whereas in uninfected *T. atopovirilia* two fragments of different size are amplified. In the multiparasitism combinations, we could easily distinguish the F1 females of the different species by the female body color. Females of *T. kaykai* have a yellow body color and *T. deion* females are brown. The *T. pretiosum* and *T. atopovirilia* females used in the experiments are respectively yellow and black.

The F1 females from the recipient line were tested for the presence of *Wolbachia* by PCR using *wsp* primers (Braig *et al.* 1998). To confirm the horizontal transfer, amplified *wsp* genes were sequenced in the donor lines and in the newly infected females. The presence of daughters in the offspring of an F1 virgin originating from a recipient line indicated horizontal transfer of *Wolbachia* and subsequent PI.

In the test for double infection when infected *T. pretiosum* and infected *T. atopovirilia* larvae share the same host egg, the amplified *wsp* fragments of *Wolbachia* in both species were distinguished using the restriction enzymes MboI and MboII. A combination of the restriction patterns in F1 *T. pretiosum* or *T. atopovirilia* females confirms a double infection.

4.2.4 *Molecular techniques*

DNA extraction was performed using one wasp homogenized in 50 µl 5% Chelex-100 and 2 µl proteinase K (20 mg/ml) and incubated for at least 4 hours at 56^{o}C, followed by 10 min at 95^{o}C. PCR reactions were performed in a total volume of 25 µl using a Techne thermocycler, 2.5 µl DNA template, 2.5 µl 10x PCR-buffer, 0.5 µl dNTP's (each in a 10 mM concentration), 0.5 µl forward and reverse primers, 0.07µl TAQ polymerase (5 units/µl) and 18.43 µl of sterile distilled water. Primers sequences and cycling programs were: (1) *wsp*-Forward primer 5'TGGTCCAATAAGTGA TGAAGAAAC-3' and *wsp*-Reverse 5'-AAAAATTAAACGCTACTCCA-3' (Braig *et al*. 1998). Cycling program: 3 min at 94^{o}C followed by 40 cycles of 1 min. at 94^{o}C, 1 min. at 50^{o}C and 1 min. at 72^{o}C with 5 min at 72^{o}C after the last cycle; (2) TTG 49-forward primer 5'-GTAGTCTGGTTTTCGATTCCCA-3' and TTG 49-reverse primer

5'-TCCCCGACCT ATCGATTTTCC-3' (Stouthamer unpublished). Cycling program: 5 min at 94^{o}C followed by 45 cycles of 1 min. at 94^{o}C, 1 min. at 63^{o}C and 1 min. at 72^{o}C with 5 min at 72^{o}C after the last cycle; (3) TAC 47-forward primer 5'-CTACGGCGACAATTGC CAC-3' and TAC 47-reverse primer 5'-CATCTTGGTCGAACCGAGCAG-3' (Stouthamer unpublished). Cycling program: 5 min at 94^{o}C followed by 30 cycles of 1 min. at 94^{o}C, 1 min. at 65^{o}C and 1 min. at 72^{o}C with 5 min at 72^{o}C after the last cycle; and (4) ITS2-forward primer 5'-TGTGAACTGCAG GACACATG-3' and ITS2-reverse primer 5'-GTCTTGCC TGCTCTGCTCTGAG-3' (Stouthamer *et al.* 1999*b*). Cycling program: 3 min at 94^{o}C followed by 33 cycles of 40 seconds at 94^{o}C, 45 second at 53^{o}C and 45 seconds at 72^{o}C with 5 min at 72^{o}C after the last cycle. All PCR products were run on a standard 1.5% agarose gel.

Cloning, sequencing and alignments of the *wsp* genes of *Wolbachia* in donor lines and in newly infected females were done. PCR products were purified with a QIAquick PCR purification kit (Qiagen®) and ligated into a Pgem-T® Vector (Promega). After transformation a PCR reaction was performed to confirm if a correct piece of DNA had been cloned. To purify the plasmid we used a QIAprep Miniprep kit (Qiagen®). Sequencing was performed in an Applied Biosystems automatic sequencer. *Wolbachia* sequences were aligned manually using the ESEE 3.0s sequence editor (Cabot 1995).

Restrictions of the *wsp* genes using the enzymes MboI and MboII were carried out to confirm double infection in *T. atopovirilia* and *T. pretiosum* F1 females after they shared the same host egg. The sizes of the different digestions products were estimated using the Webcutter 2.0 program (Heiman 1997). To perform a restriction of the *wsp* fragments, 10-µl volume (5 µl PCR product, 1 µl (10X) reaction buffer, 1 µl restriction enzyme and 3 µl distilled water) was used and incubated for 1 h at 37^{0}C. The digestion products were run on a 1.5% agarose gel. In *T. pretiosum* the use of the enzyme MboI generated in two cutting sites and three restriction fragments (266, 203 and 131 bp) of the *wsp* fragment and one cutting site and two restriction fragments (397 and 203 bp) in *T. atopovirilia*. With MboII the restriction of the *wsp* fragment resulted in one cutting sites and two restriction fragments (318 and 282 bp) in *T. pretiosum* and two cutting sites and three restriction fragments (218, 204 and 76 bp) in *T. atopovirilia*. For both enzymes, a restriction of the amplified *wsp* product from DNA template that was a mix of infected *T. atopovirilia* DNA and infected *T. pretiosum* DNA clearly showed a

combination of the restriction patterns of both *wsp* fragments.

4.3 RESULTS

Intra- and interspecific horizontal transfer of PI *Wolbachia* both occurred but not always. Depending on the super- or multiparasitism combination, 0-39% of the females acquired an infection inside the host egg (Table 4.1). Sequencing of the *wsp* fragments confirmed that *Wolbachia* in the newly infected *T. kaykai* and *T. deion* lines originated from the donor lines. The horizontal transfer rate might be underestimated in our tests because of a bacterial density below the threshold value necessary for the detection by PCR with *wsp* primers in some of the recipient F1 females.

4.3.1 *Intraspecific horizontal transfer of PI* Wolbachia

Both originally uninfected *T. kaykai* and *T. deion* larvae acquired an infection after sharing the host egg with infected conspecifics. Subsequent PI is much more efficient in newly infected *T. kaykai* females than in newly infected *T. deion* females. In *T. kaykai* 39 % of the originally uninfected F1 females became infected inside the host egg (17 of 44 were tested positive with *wsp*) and 88% of them produced some daughters from their unfertilized eggs. These results are similar to previous work described by Huigens *et al*. (2000) (= Chapter 3). In *T. deion* 36 superparasitized host eggs resulted in only 11 all-female F1 broods consisting of 17 potential newly infected females. Twenty–nine percent of these F1 females were infected (5 of 17) and 1 of these newly infected virgins produced a few daughters. Intraspecific horizontal transfer in *T. kaykai* and in *T. deion* occurred independent of the order in which two *Trichogramma* females were allowed to oviposit in the host egg (respectively $\chi^2_{0.05,1} = 0.021$; p=0.886; n = 37 for *T. kaykai* and $\chi^2_{0.05,1} = 2.21$; p=0.137; n = 11 for *T. deion*). Only in *T. atopovirilia* we could not detect intraspecific horizontal transfer: Forty-seven all-female broods resulted in 80 potential newly infected F1 females but none of them were infected or produced daughters as a virgin (Table 4.1).

Table 4.1 Inter- and intraspecific horizontal transfer of PI *Wolbachia* in *Trichogramma* sp.

Donor species females[1]	**Recipient Species**	**Host egg (Number)**	**Recipient females tested**	**Horizontal transfer**[1]	**PI in newly infected**
T. kaykai	*T. kaykai*	M (37)	44	39%[a]	88%[b]
T. deion	*T. deion*	T (11)	17	29%[ab]	20%[a]
T. atopovirilia	*T. atopovirilia*	M (47)	80	0%[2]	0%[2]
T. kaykai	*T. deion*	M (21)	26	12%[ab]	0%[a]
T. kaykai	*T. deion*	T (12)	13	8%[ab]	0%[a]
T. deion	*T. kaykai*	M (35)	59	19%[b]	0%[a]
T. deion	*T. kaykai*	T (21)	30	20%[ab]	83%[b]
T. pretiosum	***T. atopovirilia***	M (30)	95	0%[2]	0%[2]
T. atopovirilia	***T. pretiosum***	M (30)	120	0%[2]	0%[2]

[1] Chi square test. [2] Data not statistically analyzed. M = *Mamestra brassicae* and T = *Trichoplusia ni*. Bold entries indicate infection

4.3.2 *Interspecific horizontal transfer of PI* Wolbachia

Interspecific horizontal transfer occurred from *T. kaykai* to *T. deion* and vice versa. Only newly infected *T. kaykai* females exhibited the parthenogenesis phenotype. Of *T. kaykai* 19 % of the F1 females acquired *Wolbachia* from *T. deion* (17 of 89) and 29 % of them produced at least one daughter. Only 10% (4 of 39) of the *T. deion* F1 females acquired the *Wolbachia* from *T. kaykai*, but none of newly infected virgin produced some daughters. The host egg species, *T. ni* or *M. brassicae*, did not affect the interspecific horizontal transfer, or the percentage of the F1 females that acquired an infection inside the host egg, from *T. deion* to *T. kaykai* ($\chi^2_{0.05,1} = 0.36$; p=0.436; n = 56) nor vice versa ($\chi^2_{0.05,1} = 0.25$; p=0.614; n = 33). Like in the intraspecific transfer in these two species, interspecific horizontal transfer from *T. kaykai* to *T. deion* occurred independent of the order in which two *Trichogramma* females were allowed to parasitize the host egg ($\chi^2_{0.05,1} = 1.01$; p=0.316; n = 33). However, horizontal transfer occurred significantly more from *T. deion* to *T. kaykai* when *T. deion* was the first female to oviposit ($\chi^2_{0.05,1} = 12.08$; p<0.001; n = 56).

We did not find any evidence for double infection in infected *T. pretiosum* and *T. atopovirilia* F1 females after they shared the same host egg. Hundred-twenty *T.*

pretiosum females only carried the '*pretiosum Wolbachia*' and 95 *T. atopovirilia* females only the '*atopovirilia Wolbachia'* (Table 4.1) .

4.3.3 Subsequent vertical transmission after interspecific horizontal transfer

We determined vertical transmission of *Wolbachia* and PI in subsequent generations in two *T. kaykai* lines that were newly infected with a "*deion Wolbachia*". In both cases, we detect infection and PI in the F2 but thereafter the infection already seems to have been lost in the F3 - the sex ratio (% females) decreased (Table 4.2) - and in the subsequent generations PI could be observed any longer. In one line, 15 infected virgin F5 females only produced sons as well as 10 virgin F4 females and 10 virgin F9 females in the other line (Table 4.2).

Table 4.2 Vertical transmission of *Wolbachia* and PI in subsequent generations in two newly infected *Trichogramma kaykai* lines carrying a "*deion Wolbachia*".

Donor Species	**Recipient species**	**Sex ratio (% females)**				
		F2	**F3**	**F4**	**F5**	**virgin females**
T. deion	*T. kaykai*	0.82	0.37	0.79	0.85	F5: 0.00 (n=15)
T. deion	*T. kaykai*	0.87	0.74	0.76		F4: 0.00 (n=10) F9: 0.00 (n=10)

Bold entries indicate infection

4.4 DISCUSSION

These results are, to our knowledge, the first to show a natural interspecific horizontal transfer of PI *Wolbachia*, thereby partly explaining discordances between *Wolbachia* and *Trichogramma* phylogenies (Schilthuizen & Stouthamer 1997). Both intra- and interspecific transfer should also occur in nature in *Trichogramma*, since these wasps are host-generalists and multiparasitism occurs (Pinto 1999). This study confirms the frequent intraspecific horizontal transfer previously observed in *T. kaykai* (Huigens *et al.* 2000 (=Chapter 3)). The interspecific horizontal transfer of PI *Wolbachia* between the sympatric species *T. kaykai* and *T. deion* should occur in nature because the two species have been found together in a single host egg in the Mojave desert: e.g. at Randsburg road, Kern County, California 3% of the parasitized *Manduca sexta* eggs was multiparasitzed by both species (Huigens unpublished data).

We certainly do not always find successful horizontal transfer in our experiments. For horizontal transfer to be successful, *Wolbachia* first need to attain a high density in the newly infected female, infect the ovaries, and subsequently be transferred vertically and

induce PI. Unsuccessful horizontal transfer is most likely due to an incompatibility between *Wolbachia* and the host's nuclear/cytoplasmic background (Heath *et al.* 1999; Vavre *et al.* 1999). *Wolbachia* might be unable to adapt to a new nuclear background when it is not confronted with a new set of nuclear genes very frequently. Certainly in *T. kaykai* and most likely also in *T. deion*, *Wolbachia* is frequently confronted with a new nuclear background. In *T. kaykai*, we find 6-26% of the females to be infected (Stouthamer *et al.* 2001) and in *T. deion* only 2 of 229 broods were the offspring of an infected female (Chapter 6). We know that most infected *T. kaykai* females also mate with uninfected males in the population (Kazmer 1992; Chapter 7). *Wolbachia* from populations in which there is frequent gene flow into the infected population are therefore selected to adapt relatively easy to new nuclear backgrounds. After horizontal transfer such PI *Wolbachia* can be transmitted vertically and induce PI in a new host whereas PI *Wolbachia* from fixed populations cannot adapt to such a new situation. The fact that both the '*deion Wolbachia'* and the *'kaykai Wolbachia'* can be transmitted to another species supports this idea. The infected *T. atopovirilia* and *T. pretiosum* lines used in our experiments might be from a population fixed for the infection, i.e. all females in the population are infected, explaining why these *Wolbachia* are not easily transmitted horizontally. At least, they do not even rise up to densities detectable with the PCR method used. In the future, double infection resulting from infected females sharing the same host egg should be tested with *Wolbachia*-host associations where infected and uninfected individuals coexist, e.g. infected *T. kaykai* and infected *T. deion*. Another explanation for the fact that we only observe interspecific horizontal transfer between *T. kaykai* and *T. deion* might be the host phylogeny. These two north american species are closely related (Schilthuizen & Stouthamer 1997; Pinto 1999) in contrast to *T. pretiosum* and *T. atopovirilia* (Pinto 1999; Almeida unpublished results). The same goes for the *Wolbachia*'s in the species. Horizontal transfer of PI *Wolbachia* between more distant host species most likely occurs in nature (Schilthuizen & Stouthamer 1997) but might be a rare event, undetectable in our experiments.

Bacterial density is important for successful horizontal transfer of PI *Wolbachia* (Grenier *et al.* 1998). *Wolbachia* density effects have previously been shown on CI expression in *Nasonia* and *Drosophila* (Breeuwer & Werren 1993; Karr 1994; Bourtzis *et al.* 1996). In this study, the fact that newly infected virgin females never produce exclusively daughters and the large variation in sex ratio of their offspring suggest a

density effect (see also Huigens *et al.* 2000 (= Chapter 3)). We only tested for horizontal transfer in virgin recipient F1 females and therefore indirectly selected for high bacterial density. Insufficient bacterial density in newly infected virgins to induce PI immediately causes the *Wolbachia* not to be transmitted to subsequent generations. In future work we should test mated recipient F1 females. In that case fertilized eggs with a relatively low *Wolbachia* titer will become females, this will allow the *Wolbachia* to attain a high enough titer to be transmitted and to express itself over the subsequent generations.

When we compare the PI *Wolbachia*-*Trichogramma* association with the feminizing (F) *Wolbachia*-isopod association, we see some clear similarities. Rigaud *et al*. (2001) described the same pattern of interspecific horizontal transfer of *Wolbachia* when they semi-naturally transferred F *Wolbachia* between two closely related isopod species. Genetic sons of newly infected females were successfully feminized but maintenance of infection and feminization in future generations remains to be studied. Interspecific transfer between phylogenetically distant isopod species was however unsuccessful. In both *Wolbachia*-host associations, *Wolbachia* seem to form a monophyletic group. This applies to *Trichogramma*-*Wolbachia* and also to most isopod-*Wolbachia*. The *Wolbachia* seem to have a common descent and the discordance between the *Wolbachia*- and host phylogenies shows that *Wolbachia* have shifted between species in both host groups (Schilthuizen & Stouthamer 1997; Bouchon *et al.* 1998; Cordaux *et al.* 2001). In both associations the host populations are not fixed for infection (Stouthamer 1997; Rigaud 1997) which should facilitate horizontal transfer as described above. Also, both *Trichogramma* wasps and isopods have a gregarious behavior that offers excellent opportunities for natural intra- and interspecific horizontal transfer. These factors together may be the cause of the relatively high rate and success of horizontal transfers in both *Wolbachia*-host associations.

In conclusion, intra- and interspecific horizontal transfers of *Wolbachia* should occur in nature between organisms that interact in close confinement. We may have underestimated (i) the rate of natural horizontal transfer of PI *Wolbachia* in *Trichogramma* due to our PCR detection method and (ii) the subsequent vertical transmission because we only detect PI in the cases where bacterial density is high in newly infected virgins. Subsequent vertical transmission most likely limits the successful spread of newly acquired infections (Cook & Butcher 1999; Heath *et al.* 1999; Rigaud 1997, Pintureau *et al*. 2000*b*), which is also indicated by the loss of

infection in two *T. kaykai* lines newly infected with a '*deion Wolbachia'*. Such unsuccessful horizontal transfer is most likely due to incompatibilities between *Wolbachia* and the host's nuclear/cytoplasmic background. Such incompatibilities clearly exist in nature, otherwise experimental horizontal transfers must be extremely easy to obtain and research has shown that this is generally not the case. Although subsequent vertical transmission after horizontal transfer may occur at a very low rate (almost undetectable in laboratory experiments), on an evolutionary time scale it might be frequent enough to explain the discordance between *Wolbachia*- and host phylogenies.

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Dit moet titelpagina **Chapter 5** worden!!!!!!!!!!!

Reduced competitive ability due to *Wolbachia* infection in the parasitoid wasp *Trichogramma kaykai*

ABSTRACT Several hymenopteran parasitoids are infected with parthenogenesis-inducing (PI) *Wolbachia*. Infected wasps produce daughters instead of sons from unfertilized eggs. So far little is known about the direct effects of PI *Wolbachia* on their host's fitness. Here, we report a reduced competitive ability due to *Wolbachia* infection in a minute parasitoid wasp, *Trichogramma kaykai*. Immature survival of infected individuals in a host parasitized by a single infected female, laying a normal clutch of eggs, was lower than those parasitized by a single uninfected individual. When offspring of infected and uninfected females shared the same host, the infected immatures had significantly lower survival rates than their uninfected counterparts. The survival rate of infected immatures was higher when they competed with other infected immatures from a different infected parent than in competition with uninfected immatures of conspecific wasps. Thus, the host *Trichogramma* can suffer a substantial reduction in fitness when infected with the PI *Wolbachia*. We discuss why such a reduction is to be expected when populations of infected and uninfected individuals co-occur and how the reduced competitive ability of PI *Wolbachia* influences the spread of the bacteria in the field.

5.1 INTRODUCTION

Genetic elements that manipulate the sex ratio of their host do so to enhance their own transmission (Werren *et al.* 1988). One such manipulative element is *Wolbachia*, a cytoplasmic inhabiting bacterium first described by Hertig (1936). This reproductive parasite is transmitted vertically only through egg cells, thus, it enhances its transmission by manipulating the reproduction of its host to favor the production of *Wolbachia* infected egg cells. *Wolbachia* manipulates this production in one of five ways: cytoplasmic incompatibility (CI) (Yen & Barr 1971), feminization (Rigaud *et al.* 1991; Rousset *et al.* 1992), male-killing (Hurst *et al.* 1999), increased fecundity of its host (Girin & Bouletreau 1995), and induction of parthenogenesis (Stouthamer *et al.* 1990).

To date, most research has focused on *Wolbachia*'s evolution, phylogeny and the mechanisms by which they exert their main effects (O'Neill *et al.* 1997). More recently, interest has arisen in the consequences of their effects on a host's reproductive success. *Wolbachia*'s presence in large numbers within host tissues likely inflicts physiological

costs on their hosts (Stouthamer *et al.* 1999). In most cases, such costs do not appear to reduce their host's fitness (Hoffmann *et al.* 1994; Giordano *et al.* 1995; Bourtzis *et al.* 1996; Poinsot & Mercot 1997; Hoffmann *et al.* 1998) and, in some cases, *Wolbachia* even enhance it (Wade & Chang 1995; Girin & Bouletreau 1995; Stolk & Stouthamer 1995; Poinsot & Mercot 1997; Vavre *et al.* 1999). Even though it is generally assumed that physiological costs are minimized in populations where all individuals are infected, this does not imply the absence of a cost. Parasites consume energy, independent of selection pressures favoring their benevolence (Bull *et al.* 1991). For example, fecundity, adult survival, and locomotory activity are reduced in *Leptopillina heterotoma* populations fixed for CI-*Wolbachia* (Fleury *et al.* 2000), and male *Drosophila simulans* infected with CI-*Wolbachia* produce fewer sperm cysts and are less fertile than their uninfected counterparts (Snook *et al.* 2000).

At least two factors are thought to be important in influencing the cost that *Wolbachia* inflects on their host. First, in cases where the infection has not reached fixation within a host population, as is sometimes the case with PI *Wolbachia*, a genomic conflict is expected between *Wolbachia* and their host's nuclear genome: PI *Wolbachia* favors a 100% female bias whereas the nuclear genes favor a sex ratio with at least some males (Stouthamer 1997). In such situations, an arms race may ensue between the nuclear genes, which try to suppress *Wolbachia* or their effect, and those of the *Wolbachia*, which try to enhance their transmission (Stouthamer 1997). Consequently, a higher cost is expected in infected individuals when host populations comprise both infected and uninfected individuals. A second factor affecting *Wolbachia*'s cost inflicted on their host is *Wolbachia*'s mode of transmission, i.e., whether their transmission is largely vertical or both vertical and horizontal. Recently Huigens *et al.* (2000) showed that PI *Wolbachia* are frequently transferred horizontally in *Trichogramma kaykai*. When *Wolbachia* are only transmitted vertically within a host population, lower symbiont virulence should be favored since the host's genome and that of *Wolbachia* have presumably become more adapted to one another (Herre 1993). However, with horizontal transmission, higher bacterial densities are favored since *Wolbachia's* transmission is to some extend positively correlated with a high *Wolbachia* titer. Therefore, increased virulence to the host is expected (Ewald 1994).

Here, we focus on PI *Wolbachia*'s effect on the fitness of infected *T. kaykai* (Hymenoptera; Trichogrammatidae) when the wasp's larvae compete for food with

uninfected conspecific larvae within the same host (a butterfly egg). *Trichogramma kaykai* is a parasitoid wasp that lays a clutch of 4-5 eggs in an egg of the butterfly, the Mormon Metalmark, *Apodemia mormo deserti* (Lepidoptera; Lycaenidae) (Pinto *et al*. 1997). This butterfly lays mostly solitary eggs in the nodes of the desert trumpet, *Eriogonum inflatum* (Polygonaceae), a patchily distributed plant in the Mojave Desert of the American Southwest. PI *Wolbachia* infect 6 to 26% of the female *T. kaykai* in these populations (Stouthamer *et al.* 2001). These minute wasps (<1mm long) have a haplodiploid sex determination: unfertilized, haploid eggs develop into males and fertilized, diploid eggs develop into females. However, when a female is infected with PI *Wolbachia*, her unfertilized eggs also develop into females (Stouthamer & Kazmer 1994). Under laboratory conditions females cured of their PI *Wolbachia* infections generally have higher lifetime fecundities (Stouthamer & Luck 1993; van Meer 1999) and lower pre-adult mortalities (van Meer 1999; Tagami *et al.* 2001) than do their infected counterparts. However, the relevance of lifetime offspring production under laboratory conditions as a measure of fitness in the field remains unclear. Field fitness may be determined entirely by the wasp's ability to find hosts. We expect few hosts are found in the field during a female's lifetime.

Here, we study the ability of infected and uninfected *T. kaykai* immatures to compete within the same butterfly egg (host). Such a co-occurrence, i.e. superparasitism, takes place under field conditions in *T. kaykai* (Kazmer 1992; Chapter 6). Moreover, superparasitism is a common feature in the biology of *Trichogramma* spp. in general and occurs in many other hymenoptera as well. It is thought to be adaptive when unparasitized hosts are scarce (van Alphen & Visser 1990). In such competitive situations, the physiological cost of containing PI *Wolbachia* can become an important constraint when infected larvae compete with their uninfected conspecifics within the same host egg. We show that such infections reduce an immature *T. kaykai*'s ability to compete with its uninfected counterpart.

5.2 MATERIAL AND METHODS

5.2.1 *Parasitoid and host material*

To determine the fitness costs of harboring PI *Wolbachia* under superparasitism conditions, i.e. intraspecific competition, we compared the immature survival of infected versus uninfected *T. kaykai* sharing the same host egg under two different circumstances; 1) those in which the immature stages have the same infection status and 2) those in which the immature stages have a different infection status. We used isofemale lines derived from individual females that emerged from *A. m. deserti* eggs collected at two locations in Kern County, California: four infected (LC 19-1, LC 10-1, LC 110 and LC 190) and three uninfected lines (LC 105A, LC 170 and LC 177) collected at Last Chance Canyon, El Paso Mountains (1996) and one infected line (PN 1998) collected at Panamint Valley (1998). We used *Trichoplusia ni* (Hübner) eggs as laboratory hosts. This noctuid moth egg is used extensively by *Trichogramma* species (Appendix I in Pinto 1999). *Trichogramma* spp. generally seem to be more habitat than host specific (Pinto & Oatman 1988). Moreover, although a clutch of as many as five *T. kaykai* wasps can emerge from one *T. ni* egg, the normal clutch size is two to three *T. kaykai* eggs per *T. ni* (host) egg. We conducted our experiment at 26.7 °C, 50% RH and 18L: 6D photophase.

5.2.2 *Clutch size, sex allocation and immature survival in non-superparasitized hosts*

To determine the accuracy with which we predicted the number of wasp eggs allocated to a host (i.e., the clutch size laid in a host), we exposed a 24-h-old, mated, and uninfected *T. kaykai* female (LC 105A line), deprived of hosts, to a *T. ni* egg. In this experiment as in those that follow, we observed the ovipositions and recorded the number of *T. kaykai* eggs oviposited in the host using behavioral criteria described by Suzuki *et al.* (1984). Twenty-four hours later, we squashed the host egg under a compound microscope and counted the number of wasp eggs present.

To determine sex allocation and immature survival of uninfected wasps developing in hosts with only their siblings, i.e., clutch mates, we exposed a 24-h-old, mated, and uninfected *T. kaykai* female, deprived of hosts, to a *T. ni* egg and recorded the number and sex of the eggs she oviposited in the host. We isolated the host egg and counted and sexed the wasps emerging from them to determine the accuracy with which we predicted the sex of the oviposited egg and to estimate their immature survival rates. We

conducted the same behavioral observations for virgin *Wolbachia* infected wasps. We only scored the survival and sex of the eggs oviposited in clutches of 1 or 2 eggs, since we wanted to determine immature survival under conditions of normal competition amongst siblings of the same infection status inside the host egg.

5.2.3 *Immature survival in superparasitized hosts*

5.2.3.1 *Mixed infection status among competitors*

To induce intraspecific competition (superparasitism) between the offspring of infected and uninfected wasps, we allowed an infected virgin female and an uninfected mated female to oviposit in the same host egg with an interval of two hrs between ovipositions. (Infected virgin females produce only infected female offspring). We recorded the number and sex of the *T. kaykai* eggs oviposited in the host. We alternated the different infected and uninfected lines and the order of exposure of the wasps with different infection status to the host in the different replicates. After superparasitism, each host was isolated until the adult wasps emerged. We determined the infection status of the emerging females by allowing them to produce offspring. Those producing both sons and daughters were assumed to have originated from an uninfected line whereas those producing only daughters were assumed to have originated from an infected line. We assumed that all of the emerging sons arose from the uninfected lines. Under superparasitism, horizontal transfer of *Wolbachia* can occur; however, the newly infected females can still be recognized as originating from an uninfected line because they produce both sons and daughters (Huigens *et al.* 2000 (= Chapter 3)).

5.2.3.2 *Single infection status among competitors*

To determine the survival of the wasps when they compete with larvae of the same infection status, we allowed two infected or two uninfected females to parasitize the same host with an interval of two hours between the ovipositing females. We also determined the number and sex of the eggs that were allocated by both these females and recorded the number and sex of the wasps that emerged from the superparasitized hosts.

5.2.3. *Statistical analysis*

We compared the fraction of eggs oviposited by each female that emerged, i.e., that survived their immature development within the host egg, using a generalized linear model with a binomial distribution and a logit link (fitted with PROC GENMOD; SAS version 8; SAS Institute 1997). We treated estimates of immature survival of individuals from the same host as correlated and those from different hosts as independentby using the Generalized Estimating Equations approach (Diggle *et al.* 1994), available within PROC GENMOD. A wasp's infection status and sex were the treatment factors, the total number of eggs laid in a superparasitized host and the order in which the ovipositing female was exposed to the host were treated as covariates. In some comparisons we also assessed the effect of the isofemale line of the ovipositing female,

We conducted seven main comparisons (GENMOD uses Wald tests (χ^2)), two for offspring emerging from a host parasitized by a single mother (non-superparasitized hosts) and five for offspring emerging from hosts parasitized by two mothers (superparasitized hosts). For the two comparisons in non-superparasitized hosts, 1) we first compared the survival of male versus female offspring that emerged from a host. 2) We next compared the survival of offspring from an uninfected parent with those from an infected parent and assessed the effect of the isofemale line of the ovipositing female.

For the five cases involving superparasitized hosts, 3) we first determined if the mortality between sons and daughters of uninfected mothers differed when they shared a host with offspring of an infected mother. 4) After this, we determined whether mortality differed between infected and uninfected daughters in hosts parasitized by both an infected and an uninfected mother. Here, we also assessed the effect of the isofemale line of the ovipositing female, and treated the total number of eggs laid in a host by both females, and the order in which the ovipositing female was exposed to the host as covariates. 5) We then determined if the mortality between sons and daughters of uninfected mothers differed when they shared a host with offspring from another uninfected mother. 6) Next, we determined whether the mortality of uninfected daughters was influenced by the infection status of the other offspring present in a host. In these cases we used hosts parasitized by two uninfected females versus those parasitized by an infected and an uninfected mother. 7) Finally, we determined if the mortality of infected daughters was influenced by the infection status of the other

offspring in the host. In these cases we used hosts parasitized by two infected females versus those parasitized by an infected and an uninfected mother.

5.3 RESULTS

5.3.1 *Clutch size and sex of an oviposited egg*

We correctly predicted the number of wasp eggs allocated to a host in 97.0 % (n=67) of the cases when using Suzuki *et al*.'s (1984) behavioral criteria. To determine the accuracy with which we predicted the sex of an oviposited egg, we assumed that 100% of the uninfected male and female eggs allocated to a host survived and that those that were scored as "died inside the host" were actually our mistakes in predicting the number or sex of the allocated eggs. Under this "worst case" scenario, we predicted female eggs with an accuracy of 98.4% and male eggs with an accuracy of 94.9 %. We never observe a male emerging from a host that we had predicted to be a female. Thus, we are able to predict correctly the sex of the allocated eggs with a high degree of accuracy using Suzuki *et al*.'s (1984) criteria.

5.3.2 *Immature survival in non-superparasitized hosts*

1) Immature survival in non-superparasitized hosts was similar among the males (0.948 ± 0.036) (= mean ± se) and the females (0.984 ± 0.012) of the uninfected lines (χ^2 (df=1)= 2.79, n=164, P= 0.094).

2) Uninfected immatures (0.971 ± $3.10x10^{-04}$) showed significantly higher survival rates than their infected counterparts (0.811 ± 0.005) in non-superparasitized (χ^2 (df=1)= 12.39, n=445, P= <0.001) (Figure 5.1). Immature survival in non-superparasitized hosts was similar among the uninfected *T. kaykai* lines (χ^2 (df=2)= 0.25, n=164, P= 0.770). This contrasted with the heterogeneous survival among the immatures of the infected lines when they only competed with their siblings. Immatures survival among the four infected lines from Last Chance Canyon was similar (χ^2 (df=3)= 2.40, n=177, P= 0.440) but it differed among the lines when we included the infected, isofemale line from Panamint Valley (χ^2 (df=4)= 12.82, n=281, P= 0.012).

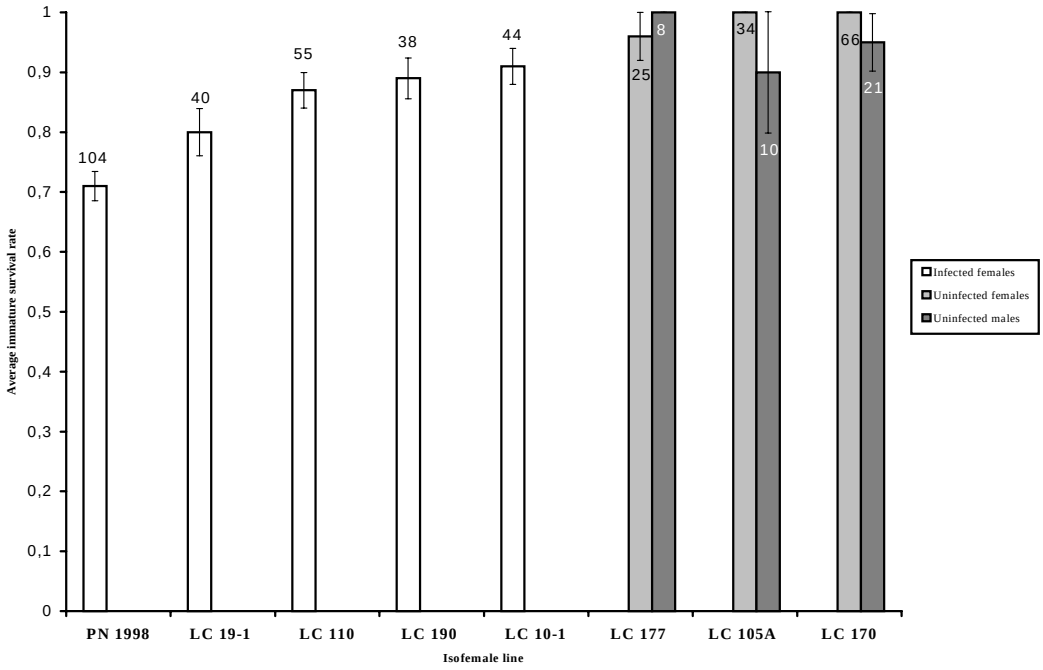

Figure 5.1 Average immature survival rates of infected and uninfected lines in host eggs where there only is a competition between sibs. Uninfected immatures are divided into males and females. Number of wasp eggs are mentioned above the bars.

5.3.3 *Immature survival in superparasitized hosts*

3) The survival of immature males (0.935 ± 0.028) was slightly higher than that of immature females (0.833 ± 0.033) when they competed with infected immatures (χ^2 (df=1)= 4.08, n=195, P= 0.043)) (Figure 5.2). Therefore, we considered the sex of uninfected immatures in the following analyses and compared the survival of uninfected immature females with that of the infected immature females.

4) Uninfected immature females (0.833 ± 0.033) showed significantly higher survival rates than infected immatures (0.320 ± 0.033) when they competed within superparasitized hosts (χ^2 (df=1)= 42.8, n=266, P< 0.001) and their survival rates were not significantly influenced by the identity of the line (χ^2 (df=7)= 3.84, n=266, P= 0.799).

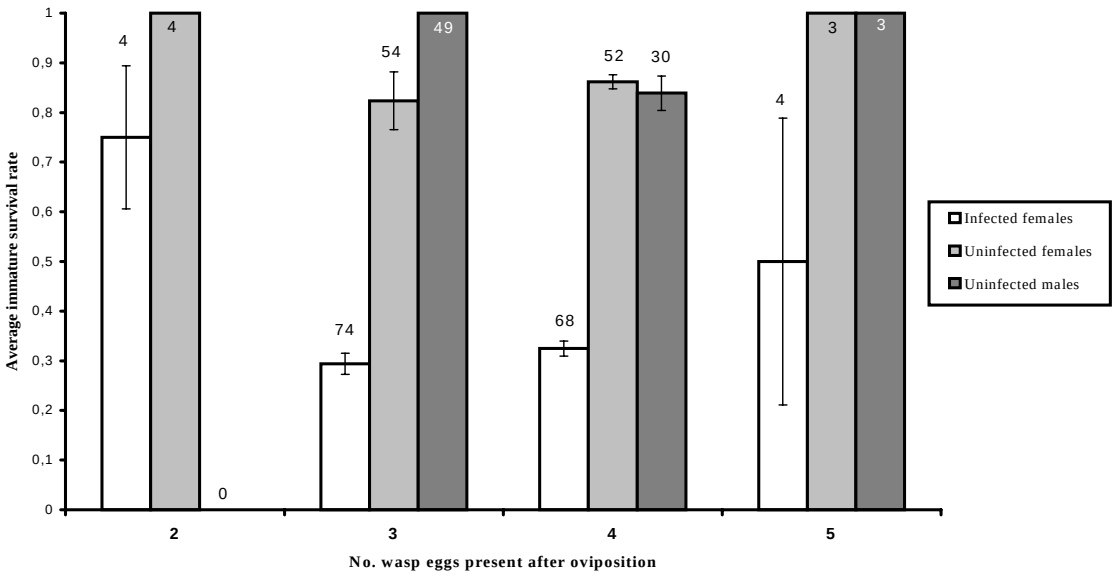

Figure 5.2 Average survival rates of infected and uninfected immatures in host eggs when there is a mixed infection status amongst the competitors. Either 2, 3, 4 or 5 wasp eggs are initially present after oviposition by both an infected and an uninfected female. The uninfected immatures are divided into males and females. The number of eggs allocated of each type are mentioned above the bars.

The survival rate of infected immatures in these competitive situations was similar at egg densities that ranged from 2 to 5 eggs per host (Figure 5.2) (χ^2 (df=1)= 0.167, n=266, P= 0.685), although the regression coefficient (= -0.105) hinted that immature females suffered more when competition within the host intensified. Furthermore, survival of infected, immature females was not significantly affected by whether their mother oviposited first (0,381 ± 0.043) or second (0,279 ± 0.050) (χ^2 (df=1)= 3.168, n=266, P=0.075).

5) Immature survival of males (0.679 ± 0.071) and uninfected females (0.639 ± 0.029) was similar when they competed with other uninfected immatures within the same host (χ^2 (df=1)= 0.142, n=104, P= 0.706) (Figure 5.3).

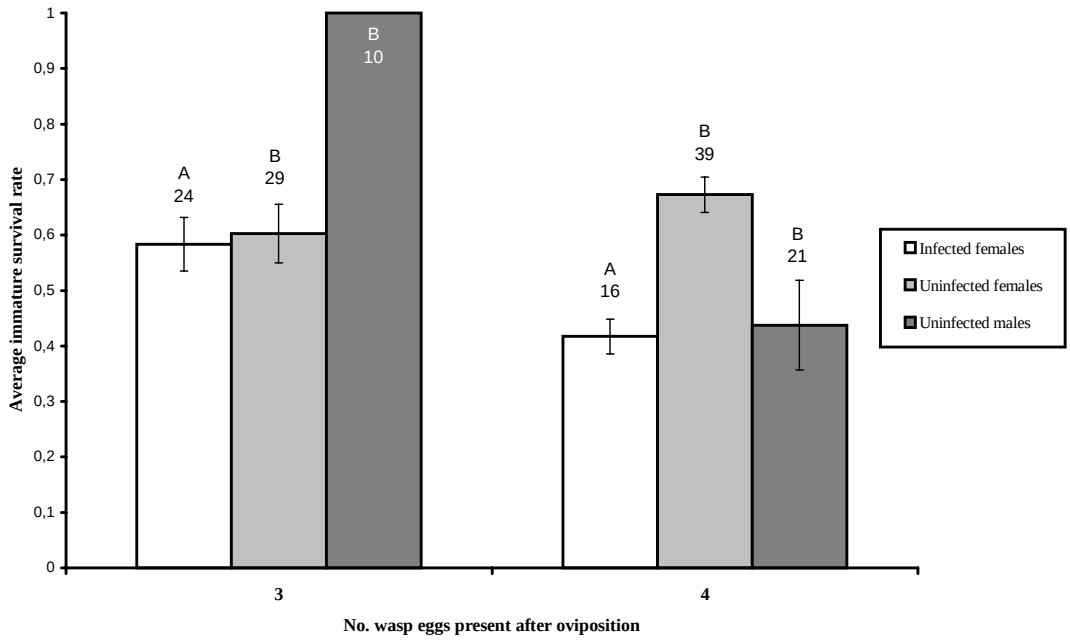

Figure 5.3 Average survival rates of infected and uninfected immatures in host eggs when there is a the same infection status amongst the competitors. Either 3 or 4 wasp eggs are initially present after oviposition by A) two infected females or B) by two uninfected females. The uninfected immatures are divided into males and females. The number of eggs allocated of each type are mentioned above the bars.

6) Uninfected immature females fared significantly worse when they competed with uninfected (0.639 ± 0.029) as opposed to infected immatures (0.833 ± 0.033) (χ^2 (df=1)= 11.82, n=185, P< 0.001).

7) Similarly, infected immatures had higher survival when they share a host with infected (0.534 ± 0.026) as opposed to uninfected conspecifics (0.320 ± 0.033) (χ^2 (df=1)= 6.12, n=198, P= 0.013).

5.4 DISCUSSION

In our experiments, infected *T. kaykai* generally showed lower survival rates as immatures than did their uninfected counterparts, even when they were the sole clutch in a host. However, the immature survival of infected offspring was heterogenous between the different infected lines. This suggests that some lines incur a higher cost of harboring *Wolbachia* than others, even when the immatures develop in a normal clutch consisting of only their siblings, that is, under conditions of normal competition. Developmental problems in some of the unfertilized, infected eggs would seem to be the

most likely explanation for this mortality. It is similar to that observed in a line of infected *Trichogramma deion* and *T. kaykai* by Tagami *et al*. (2001), although it was higher in their lines than in ours.

Also, immature survival was generally less in hosts that received a sequence of two clutches from each of two mothers than in hosts receiving a single clutch. The reduced survival in hosts with two clutches occurred regardless of the infection status of the two mothers, that is, whether both mothers were infected or uninfected. However, survival of infected immatures was much poorer when they shared the host with uninfected- than with infected immatures. Thus, infected offspring suffered both greater developmental mortality and an additional increment of mortality if they competed with immatures arising from a second clutch that had been laid by an uninfected conspecific. We eliminated the time between clutches as a factor by holding the interval between their deposition constant at two hours and altered their order in the sequence.

Several factors potentially explain the reduced survival of infected offspring. First, Hohmann & Luck (2000) found that single clutches of infected *T. kaykai* from the Panamint Valley line (PN 1998) in non-superparasitized host averaged about a half a day longer to hatch as larvae than did uninfected clutches. Strand (1986) noted that the interval between the hatching times of larvae from two clutches arising from superparasitism affects the survival probability of larvae arising from the second clutch: the greater the interval the lower the probability that the later hatching larvae survive. No newly hatched larvae survived when they competed with larvae 8 hrs or older (Strand 1986).

A female *Trichogramma*, when ovipositing in a host, injects venom before she oviposits her eggs, all of which happens during a single ovipositor insertion, which results in a single clutch of multiple eggs (Klomp & Teerink 1967; Suzuki *et al.* 1984; Strand 1986). The injected venom digests the cellular contents of the host egg, including the unscleratized parts of the host embryo (Strand 1986). The *Trichogramma* offspring when in the host are protected from digestion by their chorion (Strand 1986; Volkoff *et al.* 1995; Jarjees *et al.* 1998). They emerge from their chorion after host digestion is completed (Strand 1986) and, several hrs later, wriggle free of their exuviae and begin ingesting the predigested contents of the host (Volkoff *et al.* 1995; Jarjees *et al.* 1998). The eggs, comprising a clutch, normally emerge from their exuviae within one to one and a half hours from each other and, depending on temperature, ingest the host egg's

contents within 1 to 4 hrs after rupturing their exuviae (Klomp & Teerink 1978; Volkoff *et al.* 1995; Jarjees *et al.* 1998). During this ingestion process, larvae that do not ingest sufficient food die after regurgitating their food, which is then ingested by the remaining larvae (Klomp & Teerink 1978). Thus, a delay in hatching by infected larvae will place them at a disadvantage (Strand 1986).

Even in uninfected larvae, a delay in hatching of several hours is sufficient to increase the risk of larval death if it is a member of a second clutch, as they are unable to obtain sufficient food (Klomp & Teerink 1978; Strand 1986). A female *Trichogramma* normally detects a previously parasitized host when she initially examines it (Salt 1940), by the presence of an external mark. She can also detect a previously parasitized host if the external mark is removed (Salt 1940) by the degree of necrosis associated with the digestion of the host egg (Strand 1986). As might be expected, the probability that a second wasp will reject a previously parasitized host increases with time (Strand 1986; Luck *et al.* 2000). Nevertheless, superparasitism is favored under conditions of host scarcity if the elapsed time since allocation of first clutch is short (van Alphen & Visser 1990).

The disadvantage of delayed hatching coupled with the developmental problems in some infected lines likely explains much of the pattern observed in the competitive situations between infected and uninfected larvae.

A third factor suspected to affect the survival of *Wolbachia*-infected larvae is their apparent requirement for more nutrients when compared to that of uninfected larvae. Silva (1999) found that infected *T. cordubensis* females emerging from the small *Ephestia kuehniella* eggs, where usually one adult wasp emerges from, are significantly smaller than their uninfected counterparts. Barrett & Schmidt (1991) investigated the amount of nutrients required by and available to *T. minutum* to produce offspring from a range of hosts that differed in size. They found that *Sitotroga cerealella* eggs - in size comparable to those of *E. kuehniella* - contained an average of 2.2 μg of total amino acids producing a wasp averaging 1.0 μg of amino acids whereas a large host, *Manduca sexta*, contained 94 μg of amino acids and produced wasps averaging 8.2 μg of amino acids. Barrett & Schmidt's (1991) results suggest that *Trichogramma* are less constrained by the resources present in the larger hosts. In the superparasitized *T. ni* eggs the amount of nutrients present may contribute to the high mortality of the infected larvae.

The effect of reduced competitive ability of infected individuals on the infection rates in the field remains unclear. A lower survival of the infected eggs and the horizontal transfer of *Wolbachia* are favored under exactly the same conditions. We can calculate an average survival of the infected and uninfected immatures when they share the same host egg in our experiments; Survival of infected immatures is 0.320; of the uninfected male immatures 0.936; and of the uninfected female immatures 0.833. Huigens *et al.* (2000) (=Chapter 3) found a horizontal transfer of *Wolbachia* in 37.5% of the superparasitized hosts. The efficiency of horizontal transfer was found to be 0.91 (number of newly infected females emerging / total number of uninfected female wasps emerging). Uninfected males could also become infected inside the host but do not contribute to the infected population. Uninfected males could also become infected inside the host but do not contribute to the infected population. Therefore it is important to include the sex ratio (% females) of uninfected broods allocated in the non-superparasitized host, i.e. approximately 75% (Stouthamer *et al.* 2001). The net result of the horizontal transmission and the lower competitive ability of the infected wasps on the infection frequency of wasps emerging from a host can be determined using the following calculations in which:

I_t = Number of infected eggs allocated to a host
I_{t+1} = Number of infected female wasps that emerge
U_t = Number of uninfected eggs allocated to the host
U_{t+1} = Number of uninfected female wasps that emerge
S_I = Survival rate of infected eggs
S_{Uf} = Survival rate of uninfected female eggs
H = Horizontal transfer rate of *Wolbachia*
E = Efficiency of horizontal transfer of *Wolbachia*
SR_U = Sex ratio of uninfected broods (=% females)

$$I_{t+1} = S_I * I_t + S_{Uf} * U_t * E * H * SR_U$$
$$U_{t+1} = S_{Uf} * U_t * SR_u * (1 - E * H)$$

When we take, for example, $I_t = 1$ and $U_t = 1$, then $I_{t+1} = 0.51$ and $U_{t+1} = 0.41$. Thus, competition and horizontal transfer within a superparasitized host egg still results in the

production of a higher number of infected than uninfected females per superparasitized host. Superparasitism contributes more infected than uninfected females to the subsequent generation and contributes to the spread of the *Wolbachia* infection in the population.

The reduced competitive ability of infected larvae shows that *Wolbachia* infected *T. kaykai* potentially suffer severe reductions in the offspring production in the field. Why these bacteria appear to take such a toll on their hosts in competitive situations may be a result of a nuclear cytoplasmic conflict causing a much higher *Wolbachia* density than strictly needed for efficient transmission of the infection or it may be an unavoidable physiological cost of being infected. PI *Wolbachia* bacteria become an important constraint for wasp development when there is a scarcity of host egg nutrients. Besides the lower lifetime fecundity and increased pre-adult mortality in *Wolbachia* infected *Trichogramma* (Stouthamer & Luck 1993; van Meer 1999), this reduced competitive ability is the third effect found to reduce the fitness of PI *Wolbachia*'s host.

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Dit moet titelpagina **Chapter 6** worden!!!!!!!!!!!

Does the Paternal Sex Ratio chromosome play a role in the coexistence of *Wolbachia*-infected and uninfected forms in the parasitoid wasp *Trichogramma deion*?

ABSTRACT Parthenogenesis-inducing (PI) *Wolbachia* are known from many wasp, thrips and mite species. In most cases, the infection with these cytoplasmic bacteria has gone to fixation in the population, i.e. all females are infected, except in *Trichogramma* sp. In these minute parasitoid wasps infected and uninfected forms coexist. Recently, a paternal sex ratio (*PSR*) chromosome was discovered in *T. kaykai* that only occurs in males and converts fertilized eggs into *PSR*-carrying males, thereby preventing the *Wolbachia*-infection from spreading to all the females in the population. Because the infection with PI *Wolbachia* is also limited in another sympatric species, <1% of the females in *T. deion*, we expect *PSR* to be present in this species as well. Therefore, we collected large numbers of both wasp species and determined the species identity of males expressing the *PSR* phenotype. All *PSR* broods studied belonged to *T. kaykai*, indicating *PSR* does not occur in *T. deion*. In laboratory tests we found that *PSR* can successfully be transmitted from *T. kaykai* to *T. deion*, namely 71.4% of the *T. kaykai PSR* males horizontally transmitted the *PSR* phenotype to *T. deion* whereas the intraspecific transmission in *T. kaykai* was 81.6%. Therefore, some factor must prevent the successful spread of *PSR* in natural *T. deion* populations. Modelling shows that if *PSR* was the cause of the low infection frequency in *T. deion*, it would require sib mating frequencies in *T. deion* that were substantially lower than in *T. kaykai*. There is no reason to believe that this is the case. We therefore conclude that another factor than *PSR* causes the coexistence of infected and uninfected forms in *T. deion*, e.g. mendelian nuclear suppressor genes.

6.1 INTRODUCTION

Selfish genetic elements have been defined as those elements having characteristics that enhance their own transmission relative to the rest of an individuals genome, and that are either neutral or detrimental to the organism as a whole (Werren *et al*. 1988). Research over the last decade has shown the presence of a large number of selfish elements manipulating their host's offspring sex ratio to enhance their own transmission (O'Neill *et al.* 1997). The biased sex ratios caused by these distorters, deviate from the optimal sex ratios for the nuclear genes of their hosts. The conflict between the nuclear genes and the sex ratio distorters has led in some cases to the

evolution of nuclear genes suppressing the effects of the sex ratio distorters (Cavalcanti *et al*. 1957, Rigaud & Juchault 1993). This conflict over sex ratio and sex determination is thought to be a major cause of the wide variety of sex determination systems that are known between, and sometimes, within species (Hurst *et al.* 1997; Werren & Beukeboom 1998).

As in most Hymenoptera, the minute parasitoid wasp *Trichogramma* has a haplodiploid sex determination system. Fertilized eggs develop into females (diploid, 2n=10) whereas unfertilized eggs develop into males (haploid, n=5) (Stouthamer & Kazmer 1994). A *Trichogramma* female is able to determine the sex of an offspring, by controlling the fertilization. In the Mojave Desert three *Trichogramma* species, *T. kaykai*, *T. pratti* and *T. deion*, are found parasitizing eggs of the butterfly *Apodemia mormo deserti* (Pinto *et al.* 1997). Per parasitized host egg three to five wasps emerge. The optimal sex ratio for the nuclear genes is given by the Local Mate Competition theory (Hamilton 1967), which predicts that a mother should produce a single male with the rest of the brood consisting of females when she is the only foundress. *Trichogramma* females produce generally a single male and two to four female offspring per *A. mormo* egg. (Stouthamer & Kazmer 1994).

In many populations of *Trichogramma* wasps infections with PI *Wolbachia* bacteria enable infected females to produce exclusively female progeny both from unfertilized and from fertilized eggs (Stouthamer & Kazmer 1994). While in most species infection frequencies with *Wolbachia* remain under 5%, in populations of the *T. kaykai* parasitizing *Apodemia* eggs in the Mojave Desert the infection frequencies range from 6-26% (Stouthamer *et al.* 2001). Theory predicts that the infection with *Wolbachia* should either spread to fixation, i.e., all females are infected, or should disappear from the population. Such fixation we find in all other species hosting PI *Wolbachia* (Stouthamer 1997; Huigens & Stouthamer 2003 (= Chapter 2)). *Trichogramma* obviously forms an exception to this rule. A prolonged coexistence of infected and uninfected forms in a population is only possible when some suppressing factor is present (Stouthamer 1997). After searching and failing to find nuclear suppressor genes we discovered a second sex ratio distorter in *T. kaykai* causing males to father exclusively male offspring in the females they mate with (Stouthamer *et al*. 2001). This factor is known as *PSR* (Paternal Sex Ratio). A *PSR* chromosome, first described in the parasitoid wasp *Nasonia vitripennis* (Werren, 1991), only occurs in males and is an

example of an extremely selfish genetic element that converts fertilized diploid eggs, that would normally develop into females into haploid *PSR* males. This happens through the destruction of the paternal set of chromosomes after fertilization of the egg and only the *PSR* chromosome is transmitted (van Vugt *et al.* 2003). The *PSR* is a B-chromosome which is present in addition to the normal set of A-chromosomes (n = 5 + *PSR*). Every generation *PSR* accompanies a new A-chromosome complement from the mother. The only genetic element that is transmitted through the generations is the *PSR* chromosome (Figure 6.1).

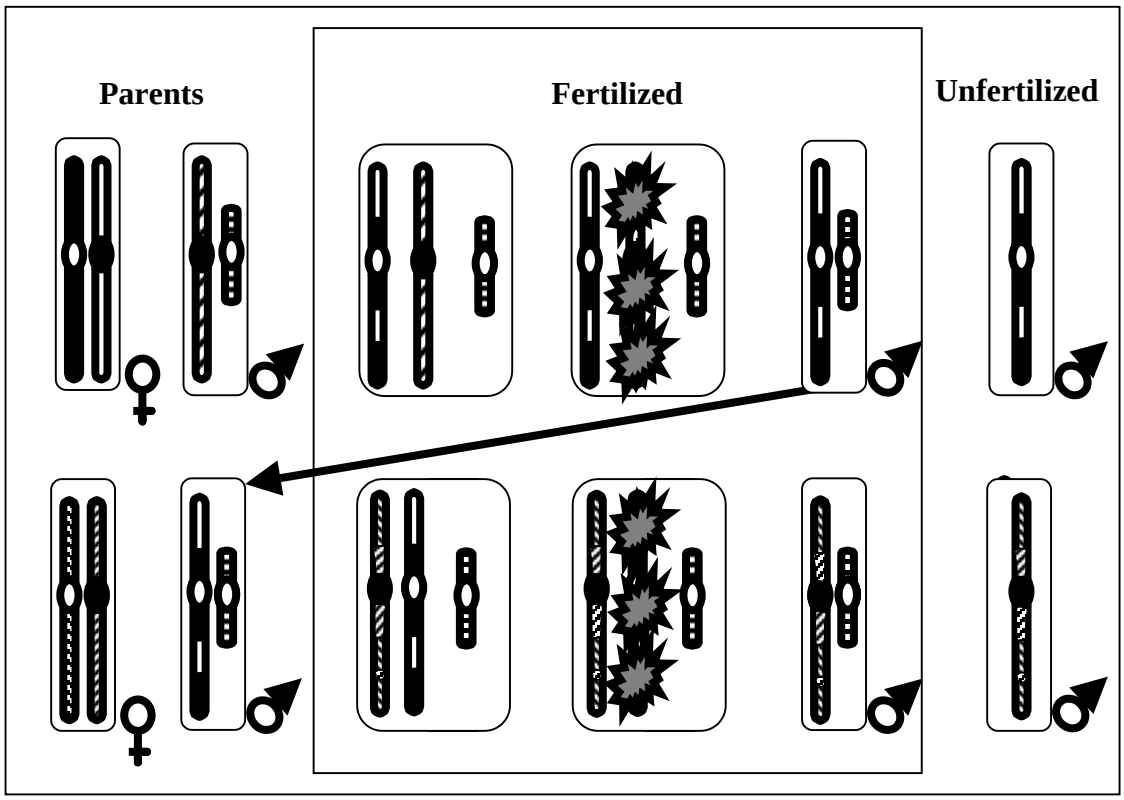

Figure 6.1. Mode of action of the *PSR* chromosome in *T. kaykai*. When a female (2n) and a *PSR*-carrying male (n+1) mate, the unfertilized eggs develop into normal males containing a halpoid set of maternal chromosomes. In fertilized eggs, however, the paternal chromosome set is destroyed and the maternal chromosome set together with the *PSR* chromosome remain. Such fertilized (haploid) eggs therefore develop into *PSR*-carrying males.

PSR was discovered in *T. kaykai* and has been found in frequencies of up to 15% of the males in field populations (Stouthamer *et al.* 2001). In populations where *PSR*, PI *Wolbachia* and normal reproduction occur four different mating combinations may occur leading to different brood compositions (Table 6.1).

Table 6.1 Four possible mating combinations that can occur in field populations of *Trichogramma* wasps between normal males and females, *PSR* males and *Wolbachia*-infected females.

Parents		**Offspring**		
female	**male**	**fertilized egg**	**unfertilized egg**	**sex ratio offspring**
normal	normal	normal female	normal male	1 male : 3-4 females
infected	normal	Infected female	infected female	all-female (4)
normal	*PSR*	*PSR* male	normal male	All-male (4)
infected	*PSR*	*PSR* male	infected female	1 female : 3-4 males

The presence of *PSR* explains why the *Wolbachia* infection in *T. kaykai* is kept at a relatively low equilibrium frequencies in the wasp population (Stouthamer *et al.* 2001). This occurs because a relatively larger fraction of the infected females mate with a *PSR* male than uninfected females do. Many uninfected females emerge from a host that also contains a brother and sib mating takes place. Only those uninfected females that do not sib-mate run the risk of mating with a *PSR* male, whereas all infected females that mate run the risk of mating with a *PSR* male. Therefore, the offspring production by infected females is more reduced than that of the uninfected females, this interaction leads to an equilibrium infection and *PSR* frequencies that are a function of, among others, the frequency of sib mating.

The *T. deion* population in the Mojave Desert also consists of infected and uninfected individuals coexisting in the population (Stouthamer 1997), however it is not known what keeps the *Wolbachia* infection frequency at low levels. *PSR* should pass species boundaries rather easily because of its mode of action, as indeed has been shown in laboratory experiments with *Nasonia sp.* (Werren 1991; Dobson & Tanouye 1995), and is therefore a good candidate for balancing the infection frequency in *T. deion* as well. Here, we determine if *PSR* can be passed on from *T. kaykai* to *T. deion* and if *PSR* is present in *T. deion* field populations thereby explaining the coexistence of infected and uninfected forms in this species.

6.2 MATERIAL AND METHODS

6.2.1 *Field work and test for the presence of sex ratio distorters*

Apodemia mormo deserti eggs were collected on the buckwheat *Eriogonum inflatum* throughout the Mojave Desert during the period from April to July 1998 to 2001. The collection sites were (coordinates given in order of latitude, longitude): Amboy, San Bernardino Co. (N 34° 34.05', W 115° 47.10'); Barstow-Daggett, San Bernardino Co. (N 34° 51.16', W 116° 53.47'); Bell Mountain Road, San Bernardino Co. (N 34° 36.44', W 117° 13.28'); Danby Gaspipe, San Bernardino Co. (N 33° 39.19', W 115° 21.13'); Dillon Road, San Bernardino Co. (N 34° 55.00', W 116° 23.15'); Essex Road, San Bernardino Co. (N 34° 46.04', W 115° 12.36'); Granite Mountains, San Bernardino Co. (N 34° 43.16', W 115° 12.36'); Ibex Hills, San Bernardino Co. (N 35° 53.37', W 116° 15.40'); Last Chance Canyon, Kern Co. (N 35° 22.35', W 117° 54,49'); L & B Canyon, Kern Co. (N 35° 22.63', W 117° 54.07'); Morongo Canyon, San Bernardino Co. (N 34° 00.33', W 116° 33.09'); Ocotillo, Imperial Co. (N 33° 44.56', W 116° 01.26'); Panamint Valley, Kern Co. (N 35° 58.36', W 117° 54.07'); Rasor Road, San Bernardino Co. (N 35° 8.222, W 116° 12.65'); Sheephole Mountains, San Bernardino Co. (N 34° 14.01',W 115° 43.14'); Sidewinder Mountains, San Bernardino Co. (N 34° 34.91', W 116° 58.33'); Silurian Valley, San Bernardino Co. (N 35° 48.58', W 116° 19.24'); Summit Range, San Bernardino Co. (N 35° 28.00', W 117° 35.09')

In the lab individual eggs were placed in vials and kept until the wasps emerged. First, the broods containing at least one female were identified to species, using the body color of females. *T. kaykai* females are yellow, *T. deion* females brown and *T. pratti* females black. Females from all-female broods were tested for PI *Wolbachia* infection. When they produced daughters as a virgin they were classified as PI *Wolbachia*-infected (Table 6.1). Those broods containing mainly males were tested for the presence of *PSR*, by crossing individual males with PI *Wolbachia*-infected females. If the males did not carry *PSR*, the offspring was all-female. If *PSR* was present, the offspring consists of females and males (Table 6.1). The males from all-male broods could not be identified to species without killing them. Therefore, half the males from an all-male brood were crossed individually with infected *T. kaykai* females and the other half with infected *T. deion* females. The males used in these tests were stored separately in 100% ethanol to preserve their DNA. Subsequently, they were used for

species identification based on the DNA sequence of their internal transcribed spacer 2 region (Stouthamer *et al*. 1999).

6.2.2 *Models describing the coexistence between PI* Wolbachia-*infection and* PSR

Models describing the frequencies of *PSR* in populations consisting either of only uninfected individuals, or of mixed populations of infected and uninfected individuals have been described in Stouthamer *et al.* (2001). While the figure showing the relationship between sib mating frequencies, egg-fertilization rate and the two sex ratio distorters in that paper is correct, the published formula describing the relationship is not. The correct formulas are:

$$p=r/(r+s-sr)$$

$$I= 1-[(1-p)(1-p) + (1-p)(1-s)p]/\{(1-p)(r) + (p-1+r)(1-r)(1-s) + (1-p)[(1-r)(1-s)p +(1-p)]\}$$

Where *p* equals the frequency of *PSR* among males, *I* equals the *Wolbachia* infection frequency among females, *s* equals the sib mating frequency among uninfected females (i.e. the fraction of uninfected females that mates with their brother) and *(1-r)* equals the fertilization frequency of eggs. We assume that all infected females mate and fertilize their eggs at the same frequency as uninfected females. In addition, we also assume that if an infected female mates with a *PSR* male, her daughters will always mate with one of their *PSR* brothers. These infected females will generally emerge in broods consisting of a single female with several *PSR* brothers (Table 6.1). For *T. kaykai* the fertilization frequency *(1-r)* is 0.75 and the sib mating frequencies have been estimated to be between 0.55 and 0.65 (Stouthamer *et al.* 2001), using the model above, from now on called model I, this would predict the infection frequency to be 6-13% and the *PSR* frequency to be 32-38% (Figure 6.2). While the predicted infection frequency from the model for *T. kaykai* was indeed close to the field infection rate the predicted *PSR* frequency was much higher than that actually found in the field. We already knew that infection causes the females to produce fewer offspring over their lifetime when they have access to unlimited numbers of host eggs (Stouthamer & Luck 1993). This is not too important for the field because we expect them to encounter only a limited number of hosts in the field, however recently we found that unfertilized eggs laid by infected females have a substantially higher mortality rate than either fertilized infected

eggs, or uninfected eggs (Tagami *et al.* 2001). Consequently, we need to incorporate a higher egg mortality for the infected females in the model. In addition, we know that a fraction of the infected females in the field does not mate, while also a fraction of the infected females does not mate. Based on a sample from L & B Canyon, Kern Co. California these values are 2.7% of the uninfected females remain virgin (Chapter 7). The sib mating frequency was estimated to be about 70% in that population (Chapter 7). If we incorporate the non-mating of some uninfected females into the model and assume that the 0.027 fraction remaining unmated is typical for wasps in that population then it means that the uninfected females that do not sib mate (30%) account for the 0.027 frequency in the overall uninfected population, this results in lack of mating in the non-sib mating females of 0.027*10/3= 0.09. Infected females will also generally emerge in patches with only infected females and will have a similar rate of remaining a virgin. We can incorporate these variables in model I to calculate the *PSR* and infection frequencies in a mixed population where a fraction *f* of the females that do not sib-mate remain virgins. In addition the infected females produce only a fraction ω of the offspring an uninfected female produces. We now developed a new model, model II, based on recurrent equations that calculates the different types of matings and females and males in the field. We define UU_t as the fraction of females that is uninfected and has mated with an uninfected male, UP_t is the fraction of females that is uninfected and has mated with a *PSR* male, U_t is the fraction of females that is uninfected and remains a virgin, IU_t is the fraction of females that is infected and has mated with a normal male, IP_t is the fraction of females that is infected and has mated with a *PSR* male and I_t is the fraction of females that is infected and has remained a virgin. There are two types of males, normal males ($1-p_t$) and *PSR* males with a frequency of p_t.

$w_f UU_{t+1} = UU_t(1-r)s + UU_t(1-r)(1-s)(1-p_t)(1-f)$

$w_f UP_{t+1} = UU_t(1-r)(1-s)(1-f)p_t$

$w_f U_{t+1} = UU_t(1-r)(1-s)f$

$w_f IU_{t+1} = IU_t(1-p_t)(1-f)\,\omega + I_t(1-p_t)(1-f)\,\omega$

$w_f IP_{t+1} = IU_t p_t(1-f)\,\omega + I_t p_t(1-f)\,\omega + IP_t(r)\,\omega$

$w_f I_{t+1} = I_t f\omega + IU_t f\omega$

$w_m N_{mal\ t+1} = UU_t(1-f)r + UP_t r + U_t$

$w_m P_{mal\ t+1}$ = $UP_t(1\text{-}r) + IP_t(1\text{-}r)\ \omega$

w_f = total females in generation (t+1) = $UU_t(1\text{-}r) + IU_t\omega + I_t\omega + IP_tr\omega$

w_m = total number of males in generation (t+1) = $UU_t(1\text{-}f)r + UP_t + U_t + IP_t(1\text{-}r)\ \omega$

This model is used to determine the possible *PSR* and infection frequencies when both of them occur together in the population. In addition, we can modify this model to predict if *PSR* can maintain itself in a population if the infection frequency in the population is maintained at a low level by other factors. This is done by setting the frequency of *IU* each generation at a fixed level.

6.3 RESULTS

6.3.1 *PI* Wolbachia-*infection and* PSR *rates in* Trichogramma kaykai *and* T. deion

Trichogramma kaykai was more abundant on *A. m. deserti* than the two other species, *T. deion* and *T. pratti*. In a number of cases we found wasps of two different species emerging from a single host egg (Table 6.2). Both in *T. kaykai* and *T. deion* PI *Wolbachia*-infected and uninfected forms coexist. In *T. pratti* we have yet to find evidence for the presence of any kind of sex ratio distorter. Average infection rate in *T. kaykai* is 6.64% and in *T. deion* 0.88%.

Table 6.2 *Trichogramma kaykai, T. deion* and *T. pratti* on their lepidopteran host *Apodemia mormo deserti* in the Mojave Desert (1998-2001).

Species[1]	**N° of broods**	**Broods shared with *T. kaykai***	**Broods shared with *T. deion***	**Broods shared with *T. pratti***	***PSR*** [2] **%**	**Inf.**[3] **%**
T. kaykai	2750	-	10	2	2-21%	3-33%
T. deion	229	10	-	0	-	3-6%
T. pratti	99	2	0	-	-	-

[1] Species have been identified morphologically by John Pinto and Gary Platner, based on female body color or with a molecular method based on the ITS2-gene (Stouthamer *et al.* 1999). [2] *PSR* % was calculated as the % of F1 males expressing the *PSR* phenotype. [3] Infection % was calculated as the % of the F1 females expressing the PI *Wolbachia* phenotype.

At 10 collection sites *PSR* was detected in *Trichogramma* wasps during the period 1998-2001. Sixty-four *PSR* broods were found (Table 6.3), 48 of these were all-male broods, 14 were male-biased and 2 consisted of an equal number of males and females.

Table 6.3 The incidence of sex ratio distorters in populations of *Trichogramma deion* and *T. kaykai* in the Mojave Desert over the years 1998-2001.

	T. deion				***T. kaykai***				
Year/Location	**N° broods**	**N° infected broods**	**Inf[1] %**	***PSR*[2] %**	**N° broods**	**N° *PSR* broods**	**N° infected broods**	**Inf %**	***PSR* %**
1998									
Panamint Valley	7	0	0	0	115	3	10	8.7	14.4
Barstow Dagget	15	0	0	0	237	4	15	6.3	9.0
Ibex Hills	17	1	5.9	0	116	7	13	11.2	9.0
L& B Canyon	39	1	2.6	0	94	1	4	4.3	5.0
Rasor Road	5	0	0	0	27	1	2	7.4	6.0
Sheephole Mnts	47	0	0	0	137	1	5	3.7	2.4
Silurian Valley	11	0	0	0	132	2	22	16.7	4.6
Dillon Road	13	0	0	0	6	0	0	0	0
Amboy	1	0	0	0	5	0	0	0	0
Morongo Canyon	0	0	0	0	1	0	0	0	0
Summit Range	0	0	0	0	5	0	0	0	0
Ocotillo	11	0	0	0	11	0	0	0	0
Subtotal	166	2			886	18	71		
1999									
Sidewinder Mnts	0	0	0	0	28	0	2	7.1	0
Bell Mountain Rd	0	0	0	0	28	1	1	3.6	1.0
Subtotal	0	0			56	1	3		
2000									
Last Chance Cnyn	1	0	0	0	32	3	2	6.3	21.1
Essex	22	0	0	0	27	1	0	0	12.9
Panamint Valley 1	4	0	0	0	74	4	7	9.5	16.7
Bell Mountain Rd	0	0	0	0	88	3	7	8.0	9.0
Granite Mnts	2	0	0	0	2	0	0	0	0
Silurian Valley	0	0	0	0	2	0	0	0	0
Sidewinder Mnts	4	0	0	0	323	13	23	5.7	11.0
Subtotal	33	0			548	20	39		
2001									
Sidewinder Mnts	11	0	0	0	192	4	20	10.4	7.9
Panamint Valley	3	0	0	0	156	0	5	3.2	0
L&B Canyon	7	0	0	0	669	13	35	5.2	6.7
Bell Mountain Rd	4	0	0	0	220	3	12	5.5	5.0
Ibex Hills	0	0	0	0	17	0	2	11.8	0
Rasor Road	0	0	0	0	3	0	1	33.3	0
Danby Gaspipe	1	0	0	0	0	0	0	0	0
Silurian Valley	0	0	0	0	2	0	0	0	0
Sheephole Mnts	4	0	0	0	1	0	0	0	0
Subtotal	30	0			1260	20	75		
Avg. 1998-2001			0.88	0				6.64	7.53
Total 1998-2001	229	2			2750	64	188		

[1] Inf %: percentage of broods with F1 females expressing the PI *Wolbachia* phenotype; [2] *PSR* %: percentage of F1 males emerging from *A. m. deserti* eggs that expressed the *PSR* phenotype.

All of the *PSR* males from the equal and male-biased broods emerged together with a *T. kaykai* female. After the presence of the *PSR* phenotype had been established, one male of a *PSR* brood was identified using the PCR based method. All the *PSR* males tested were *T. kaykai. PSR* was not found in field-collected *T. deion*. In the laboratory tests transmission of *PSR* to *T. kaykai* females occurred 89 times, the transmission rate was 81.6 % (The N° of crosses which resulted in males in the offspring divided by the total N° of crosses that resulted in any kind of offspring). A total of 49 crosses between *T. deion* females and *T. kaykai* males from *PSR* broods produced offspring. The rate of interspecific transmission was 71.4%. The interspecific transmission rate was not significantly different from the intraspecific transmission rate ($\chi^2_{0.05,1} = 2.09$, $p = 0.148$; $n = 158$). The transmission rate of *PSR* to the first generation must be practically perfect because in all-male broods we expect approximately 75% of the males to be the result of fertilized eggs, and consequently only 75% of males from such broods are carriers of the *PSR* chromosome.

In those crosses where horizontal transmission of *PSR* occurred, the *PSR-deion* lines were maintained for several generations to ensure the stability of the trait. In all cases the sex ratio produced by these lines remained male-biased.

6.3.2 *Models describing the coexistence between PI* Wolbachia-*infection and* PSR

The relationship between *PSR* frequency and *Wolbachia* infection frequency has been modeled in Stouthamer *et al*. (2001). The assumptions underlying this model I are: 1. there is no difference in offspring production between infected and uninfected females, 2. *PSR* males are equal in their ability to locate females and mate with them as normal males, 3. all infected and uninfected females mate, 4. females fertilize 75% of their eggs. The sib mating frequency of the wasps in the field has been estimated to be between 55 and 65% (Stouthamer & Kazmer 1994), however more recent estimates place the frequency at around 70% (Chapter 7). Assuming these values are correct then model I estimates the infection frequency at 12.5 % and the *PSR* frequency at 32%, which are much higher than those found in the field where both infection and *PSR* are at about 7%. Model I was adjusted to incorporate the fact that some of the infected and uninfected females remain virgins, as well as the fact that infected females produce fewer offspring than uninfected females. This model II can be used to predict the equilibrium infected and *PSR* frequencies using the field-derived values for fertilization

rate *(1-r)* = 0.75, sib mating frequency *s*= 0.70, frequency of virgins among uninfected females that do not sib-mate to be *f*= 0.09 (see above). The frequency of virgins among infected females is the same as that for infected females that do not sib-mate. We plot the relationships between sib mating and the sex ratio distorter frequencies for the situation that infected females produce either 100% (ω=1), 90% (ω=0.9) or 80% (ω=0.8) of the number of offspring of uninfected females. The predicted equilibrium values for various sib mating frequencies are given in figure 6.2.

Compared to model I (Figure 6.2 a), the inclusion of a fraction of 0.09 of the females that does not sib mate and remains unmated causes a dramatic shift in the parameter space where the *PSR* and infection both go to fixation.

In addition, in the region of interest of > 0.5 sib mating frequency both the *PSR* and infection frequency become higher. The inclusion of the lower offspring production of infected females has a strong influence on the infection and *PSR* frequencies.

When the relative offspring production of the infected females is reduced from ω=1 to ω=0.9, the infection frequency increases while the *PSR* frequency decreases in the sib mating frequency range > 0.5. The sex ratio distorter frequencies decline further when the ω is reduced to 0.8. Under these circumstances the infection disappears altogether in the intermediate sib mating frequencies. In this region the *PSR* attains values characteristic for uninfected populations. In the area of interest (sib mating > 0.5) shows that the predicted infection frequencies and *PSR* are lower than in model I and the other variants of model II. However, if the sib mating frequency in the field is estimated to be about 70% then the observed infection and *PSR* frequencies are still lower than predicted for *T. kaykai*. All these models show that in order to maintain a lower level of infection in the population a lower level of sib mating is required. Low levels of sib mating are accompanied by higher levels of *PSR* among males.

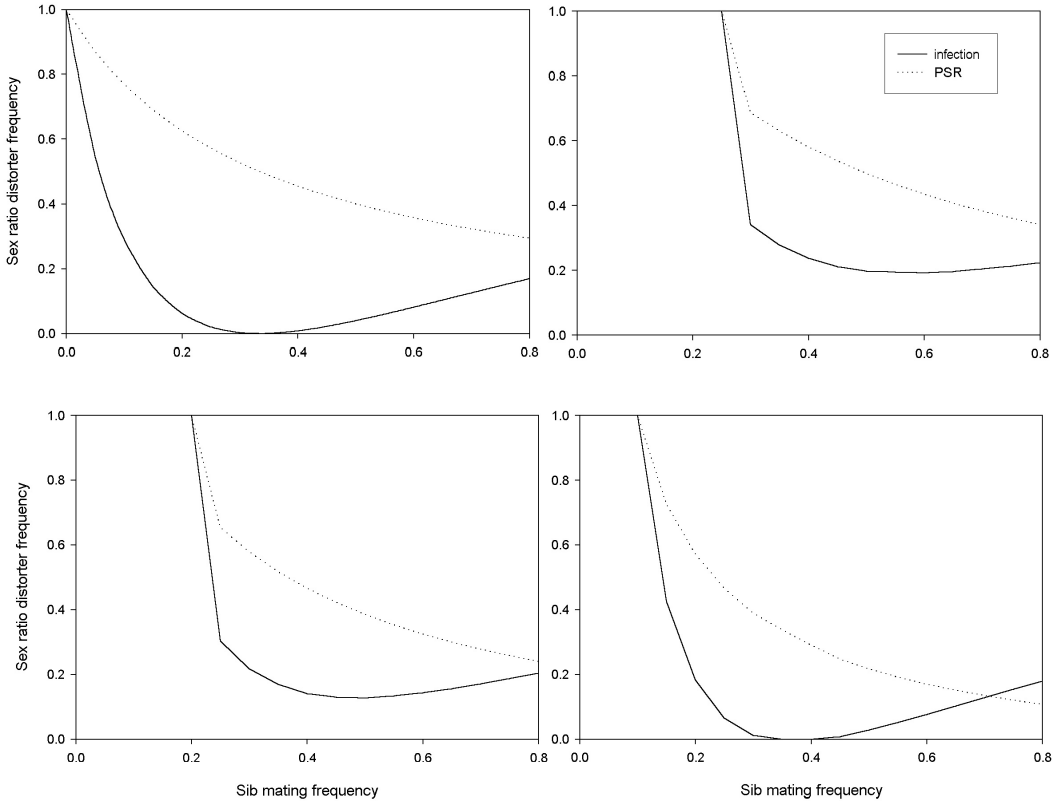

Figure 6.2 Relationship between sib mating frequency in uninfected females (fraction of uninfected females that has mated with their brother) and the frequency of the sex ratio distorters *PSR* among males and PI *Wolbachia* among females using different models. A. Model 1 in which there is the assumption of equal offspring production by PI *Wolbachia*-infected females and uninfected females, all females are assumed to mate, B. Model 2 in which we still assume equal offspring production by infected and uninfected females but now 9% of the broods are produced by virgin females. C. As in B but now we also assume that infected females produce only 90% of the offspring that an uninfected female produces. D. As in B but now we also assume that infected females produce only 80% of the offspring that an uninfected female produces.

When we plot an uninfected population in which *PSR* occurs as a function of the sib mating frequency, it shows that the *PSR* will not be able to maintain itself in populations with a high sib mating frequency. Figure 6.3 shows the relationship between *PSR* and sib mating under three different assumptions: 1) *PSR* when all females mate, 2) when a fraction of 0.09 of the females remains unmated and 3) when a fraction of 0.09 of the females remains unmated and we fix the infection rate among females at about 1%, we did this by setting the frequency of IU_t each generation at 0.01.

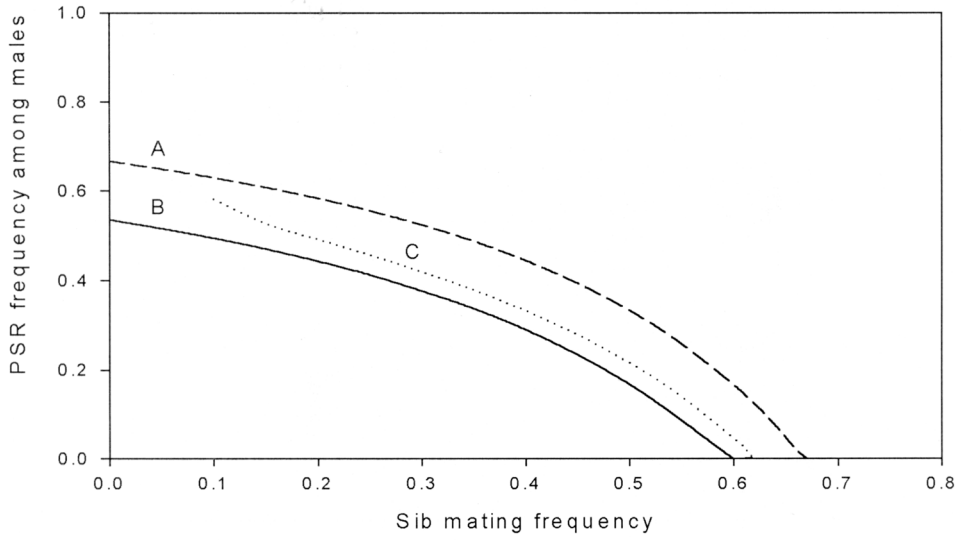

Figure 6.3 Relationship between the sib mating frequency of uninfected females and the frequency of *PSR* among males in the population. Here we assume three different populations **A**. all females are assumed to mate, **B**. 9% of the females remain virgin and **C**. the same as B but now we assume that about 1% of all the females is infected with PI *Wolbachia*, this frequency is fixed and not allowed to vary as a function of *PSR* frequency.

The assumption is in the latter case that some other factor keeps the infection at equilibrium independent of *PSR*. These calculations show that if the sib mating frequency of *T. deion* females in the field is higher than 0.6 than it is unlikely that *PSR* would persist in the population if the infection frequency is maintained at the level of 1% by some other factor

6.4 DISCUSSION

In *T. kaykai*, we can explain the persistence of infected and uninfected individuals by the presence of the *PSR* factor as has been described before by Stouthamer *et al.* (2001) but our study indicates that the low infection levels in *T. deion* must be explained by something else.

Laboratory experiments showed that the field collected *T. kaykai-PSR* males were capable of efficiently transmitting *PSR* to *T. deion*. In these experiments the interspecific transmission of *PSR* from *T. kaykai* to *T. deion* (71.4%) was comparable to the intra specific transmission from *T. kaykai* to *T. kaykai* (81.6%). In the lab experiments the *T. kaykai* males were confined with single *T. deion* virgin females.

This confinement may have led to this high frequency of interspecific mating. *T. deion* and *T. kaykai* are phylogenetically closely related (Pinto *et al.* 1997), but we do not know if they are attracted to each others pheromones in the field. Even without interspecific response to pheromones the two species will sometimes be in close contact when they emerge from superparasitized eggs. Such superparasitized eggs have been observed in field collections (Table 6.2). Therefore transmission from *T. kaykai* to *T. deion* should happen occasionally in the field.

However, all *PSR* broods collected in the field from 1998 to 2001 belonged to *T. kaykai*. Does this exclude *T. deion* as a possible carrier of *PSR*? If we assume that the *PSR* frequency in *T. deion* equals that in *T. kaykai* we can calculate the chance of finding zero *PSR*-deion broods among the 229 broods collected, given that the frequency of *PSR* is 64/2750 (= frequency in *T. kaykai*). Using the binomial distribution this chance is P= 0.004. From this we could conclude that *PSR* does not cause the low *Wolbachia* infection frequency in *T. deion*. But is the *PSR* frequency found in *T. kaykai* a reasonable estimate for the *PSR* frequency that could maintain a low *Wolbachia* infection frequency in *T. deion*? Using the models (Figure 6.2) it is clear that in all cases low infection frequencies are caused by high *PSR* frequencies. Consequently, the expectation would be that the *PSR* frequency that would be able to keep the *Wolbachia* frequency at the low level of 0.88% in *T. deion* should be higher than the *PSR* frequency that keeps the infection frequency at 6.64% as we found in *T. kaykai*. Therefore we would have expected even a better chance of encountering at least one *T. deion* brood among the field collected *PSR* broods. The fact that we do not find any *PSR* males in *T. deion* broods, makes it unlikely that *PSR* causes the low infection frequency in *T. deion*.

In addition, the models show that if indeed there is some factor keeping the infection frequency at the low level of 1%, *PSR* can only maintain itself in the population when the sib mating frequencies are substantially lower for *T. deion* than were measured for *T. kaykai* (Figure 6.3). We have no reason to assume that the sib mating frequencies in *T. deion* are lower than in *T. kaykai*. In our 229 collected *T. deion* broods from *A. m. deserti*, 87% of the females emerged together with a male in the brood, suggesting that high sib mating frequencies are probable in *T. deion*. Moreover, a sample of 312 *T. deion* broods collected throughout the USA on different hosts by Pinto (1999) showed that 83% percent of the females emerged with a male in the brood.

We expected *T. deion* to be a carrier of *PSR* for several reasons: first of all the infection frequency with *Wolbachia* in this species is also maintained at the relatively low frequencies of <20%, thus a suppressing factor of *Wolbachia* is expected (Stouthamer 1997). In addition, our laboratory experiments showed that *T. kaykai PSR* males are capable of transmitting *PSR* to *T. deion*. When some other factor keeps the *Wolbachia* infection at the low level we have found in the field, even the occasional transmission of the *PSR* from *T. kaykai* to *T. deion* does not lead to a maintenance of *PSR* in the population (assuming the sib mating frequency in *T. deion* is comparable to that of *T. kaykai*).

Consequently, some other factor must be causing the low infection frequency among *T. deion* females. We can hypothesize a) the presence of a nuclear suppressor of *Wolbachia* or its effect, b) interspecific horizontal transmission of *Wolbachia* from *T. kaykai* to *T. deion* (Chapter 4) and a low vertical transmission efficiency in natural *T. deion* populations, and c) natural curing of the infection through high temperatures or naturally occurring antibiotics. The nature of the suppressing factor in *T. deion* remains to be studied.

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Dit moet titelpagina **Chapter 7** worden!!!!!!!!!!!

Sib mating as a barrier against sex ratio distortion in a parasitoid wasp

ABSTRACT Sex ratio distorting elements that enhance their own transmission by manipulating their host's reproduction are widespread in nature. They are expected to have played a major role in the evolution of different sex ratio strategies. Here, we studied the role of mating structure in the evolution of two sex ratio distorting elements in the parasitoid wasp *Trichogramma kaykai*. A small fraction (6-26%) of the females are known to be infected with a parthenogenesis-inducing (PI) *Wolbachia* bacterium, that enables infected females to produce daughters both from fertilized and unfertilized eggs. A paternal sex ratio (*PSR*) chromosome in 2-21% (Chapter 6) of the males converts fertilized eggs into *PSR*-carrying sons, thereby preventing the *Wolbachia* infection from spreading to all the females in the population. Uninfected *T. kaykai* females exhibit female-biased sex ratios associated with brother-sister (sib) mating. Such sib mating acts as a barrier against the destructive effect of mating a *PSR*-carrying male. We estimated high levels of sib mating of 70% and an off-patch mating of 15% using a population genetic model with microsatellites as markers. Thirty-five percent of the patches were estimated to be parasitized by two *T. kaykai* females. Incorporating such levels of sib mating in a previously developed model results in stable low frequencies of infection, i.e., a coexistence between infected and uninfected individuals, and the *PSR* chromosome. However, given a 70% sib mating frequency our model predicts a higher frequency of both PI *Wolbachia* and of the *PSR* chromosome. Potential reasons for this discrepancy are discussed.

7.1 INTRODUCTION

Many organisms are carriers of selfish genetic elements that have been defined as elements that will spread through populations despite the costs they may inflict on their hosts (Werren *et al.* 1988). One area in which many of the selfish genetic elements manipulate their host is in the control over the offspring sex ratio. Examples of elements that manipulate host sex ratio include cytoplasmically inherited microorganisms and B-chromosomes. Such sex ratio distorters are thought to be a major factor in the evolution of different sex ratio strategies (Hurst *et al.* 1997; Werren & Beukeboom 1998).

Current sex ratio theory has largely had its origin in studies involving parasitic Hymenoptera. Offspring gender in Hymenoptera is determined by the ploidy level of the eggs. Unfertilized (haploid) eggs develop into males whereas fertilized (diploid)

eggs develop into females. Mated females can control the access of sperm to the eggs and therefore determine the sex of their offspring. The Local Mate Competition theory predicts that female biased sex ratios are optimal when a single founding female oviposits in a host patch where she can lay multiple eggs (Hamilton 1967). Consequently her offspring will inbreed, when after emergence the males inseminate their sisters. The proportion of females inseminated by a full-sib brother is regarded as the level of sib mating (Antolin 1999). The mating structure of a host population is particularly important for understanding the dynamics and evolution of sex ratio distorters (Stouthamer *et al.* 2001). Here, we study the role of mating structure in the coexistence of two sex ratio distorters in a parasitoid wasp population.

The gregarious wasp *Trichogramma kaykai* inhabits the Mojave Desert in Southern California where it parasitizes the eggs of the metal mark butterfly, *Apodemia mormo deserti* (C. and R. Felder) (Lepidoptera: Riodinidae). These eggs are laid in the nodes or flowers of the desert trumpet, *Eriogonum inflatum*, Torr. and Free. (Polygonaceae). The perennial desert trumpet produces a basal rosette from which a number of flowering stalks arise. It is on these stalks that the butterfly lays its eggs, mostly solitary but sometimes also in small clutches of 2-10 eggs. A *T. kaykai* female usually lays a clutch of 4-5 offspring in an *A. m. deserti* egg. Most wasp broods consist of a single male and a few females consistent with the Local Mate Competition theory (Hamilton 1967) but some butterfly eggs contain broods that are all-female, male-biased or all-male (Stouthamer *et al.* 2001).

T. kaykai is a host for two sex ratio distorters. In the field, 6-26% of the *T. kaykai* females are infected with a cytoplasmic bacterium, the parthenogenesis-inducing (PI) *Wolbachia*. As most Hymenoptera, *T. kaykai* has a haplodiploid sex determination system. Fertilized eggs develop into females (diploid, 2n=10) whereas unfertilized eggs develop into males (haploid, n=5) (Stouthamer & Kazmer 1994). However when a female is infected with a PI *Wolbachia*, both fertilized and unfertilized eggs develop into infected daughters, resulting in all-female broods in the field. The mode of action of the PI *Wolbachia* centers on an aborted first mitotic division resulting in a fusion of the two identical sets of chromosomes and therefore an unfertilized egg becomes a completely homozygous female (Stouthamer & Kazmer 1994). If an infected female is inseminated, fertilized eggs grow out to be hybrid, infected females that can also produce daughters without mating. PI *Wolbachia* are mainly vertically transmitted but

also horizontally when infected and uninfected larvae share the same butterfly egg (Huigens *et al.* 2000 (=Chapter 3)).

Such PI *Wolbachia* infection is expected to spread to fixation, i.e., all females will be infected, leading to a loss of sexual reproduction, a situation we find in most infected species (Stouthamer 1997). A stable coexistence between infected and uninfected individuals is only possible if other factors are present that suppress the infection or its effect (Stouthamer 1997). In *T. kaykai*, infected and uninfected individuals coexist because of the presence of another sex ratio distorting element in males. About 10% of the males carry a small supernumerary B-chromosome, called Paternal Sex Ratio (*PSR*) (Stouthamer *et al.* 2001). Such a *PSR* chromosome was until then only found in the parasitoid wasp *Nasonia vitripennis* (Werren 1991). When a female is inseminated by a *PSR*-carrying male, the paternal chromosomes in fertilized eggs are functionally destroyed and only the maternal chromosomes and the *PSR* factor itself remain. Such fertilized eggs develop into sons carrying a haploid set of chromosomes from the mother and the *PSR* chromosome from their father. *PSR* therefore causes uninfected and infected females to produce sons from their fertilized eggs. Infected and uninfected females inseminated by a *PSR* male therefore produce respectively male-biased broods and all-male broods (Table 7.1).

To explain the low frequencies of the two sex ratio distorters we need to understand the mating structure of uninfected *T. kaykai* in the field. The frequency of females leaving a patch without mating is an important variable determining the frequency of the *PSR* factor, because *PSR* can only be passed on when *PSR*-males mate. Uninfected females that mate on-patch -of which a large fraction is inseminated by a brother- are protected against the destructive effect of the *PSR* chromosome. Using allozymes as genetic markers, Kazmer (1992) and Kazmer & Luck (1991) studied the mating structure of *T. kaykai* populations. They found that most matings of uninfected females occur within the patch 67-69%, while most of these are also sib matings, leading to an overall sib mating frequency of 56-62%. Assuming that all females in the population mate and 10% of the males carry the *PSR* factor, this would mean that only 3.1-3.3% of the uninfected females mate off-patch with a *PSR* male. In contrast, most infected females emerge together with only their sisters and will mate with males from the population, and 10% of these are carriers of *PSR*. Therefore approximately 10% of the infected females mate with *PSR* males. The consequence is that the higher daughter production of the infected

population is suppressed more than that of the uninfected population, allowing for a stable coexistence of both forms within the population (Stouthamer *et al.* 2001).

In this study we focus on the mating structure of *T. kaykai* and its importance for the evolution of the two sex ratio distorters in natural populations. More specifically, we estimate the level of sib mating using a population genetic model in which we use microsatellites as genetic markers. Subsequently we enter the calculated sib mating frequency into models describing the equilibrium infection frequency with PI *Wolbachia* among females and *PSR* among males to determine if our field observations match the model predictions. The model always predicts higher frequencies of the sex ratio distorters than measured in the field, reasons for this discrepancy are discussed.

7.2 MATERIAL AND METHODS

7.2.1 *Mating structure*

In the field, the following *Trichogramma kaykai* broods (F1) are expected to emerge from *Apodemia mormo deserti* eggs: i) sexual broods laid by a mated uninfected female, ii) all-male broods laid by a virgin uninfected female or by an uninfected female mated with a *PSR* male, iii) all-female broods laid by a mated uninfected female that laid only fertilized eggs or an infected female (mated or virgin), and iv) male-biased broods laid by an infected female mated with a *PSR* male (Table 7.1).

Table 7.1 Possible mating combinations in *Trichogramma kaykai* and the resulting brood types. *T. kaykai* females are known to fertilize around 75% of their eggs

Parent(s)	Fertilized egg	Unfertilized egg	Brood type	Sex ratio (% females)
U♀ x U♂	U♀	U♂	Sexual	75
U♀ x P♂	P♂	U♂	All-male	0
U♀	-	U♂	All-male	0
I♀ x U♂	I♀	I♀	All-female	100
I♀ x P♂	P♂	I♀	Male-biased	25
I♀	-	I♀	All-female	100

U = uninfected; I = PI *Wolbachia*-infected. P = carrying the *PSR* chromosome.

Therefore the mating structure of uninfected and infected *T. kaykai* females is as follows: Uninfected females emerge with a few sisters and 1 or 2 brothers. When they mate on-patch (which means in this case around the single host egg from which they emerge), the largest fraction will mate with a brother (sib mating) and some with a non-sib. A small fraction of the females that do not mate on-patch will remain virgin. The

ones that mate off-patch will mate with either a normal male or a *PSR* male. Infected females emerge with only sisters or with only *PSR* brothers. The ones that emerge with only sisters will not mate on-patch. The females that mate off-patch will mate with either a normal male or a *PSR* male, the remainder will stay virgin. We assume that infected females emerging with only *PSR* brothers will always mate on-patch with a *PSR* brother (Figure 7.1).

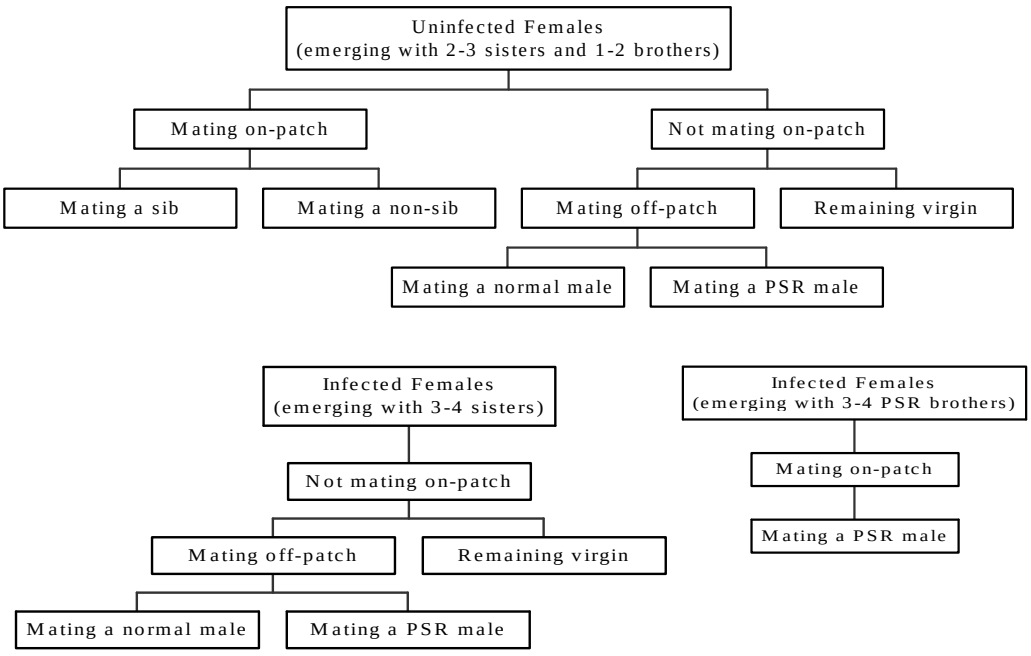

Figure 7.1 Mating structure of uninfected and infected *Trichogramma kaykai* females emerging from *Apodemia mormo deserti* eggs in the Mojave Desert. Uninfected females emerge with a few sisters and 1 or 2 brothers. Infected females emerge with either only sisters or only *PSR* brothers.

7.2.2 *Sampling*

Adult *Apodemia mormo deserti* appear early spring depending on the rainfall patterns the previous fall, winter and spring, and on the prevailing temperatures during late winter and early spring (Pratt Pers. Comm.). The metal mark overwinters as a diapausing larva and completes its development during the rainy season in late winter and/or early spring. Female butterflies begin flying in early spring, mate, and deposit their eggs on the stalks of *Eriogonum inflatum* until the beginning of July. We collected eggs of *A. m. deserti* on *E. inflatum* from April to June 2001 at one sampling site in the

Mojave Desert, L &B Canyon, El Paso Mountains, Kern County (N 35° 22.63', W 117° 54.07'), and determined the size of the *A.m. deserti* egg clutch.

We took the butterfly eggs to the lab. After emergence we counted and sexed all broods. Broods consisting of a male and several females were classified as sexual broods and stored in 95% ethanol for subsequent microsatellite analysis. The F1 females from all-female broods, broods with an equal number of males and females and male-biased broods were individually isolated and allowed to produce offspring to test for *Wolbachia* infection. When these females produced only daughters they were classified as infected. We determined the infection frequency as the number of infected F1 females divided by the total number of F1 females. The F1 males from all-male broods, broods with an equal number of males and females and male-biased broods were isolated and given the opportunity to mate with infected females to test for the presence of *PSR*. When the F2 offspring consisted of a high proportion of sons, the F1 males were scored as *PSR*-carrying. We determined the *PS*R frequency as the number of F1 males carrying *PSR* divided by the total number of F1 males. In these tests for infection and presence of *PSR* we used eggs from the moth *Ephestia kuehniella* as hosts. The experiments were conducted at 26.7 °C, 50% RH and 18L: 6D photophase.

7.2.3 *Sib mating in uninfected* T. kaykai

We estimated the level of sib mating with a population genetic model using microsatellites as molecular markers. Five microsatellite loci were sampled to genotype 52 sexual broods, containing 181 uninfected females (diploid) and 52 normal males (haploid). DNA was extracted from all individual wasps belonging to these broods. The selection was such that i) we only took broods from solitary *A. m. deserti* eggs, and ii) we took mostly broods consisting of three or four females and one male. Smaller broods might not yield sufficient information and these broods correspond well to the average brood size of *T. kaykai* in the field. A previous null allele analysis for these loci revealed the absence of null alleles in *T. kaykai* for all the five loci under study (Schilder 2000).

DNA extraction. DNA was isolated from individual wasps using a Chelex extraction buffer. Individual male wasps were shaken for one hour in 100 µl TE to remove the alcohol. Subsequently the wasp was ground in 50 µl 5% Chelex-100 and 4 µl proteïnase K (20mg/ml). This was incubated overnight at 56 °C, followed by 10 minutes at 95 °C.

PCR reactions. The reaction volume for the PCR was 25 µl: 2.5 µl Template DNA, 0.07 µl Taq (5 units/µl), 0.5 µl Forward and reverse primer (10ng, see Table 6.2), 0.5 µl dNTP's (10mM), 2.5 µl PCR-buffer (10mM) and 18.43 µl H_2O. We used five microsatellite primer combinations of which the forward primer was labeled with a certain fluorescent dye (Table 7.2).

Table 7.2 Primer sequences and PCR conditions of the microsatellite markers used in this study.

Marker	Primer sequence (5'-3')		PCR	
	Forward	Reverse	T_m	C
CT 122	NedGTGACTGCCTTATTCTGCATAC	TCGGGTCGTTGTAGCGGGC	63	45
TTG 49	FamGTAGTCTGGTTTTCGATTCCCA	TCCCCGACCTATCGATTTTCC	63	45
TTG 46	FamGATGTTTACTTCGCAGGCCGC	CTACGGGGCATACGATATGTG	65	30
TTC 53	HexGGGCATGTGCAAAAGACTAG	CGAGAGGGTTTCTTCAATGG	59	30
TAC 47	HexCTACGGCGACAATTGCCAC	CATCTTGGTCGAACCGAGCAG	65	30

PCR: PCR conditions; T_m: Annealing temperature (°C); C: Number of cycli

PCR conditions included 3 minutes denaturation at 94 °C followed by a number of cycles of 45 seconds denaturation at 94 °C, 45 seconds at the annealing temperature and 45 seconds extension at 72 °C (Table 7.2). The last cycle included an extra 3 minutes extension at 72 °C.

Electrophoresis and genotyping. The amplification products of the five loci from each DNA sample were mixed together. The resulting mixture contained respectively 2.0 µl of each amplification product and 15.0 µl of H_2O. For each sample, the products were separated by capillary electrophoresis on an ABI PRISM 310 (PE Applied Biosystems) automated fluorescent sequencer. An internal lane standard Genescan-500 Rox was run along in each lane to determine the size of the amplified microsatellite alleles. The results were analyzed using GeneScan 3.1 Software (PE Applied Biosystems).

Relatedness, Inbreeding and Sib mating. All relatedness and inbreeding calculations were performed using the Relatedness version 4.2 computer program (Goodnight 1996), which implements the formulas described in Queller & Goodnight (1989). Standard errors of the estimates were calculated using a jackknife technique, by jackknifing over groups (patches). We described all samples by determining sample size, proportion of polymorphic loci, number of alleles per polymorphic locus and observed and expected

heterozygosity. Subsequently, we performed a test for linkage disequilibrium between loci with the program GDA d12 (Lewis & Zaykin 2001) using only the female data.

To examine mating structure, we used calculations described by Kazmer & Luck (1991). We first calculated the relatedness of *T. kaykai* individuals in a patch: i) the relatedness, or the correlation of alleles among females (r_f) emerging from a single patch, and ii) the relatedness between males and females (r_{mf}) and between females and males (r_{fm}) within a patch.

Secondly, we calculated the inbreeding coefficient *F* of the population, also known as the correlation of alleles within individuals. From this, we can estimate a) the mean number of females successfully parasitizing a single *A. m. deserti* egg (N_h), b) the fraction of double- (or super-) parasitized patches (*L),* c) the level of off-patch mating (*M*) , and d) sib mating by uninfected females (F_s):

The mean number of successful foundresses can be estimated by:

$$E\ 1.1 \quad N_h = (1 + 3F) / 4\ r_{fm}$$

From this, the fraction of double-parasitized patches can be estimated by:

$$E\ 1.2 \quad L = 2 (1- (1 / N_h))$$

The frequency of off-patch mating can be estimated by:

$$E\ 1.3 \quad M = 1 – (F / r_{fm})$$

And finally, sib mating as the proportion of matings between full sibs can be estimated by:

$$E\ 1.4 \quad F_s = 4F / (1 + 3F)$$

7.2.4 *Model describing the dynamics of PI* Wolbachia *and* PSR *in* T. kaykai

The next step is to understand the natural infection and *PSR* frequencies in *T. kaykai* with the mating structure that we have just estimated. The dynamics of the two sex ratio distorters in *T. kaykai* populations have previously been described by Stouthamer *et al.* (2001). Recently, we have expanded Stouthamer *et al.*'s (2001) model (see Chapter 6). The adjustment of their model resulted in the following equations for the different females and males:

$$w_f UU_{t+1} = UU_t(1-r)s + UU_t(1-r)(1-s)(1-p_t)(1-f)$$

$$w_f UP_{t+1} = UU_t(1-r)(1-s)(1-f)p_t$$

$$w_f U_{t+1} = UU_t(1-r)(1-s)f$$

$w_f IU_{t+1}$ $= IU_t(1-p_t)(1-f)\,\omega + I_t(1-p_t)(1-f)\,\omega$

$w_f IP_{t+1}$ $= IU_t p_t(1-f)\,\omega + I_t p_t(1-f)\,\omega + IP_t(r)\,\omega$

$w_f I_{t+1}$ $= I_t f\omega + IU_t f\omega$

$w_m N_{mal\ t+1}$ $= UU_t(1-f)r + UP_t r + U_t$

$w_m P_{mal\ t+1}$ $= UP_t(1-r) + IP_t(1-r)\,\omega$

w_f = total females in generation (t+1) = $UU_t(1-r) + IU_t\omega + I_t\omega + IP_t r\omega$

w_m = total number of males in generation (t+1) = $UU_t(1-f)r + UP_t + U_t + IP_t(1-r)\,\omega$

in which, UU_t equals the fraction of uninfected females that have mated with an uninfected male, UP_t equals the fraction of uninfected females that have mated with a *PSR* male, U_t equals the fraction of uninfected females that remain virgin, IU_t is the fraction of infected females that have mated with a normal male, IP_t is the fraction of infected females that is have mated with a *PSR* male and I_t is the fraction of infected females that remain virgin. There are two types of males, normal males ($1-p_t$) and *PSR* males with a frequency of p_t.

In these equations, *p* equals the frequency of *PSR* among males, *I* equals the *Wolbachia* infection frequency among females, *(1-r)* equals the fertilization frequency of eggs and *s* equals the sib mating frequency among uninfected females (i.e. the fraction of uninfected females that mates with their brother). In this new model we added two parameters: a) In the Stouthamer *et al.* (2001) model, we assumed that all infected females mate and fertilize their eggs at the same frequency as uninfected females but here we also assume that a fraction of both infected and uninfected females remain virgin. Therefore we included *f* as the fraction of females that leave their natal patch without mating and that do not mate subsequently. b) In addition, we included ω as the infected females produce only a fraction ω of the offspring an uninfected females (Stouthamer & Luck 1993; Tagami *et al.* 2001).

With this new model we calculate through simulations *I* and *p* at equilibrium and the fraction of the four different brood types; uninfected broods ($UU_t + U_t$), infected broods ($IU_t + I_t$), *PSR* broods (UP_t) and infected broods with *PSR* (IP_t).

7.3 RESULTS

7.3.1 *Frequency of brood types and sex ratio distorters*

In total we collected 1662 *Apodemia mormo deserti* eggs. Most *A. m. deserti* eggs were solitary (76.4 %), 14.8% were from a 2-egg clutch, 3.6% from a 3-egg clutch, 1.2 % from a 4-egg clutch, 1.5% from a 5-egg clutch, 1.8% from a 6-egg clutch, and we found a one clutch of 9 eggs (5.4 10^{-03}%). These eggs resulted in 669 *Trichogramma kaykai* broods. We consider one brood as emerging from one *A. m. deserti* egg or emerging from several neighboring eggs, i.e., an egg-clutch, on the same day. Of all these broods 93.3% were uninfected, 4.8% contained infected females, 1.5% contained *PSR* males and 0.4% contained both infected females and *PSR* males (Table 7.3). In the uninfected broods, 2.7% of the females emerge without a male, 85.1% emerges together with one male and the remaining part (12.2%) emerges together with two or more males. The infection frequency amongst F1 females was 7.1% and the *PSR* frequency amongst F1 males was 6.7%.

Table 7.3 Different *Trichogramma kaykai* brood types emerging from an *Apodemia mormo deserti* patch.

Brood type	**N**	**Freq**	**S**	**AF**	**AM**	**E**	**MB**	**Av. ♀♀**	**Av. ♂♂**
Uninfected	624	93.3	538	49	17	11	9	4.63±1.07	1.15±0.73
Infected	32	4.8	-	26	-	3	3	4.19±1.15	0.28±0.77
PSR	10	1.5	-	-	5	2	3	3.70±0.67	3.10±0.74
Infected + *PSR*	3	0.4	-	-	-	1	2	5.00±1.00	1.67±1.15

S= Number of broods containing more females than males. AF= Number of broods containing only females. AM= Number of broods containing only males. E= Number of broods containing an equal number of males and females. MB= Number of broods containing more males than females.

7.3.2 *Estimation of sib mating with a population genetic model using microsatellites*

Four loci were polymorphic in our samples. TTG 46 was fixed for the same allele. The average number of alleles per locus is 10 (per polymorphic locus 12.3) (Table 7.4). Among the polymorphic loci, the TTC53 locus has the lowest expected heterozygosity (0.23) compared to the other three loci (0.82-0.87). Average expected heterozygosity was 0.55, or 0.69 when the monomorphic locus was excluded. In all cases the observed heterozygosity is much lower than the expected. The increased homozygosity is most likely due to a high proportion of sib mating in our population. We did not find any evidence for linkage disequilibrium between loci.

Table 7.4 Polymorphism (number of alleles) and heterozygosity (observed and expected) of the five microsatellite loci in our *Trichogramma kaykai* samples.

Locus	Number of females	Number of alleles	Size range (bp.)	Polymorphic/ monomorphic	H_e	H_o
TTG 49	162	14	178-220	polymorphic	0.82	0.51
CT 122	169	12	74-126	polymorphic	0.84	0.53
TAC 47	163	19	166-231	polymorphic	0.87	0.55
TTG 46	116	1	106	monomorphic	0.00	0.00
TTC 53	136	4	75-84	polymorphic	0.23	0.17
Mean (all)	149.2	10			0.55	0.35
Mean (polymorphic)	157.5	12.3			0.69	0.44

H_e= expected heterozygosity. H_o= observed heterozygosity

The relatedness between the sexes ranged from 0.436 to 0.745. Relatedness between females was 0.745 ± 0.028 (SE), between females and males 0.634 ± 0.056 and between males and females 0.436 ± 0.042. Standard errors were calculated using a jackknife technique across patches. The estimated number of successful foundresses N_h is 1.21, showing that eggs are sometimes parasitized by more than one female. Using the microsatellite data, the fraction of double-parasitized eggs *L* is estimated at 35,2 %.

The inbreeding coefficient *F* was estimated at 0.372 ± 0.039 ranging from 0.323 to 0.385 for the polymorphic loci. Therefore, we estimate an off-patch mating *M* of 14.7%. Substituting F in equation E 1.4 results in an estimate of sib mating F_s of 70.3% ± 3.6 (Table 7.5).

Table 7.5 Estimates of allelic correlations and mating structure indices for the *Trichogramma kaykai* population.

Allelic correlations				Mating structure indices			
F	**r_f**	**r_{fm}**	**r_{mf}**	**N_h**	**L (%)**	**M (%)**	**S (%)**
0.372	0.745	0.436	0.634	1.21	35.2	14.7	70.3
(0.039)	(0.028)	(0.056)	(0.042)	(0.23)	(25.9)	(17.6)	(3.6)

F= Inbreeding coefficient, r_f = correlation of alleles between females, r_{mf} = correlation of alleles between females and males, r_{fm} = correlation of alleles between males and females, N_h = mean number of foundresses per patch, L = fraction of double parasitized patches, M = fraction of off-patch matings and S = fraction of matings between full-sibs. In between brackets are the standard errors.

7.3.3 *Model describing the dynamics of the two sex ratio distorters in* T. kaykai

As described above, a fraction of 0.027 of the uninfected females remains virgin. This means that the uninfected females that do not sib mate (30%) account for the 0.027 frequency in the overall uninfected population, resulting in a lack of mating in the non-sib mating females of f = 0.027*10/3= 0.09. We assume that infected females also remain virgin at this rate of 0.09 because this fraction just represents the chance that a female that does not mate on a patch and will mate elsewhere. According to Tagami *et al.* (2001) there is a substantial reduction in the offspring production of infected females, we take ω = 0.8 *T. kaykai* females fertilize approximately 75% of the eggs, so $1-r$ = 0.75. Finally, we have to integrate the sib mating frequency of 0.70 as we found with the microsatellite markers.

Now the model estimates an infection frequency I of 12.8% and a *PSR* frequency p of 13.4%. This would mean that 0.848 of the broods is uninfected, 0.107 are infected broods, 0.032 are *PSR* broods and 0.021 are infected + *PSR*. These values show that with a sib mating of 0.70 we can explain a coexistence of the two sex ratio distorters in the population. The estimated infection and *PSR* frequencies are, however, higher than the sex ratio distorter frequencies found in the field.

7.4 DISCUSSION

Our approach describes a mating structure in *Trichogramma kaykai* similar to previous studies on mating structure in *Trichogramma*, although our estimate of sib mating is approximately 10-15% higher (Table 7.6). High levels of sib mating measured by Kazmer & Luck (1991) and those estimated in this *T. kaykai* population, are expected for gregarious parasitoids with female-biased sex ratios (Hamilton 1967). Like several other *Trichogramma* species (Antolin 1999; Godfray 1994; Hardy 1994; Godfray & Cook 1997; Fauverge *et al.* 1999), *T. kaykai* does not exhibit 100% inbreeding nor complete outbreeding avoidance. High levels of sib mating are associated with the typical mating behavior in *Trichogramma* where males emerge first and wait for the females to emerge (Pompanon *et al.* 1995). Uninfected *T. kaykai* females also mate on- and off-patch with non-sibs. On-patch mating between non-sibs, 14.9% in our population and 5-13% found by Kazmer & Luck (1991), most likely results from superparasitized *A. m. deserti* eggs. This fraction of double-parasitized host eggs is estimated from 20-35% in the three *T. kaykai* populations. A relatively large fraction of

the uninfected females still mates off-patch: approximately 15% in our population which is lower than the 31-33% off-patch mating in two other *T. kaykai* populations (Kazmer & Luck 1991).

Table 7.6 Experimental studies of mating structure in *Trichogramma* species.

Species	Method	H_e	F	r_f	r_{fm}	S	M	N_h	References
T. pretiosum	Allozyme	0.43	0.32	-	0.39	0.65	0.19	1.24	Kazmer & Luck 1991; Kazmer 1992
T. pretiosum	Allozyme	-	0.25	0.65	-	0.57	-	-	Antolin 1999
T. kaykai	Allozyme	0.38	0.24	-	0.37	0.56	0.31	1.17	Kazmer & Luck
		0.41	0.30			0.63	0.33	1.29	1991; Kazmer 1992
T. kaykai	Microsatellite	0.69	0.37	0.75	0.44	0.70	0.15	1.21	This study

He= Expected heterozygosity. F= Inbreeding coefficient. r_f= correlation of alleles between females, r_{fm}= correlation of alleles between males and females and male. S = fraction of matings between full-sibs. M= fraction of off-patch matings. N_h= mean number of foundresses per patch.

Off-patch mating most likely occurs when males disperse from the natal patch and find virgin females under field conditions. Uninfected females can remain virgin when i) they emerge from an all-female brood (7.9% of the uninfected broods) and ii) they emerge together with males but leave the natal patch without mating. In *T. papilionis,* the fraction of females leaving a patch without mating one of the males on-patch has been observed to be 10-15% (Suzuki & Hierata 1985). For off-patch mating to occur males should detect sex pheromones produced by virgin females in the field. In *T. brassicae* (Pintureau & Toonders 1983; Pompanon *et al.* 1997; Fauvergue *et al.* 1995), *T. bourarache* (Pompanon *et al.* 1997), *T. pretiosum* (Kazmer & Luck 1995), and *T. turkestanica* (Silva & Stouthamer 1997), the possible existence of such sex pheromone production by virgin females has been reported. Off-patch mating has a profound influence on the optimal sex ratio (Nunney & Luck 1988) and is generally not incorporated in models testing sex ratio theory (Werren 1980).

Multiple mating might be common in *T. kaykai*. In *T. pretiosum,* for example, females are known to copulate multiple times on the natal patch (Suzuki & Hiehata 1985) and in *T. turkestanica* second mating was found to be efficient (Pintureau *et al.* 1997). In *T. pretiosum,* multiple mating must take place on-patch, because mated females failed to attract males in the field (Kazmer 1992). We expect this to be the same in *T. kaykai*,

also because off-patch encounters with more than one male within a limited time-span seem very unlikely under the desert field conditions. Therefore, when an uninfected female leaves a patch after mating a sib or non-sib, the chance of mating a *PSR* male off-patch is most likely negligible. On-patch multiple mating should occur in *T. kaykai* because some uninfected females emerge together with two or more males in the field (12.2%). Such broods might have resulted from superparasitized host eggs. In 3 of the 52 genotyped broods we found evidence for multiple mating.

In general, inbreeding depression is not expected to be very high in haplodiploids (Crozier 1985; Werren 1993). Several species with high levels of sib mating however show considerable inbreeding depression amongst which is one *Trichogramma* species, namely *T. pretiosum* (Antolin 1999), but in others no such effect is found (Sorati *et al*. 1996). Little is kwon about the possible inbreeding depression in *T. kaykai*.

Our estimation of sib mating is in the range at which we can explain a coexistence between infected and uninfected individuals (Stouthamer *et al.* 2001). With a complete sib mating *PSR* would not be able to invade a population at all, simply because there are no females to mate with. Stouthamer *et al.* (2001) showed that when sib mating is above 67%, *PSR* could not survive in an uninfected population. When we assume the absence of *PSR*, a PI *Wolbachia* infection would spread to fixation independent of the level of sib mating.

When we take both an infection and the presence of *PSR* into account and incorporate a sib mating of 70% and a fertilization rate of 75% (Stouthamer & Kazmer 1994) in our new model, i.e., a slight modification of Stouthamer *et al*.'s (2001) model, this corresponds to a stable coexistence between infected and uninfected individuals. But the predicted infection and *PSR* frequencies are higher than the ones found in the field; either the model overestimates the actual infection and *PSR* frequencies, which is most likely, or our methodology of testing for infection and *PSR* causes an underestimation. We may underestimate the infection frequency if we classified some of the sexual broods as uninfected, while they are actually infected and contain a male because of the inefficient vertical transmission of *Wolbachia*. In the tests for *PSR*, the males might have failed to successfully mate with infected females or the infected females failed to produce offspring. Due to possible multiple mating (or superparasitism) in the field some of the sexual broods might have actually contained *PSR* males. The estimated fraction of broods that are infected and carry *PSR* deviate most from the fraction found

in the field (model 0.021 vs. field 0.004). This might be due to the fact that some of the *PSR* broods that contained only males were actually laid by infected females. In those broods the females could have either fertilized all of their eggs or the unfertilized infected eggs that would have developed into a daughter had died. This mortality would be consistent with the results of Tagami *et al.* (2001). A molecular marker for the *PSR* factor should help us in the future.

The model does not incorporate superparasitsm and on-patch multiple mating, which both occur in *T. kaykai*, and can both be important parameters for the dynamics of sex ratio distorters in *T. kaykai*. First of all, because horizontal transfer of *Wolbachia* takes place when infected and uninfected larvae share the same host egg (Huigens *et al.* 2000 (=Chapter 3)), superparasitism should result in a slight increase of the infection frequency in the host population (Chapter 5). Both superparasitism and multiple mating increase the *PSR* frequency in a population, which is in contrast with the lower *PSR* frequencies that we find in the field. On-patch multiple mating and on-patch mating between non-sibs, both resulting from superparasitized host eggs, mean that *PSR* males also have the opportunity to mate on-patch with uninfected females. In the field we do find a few broods with *PSR* males and uninfected females that most likely resulted from a superparasitism by an uninfected mated female and another uninfected female that had mated with a *PSR* male in the field (5 out of 669 *T. kaykai* broods).

Summarizing, the mating structure in this parasitoid largely determines the frequencies of the sex ratio distorters in the population. Frequent sib mating on the natal patch acts as a barrier against mating a *PSR*-carrying male for uninfected females. Because infected females do not mate on-patch, the *PSR* factor has a larger destructive effect on the infected population. This study shows how important a thorough analysis of host mating structure is to understand the dynamics of the sex ratio distorters in the host population.

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Dit moet titelpagina **Chapter 8** worden!!!!!!!!!!!

Summarizing discussion

8.1 INTRODUCTION

The cytoplasmic microbe *Wolbachia* has switched the mode of reproduction from sexuality to complete parthenogenesis in several wasp, thrips and mite species to its own advantage (reviewed in Huigens & Stouthamer 2003 (=Chapter 2)). A fixation of the infection in populations is easy to explain when infected females produce more daughters than uninfected females do (Stouthamer 1997). However, in most *Trichogramma* species only a small fraction of the females in the population is infected despite the fact that infected females produce more daughters than their uninfected conspecifics (Stouthamer 1997). In this thesis I have tried to expand our understanding of the evolutionary pathways that PI *Wolbachia* infections may follow in natural *Trichogramma* populations and more specifically I wanted to explain the coexistence of infected and uninfected forms in populations of these wasps. Therefore we have studied *Trichogramma* populations in the Mojave Desert and quantified the interaction between *Wolbachia* and its host in field populations. In this chapter I summarize and synthesize the most important results of the previous chapters to answer the following specific questions: What are the modes of transmission of PI *Wolbachia* (Chapter 3 & 4)? Are there fitness costs associated with the infection in a mixed population (Chapter 5)? What can explain the coexistence of infected and uninfected forms in *T. kaykai* and *T. deion* (Chapter 6)? What is the mating structure in uninfected *Trichogramma kaykai* (Chapter 7)?

8.2 SUMMARIZING THE MOST IMPORTANT RESULTS OF THE PREVIOUS EXPERIMENTAL STUDIES

Chapter 3: Vertical transmission of *Wolbachia* has been viewed as the main mode of transmission but here we report an unexpectedly frequent horizontal transmission of PI *Wolbachia* from infected to uninfected *Trichogramma kaykai* larvae sharing a common food source. In 21 of 56 host eggs shared by infected and uninfected larvae, originally uninfected larvae acquired an infection. The transferred *Wolbachia* are subsequently vertically transmitted to the new host's offspring. Such relatively frequent horizontal

transmission might select for higher virulence compared to a situation of pure vertical transmission of *Wolbachia*.

Chapter 4: In this chapter we show inter- and intraspecific transfer of *Wolbachia* in *Trichogramma*. Interspecific transfer occurred between two closely related species, *T. kaykai* and *T. deion*. In addition, originally uninfected *T. deion* larvae can also acquire PI *Wolbachia* from a conspecific, as we found in *T. kaykai* (Chapter 3). After both intra- and interspecific horizontal transfer, only a fraction of the newly infected females also exhibits parthenogenesis. In general, intraspecific horizontal transfer was more successful than interspecific transfer. *Wolbachia* could undergo vertical transmission in a new species but infection was lost after several generations. Horizontal transfer is certainly not common between or within all species: Within *T. atopovirilia* horizontal transfer was unsuccessful and both infected *T. atopovirilia* and infected *T. atopovirilia* remained infected with only their own *Wolbachia* after they shared the same food source. The results suggest that incompatibilities between *Wolbachia* and the nuclear/cytoplasmic background of the new host limit the spread of newly acquired infections but on an evolutionary time scale interspecific horizontal transfer might be frequent enough to explain the discordance between *Wolbachia*- and host phylogenies.

Chapter 5: Here, we report a reduced competitive ability due to *Wolbachia* infection in *Trichogramma kaykai*. Immature survival of infected individuals in a host parasitized by a single infected female, was lower than those parasitized by a single uninfected individual. When offspring of infected and uninfected females shared the same host, the infected immatures had significantly lower survival rates than their uninfected counterparts. The survival rate of infected immatures was higher when they competed with other infected immatures from a different infected parent than in competition with uninfected immatures. This shows that the host *Trichogramma* can suffer a substantial reduction in fitness when infected with the PI *Wolbachia*. Because of the reduced competitive ability of infected larvae, horizontal transfer that occurs under the same superparasitism circumstances does not greatly increase the infection rate in the population.

Chapter 6: Here, we show that a coexistence between PI *Wolbachia* infected and uninfected forms cannot always be attributed to the presence of a *PSR* factor. In *T. deion* the infection with PI *Wolbachia* is limited to 1% of the females, suggesting the presence of a balancing factor, potentially *PSR,* in this species. We collected large numbers of parasitized *Apodemia* eggs in the field seasons of 1998, 1999, 2000 and 2001 and determined the species identity of males expressing the *PSR* phenotype using a molecular identification key based on the ITS-2 sequences. The test to determine if a male expressed the *PSR* phenotype, consisted of mating the males from a male biased brood with infected females. Because we did not know the identity of the males *a priori* half the males of a potential *psr* brood were mated with *T. deion* females and the other half with *T. kaykai* females. The results showed that all *PSR* broods studied belonged to *T. kaykai.* In the tests for *PSR* 71.4% of the *T. kaykai PSR* males horizontally transmitted the *PSR* phenotype to *T. deion,* comparable to the intraspecific transmission rate of *PSR* to *T. kaykai* females, namely 81.6%. This interspecific movement of *PSR* is expected to happen in the field but we do not find *PSR* in *T. deion*. Modelling shows that low *Wolbachia* infection frequencies can only be attained when the *PSR* rates are very high. Therefore, we conclude that other factors keep the PI *Wolbachia*-infection from spreading to fixation in this species.

Chapter 7: In this study we investigate the role of mating structure in the dynamics of two sex ratio distorting elements, the PI *Wolbachia* and the paternal sex ratio (*PSR*) chromosome, in the parasitoid wasp *Trichogramma kaykai*. The *PSR* factor prevents the *Wolbachia* infection from spreading to all the females in the population. Uninfected *T. kaykai* females exhibit female-biased sex ratios associated with brother-sister (sib) mating. Such sib-mating acts as a barrier against the destructive effect of mating with a *PSR*-carrying male. We estimated high levels of sib-mating of 70% and an off-patch mating of 15% using a model based on a population genetic model with microsatellites as genetic markers. Thirty-five percent of the patches were estimated to be parasitized by two *T. kaykai* females. Incorporating such levels of sib mating in a previously developed model resulted in stable low frequencies of infection, i.e. a coexistence between infected and uninfected individuals, and of the *PSR* chromosome. Our results show how mating structure allows the two sex ratio distorters to coexist in the population. However when we enter a sib mating level of 70% in our population models

the models would still predict higher incidences of *PSR* and the PI *Wolbachia*-infection. We expect that some undercount of the infection and the *PSR* has taken place in our field samples.

8.3 GENOMIC CONFLICT

The nuclear cytoplasmic conflict, as described in Cosmides & Tooby (1981), between PI *Wolbachia* and the nuclear genes of the host is not equally strong in all host species. Only in mixed populations, i.e., infected and uninfected individuals coexist, this conflict is strong. In *Trichogramma* sp., infected wasps also suffer a fitness cost (Stouthamer & Luck 1993; van Meer 1999, Silva 1999; Huigens & Stouthamer 2003 (= Chapter 2); Chapter 5) associated with the nuclear cytoplasmic conflict and possible horizontal transfer of *Wolbachia*. In such populations many infected *Trichogramma* females mate with males in the population, as in *T. kaykai* (Kazmer 1992; Stouthamer & Kazmer 1994), and 75% of their offspring develops from fertilized eggs. Here, being infected does not involve a degeneration of sexual functions. In fixed populations, parthenogenetic reproduction may be unfavourable over the long run when negative mutations accumulate according to Muller's ratchet and populations cannot keep up with changes in the environment. However, in fixed populations infection does not result in a cost (van Meer 1999; Silva 1999; Chapter 2). When the population is fixed for the infection and sexual functions are lost, nuclear and *Wolbachia* genes have the same evolutionary interest. The nuclear cytoplasmic conflict is minimal in this case. A long co-evolution between nuclear and *Wolbachia* genes may therefore have led to neutrality, as in *Trichogramma cordubensis* and *T. oleae* (van Meer 1999; Silva 1999), or in some cases even benevolence as is known from CI-*Wolbachia* in *Tribolium confusum* where infected males produce more sperm cysts than uninfected conspecifics (Wade & Chang 1995). In case of the parasitoid wasp *Asobara tabida,* the long evolution of the association has even led to a situation where the host has become dependent on *Wolbachia*; females cured of their infection do not produce mature oocytes anymore (Dedeine *et al.* 2001). In addition, Starr & Cline (2002) recently showed that *Wolbachia* infection can counteract deleterious mutations in *Drosophila melanogaster*; infection restores oogenesis defects in a sterile *Drosophila* mutant that has lesions in a master regulatory gene of sex determination. *Wolbachia* clearly show different forms of a functional relationship between a symbiont and its host, namely

from a) reproductive parasitism through the induction ofpParthenogenesis, CI, MK and feminization, b) fitness costs of infection as I have shown in this thesis (Chapter 5), to c) a form of mutualism in the above mentioned cases.

Once an infection starts spreading in a host population, it seems to be difficult for a host organism to evolve a nuclear trait that suppresses *Wolbachia* or its effect. Almost all infected host species are completely infected and parthenogenetic; these hosts can be considered to be hijacked by the PI *Wolbachia*.

We should however not neglect the possibility that many now uninfected species may have been (partly) infected before and were actually able to overcome their infections. In this thesis I showed that parthenogenesis can be contagious but infections are not so easily acquired when received from another species. *Wolbachia* seem to have problems to overcome certain barriers in new hosts, maybe through a long co-evolution with the original host (Chapter 3 & 4). It may be an inability of *Wolbachia* to adapt to a new host but on the other hand also a host trait that actually causes *Wolbachia*'s inability to invade.

In addition, the stable coexistence of infected and uninfected individuals in *Trichogramma* wasps shows that the spread of *Wolbachia* can be limited. The low infection level in *T. kaykai* is a peculiar case. In this species infected females have a reduced fitness compared to uninfected females maybe due to the nuclear cytoplasmic conflict in which nuclear genes try to suppress the infection (Chapter 5). The costs alone are not high enough to prevent the infection from spreading in the population. In *T. kaykai*, the coexistence is not a result of a contra-adaptation by the nuclear genes but due to the presence of another selfish genetic element, the *PSR* factor (Stouthamer *et al.* 2001), that may have been introduced in the population through an interspecies crossing. A nuclear suppressor of *Wolbachia* or its effect has not evolved in *T. kaykai*, nor a nuclear trait that reduces its vertical transmission (Stouthamer *et al.* 2001). Of course the presence of the *PSR* factor as the limiting factor of infection indirectly also limits the spread of nuclear suppressors (Stouthamer unpublished model), i.e., because *PSR* prevents the infection from spreading in the population there is no strong selective advantage for a nuclear gene that suppresses the *Wolbachia* infection. On the other hand there also is a strong genomic conflict between the *PSR* chromosome and the A-chromosomes. Therefore, in theory, a nuclear suppressor of *PSR* could evolve, which may however indirectly result in fixation of the infection. Strictly speaking, the nuclear

genes of *T. kaykai* seem to be caught in a position where the selective advantage of evolving suppressors against a sex ratio distorter has been pre-empted by the presence of the opposing sex ratio distorter. This is particularly true for a nuclear suppressor of *Wolbachia.* The advantage of such a gene is the production of male offspring by infected females. This trait is however exploited more efficiently by the *PSR* factor since *PSR* induces male production in both infected and uninfected females. The coexistence of both forms in *T. deion* might be a better example in which the host may have evolved a trait that suppresses the infection. In *T. deion* it is at least not a *PSR* factor that is preventing the spread of the infection (Chapter 6). We know a suppressor of *Wolbachia* can evolve, e.g. in *Armadillidium vulgare* a masculinizing (M) gene supresses the Feminizing *Wolbachia* infection (Rigaud & Juchault, 1993).

8.4 COEXISTENCE OF INFECTED AND UNINFECTED FORMS: EVOLUTIONARY PATHWAY

One could say that PI is obviously a successful strategy for *Wolbachia* to invade a population. In many species this 100% daughter production has led to completely (infected) parthenogenetic populations, i.e., an optimal situation for *Wolbachia,* but we do not always find this outcome. Two evolutionary pathways are possible if an individual in an uninfected *Trichogramma* population becomes infected with a PI *Wolbachia* through a successful horizontal transfer (Chapter 3 & 4): 1) The infection is lost through drift or through negative fitness effects of the costs associated with the infection; 2) The infection spreads because the infected females produce more daughters than the uninfected females. If scenario two is true then the infection a) results in such an advantage that it will spread to fixation (Stouthamer 1997; Huigens & Stouthamer 2003 (= Chapter 2)), or b) spreads to an equilibrium, i.e. a stable coexistence between infected and uninfected individuals where different factors are either increasing or decreasing the infection rate in the population (Figure 8.1).

An equilibrium can be imposed by: i) *Non-Mendelian nuclear suppressors* (e.g. *PSR* factor). Intermediate levels of infection can come about when a *PSR* factor enters or evolves in the population as has been found in *T. kaykai* (Stouthamer *et al.* 2001) and extensively discussed in this thesis (Chapter 6 & 7). ii) *Mendelian nuclear suppressors*. Once the *Wolbachia* infection is spreading in the population, mutations that restore the ability of infected females to produce males have a selective advantage. Such

suppressor genes can spread and allow the population to remain in equilibrium at certain infection frequencies (see Stouthamer *et al.* 2001). No confirmed cases of nuclear suppressors against PI *Wolbachia* are known yet. iii) *Bacterial transmission*. When some of the offspring of an infected female looses the infection, stable infection frequencies can be reached. The equilibrium is however very sensitive to slight changes in vertical transmission efficiency and relative offspring production of infected females versus uninfected females (Stouthamer 1997; Stouthamer *et al.* 2001). Intraspecific horizontal transfer above the inefficient vertical transmission would increase the infection rate (Chapter 3) but this is almost completely negated by the low survival of the originally infected larvae under the same superparasitism conditions (Chapter 5). Regular interspecific transfer, followed by inefficient transmission might also result in an equilibrium of low infection frequency although this has not been modelled yet. iv) *Abiotic factors*. Natural curing of infected females through high temperature or naturally occurring antibiotics, specially in a hot environment like the Mojave Desert where temperatures can rise to about 40-50 °C. We know that infected females of several *Trichogramma* species produce an increasing fraction of sons in their offspring when they are reared at temperatures close to 30 °C (Bowen & Stern 1966; Jardak *et al.* 1979; Cabello & Vargas 1985; Pintureau *et al.* 1999). Only one study was carried out under more natural conditions: in *T. oleae*, one of the two *Trichogramma* species fixed for infection, the proportion of males + intersexes in a population contained in a greenhouse without heating increased with rising temperatures (Pintureau *et al.* 2002). *Wolbachia* in desert *Trichogramma* wasps may have been adapted to higher temperatures but not to the extremes that are sometimes reached. In hot summers a naturally occurring sweep could dramatically lower the infection level in the population after which the infection rate may rise again in colder periods. At high temperatures it may also be that many wasps die. When there is already a low infection level in the population, a hot summer may sometimes cause local extinctions of the infection which is compensated by regular horizontal transfers or immigration of infected individuals from populations with a milder climate, e.g. from higher altitudes. This would mean that the infection never has enough time to spread to higher levels.

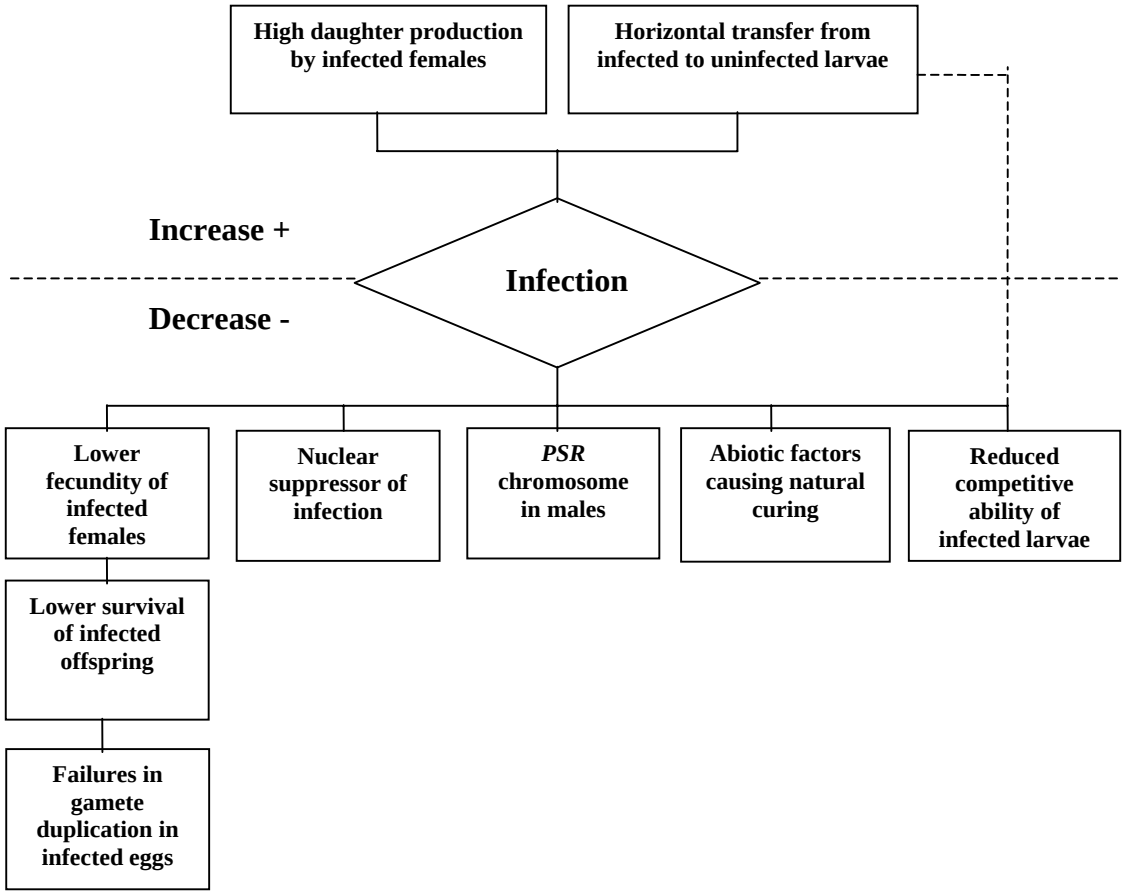

Figure 8.1 Possible factors increasing or decreasing the PI *Wolbachia* infection rate in a mixed *Trichogramma* population. Horizontal transfer from infected to uninfected larvae and reduced competitive ability of infected larvae act under the same circumstances.

In addition to these four possible explanations for stable low infection frequencies, there may be several other factors decreasing the spread of an infection but they are alone not enough to explain a coexistence of the two reproductive forms, these are: v) *Density dependent factors*. Due to the reduced offspring production of infected females, the infection rate may decrease under high host densities but may increase when hosts are scarce because they produce more daughters than their uninfected conspecifics (Stouthamer & Luck 1993). vi) *Failures in gamete duplication*. The gamete duplication in some *Trichogramma* species appears to be far from perfect. In *T. kaykai* and *T. deion*, a higher pre-pupal mortality (± 65%) of infected eggs compared to uninfected eggs was correlated with an arrestment in the early mitotic stages of the embryonic development (Tagami *et al.* 2001). Tagami *et al.* (2001) however did there experiments with a small

lepidopteran host egg, *Ephestia kuehniella*. We now want to determine if this mortality also takes place in larger host eggs that *Trichogramma* encounters in the field.

When we apply all these different hypotheses again on the situation in *T. kaykai* and *T. deion*, we know that the coexistence in *T. kaykai* is mainly due to the presence of the *PSR* factor (Stouthamer *et al.* 2001) and other factors keep each other more or less in balance and are only minor contributors (Figure 8.1). Taking the above mentioned explanations into account we can hypothesize the following for the 1% infection in *T. deion*:

i) Non-Mendelian nuclear suppressor. We can exclude the presence of a *PSR* factor as the main contributor to a low infection level (Chapter 6). ii) *Mendelian nuclear suppressor*. Such a suppressor might be present in *T. deion*. We were however not able to test this hypothesis due to the low number of infected *T. deion* broods collected but this certainly has to be done in the future. iii) *Bacterial transmission*. The vertical transmission efficiency of *Wolbachia* in *T. deion* was very high in four laboratory iso-female lines, namely 98-100% in the first three days of being able to parasitize an unlimited number of eggs at 23 °C (Stouthamer unpublished results). Such a transmission cannot explain the low infection level in *T. deion*, slight changes in the cost of the infection would result in a dramatic increase or decrease of the infection (Stouthamer 1997). We do however never find very high infection rates. The possible inter- and intraspecific horizontal transfer of *Wolbachia* would increase the level of infection even more if infected *T. deion* does not suffer from the same reduced fitness as infected *T. kaykai* (Chapter 3, 4 & 5). Of course, we do not know much about the transmission efficiency in the field which may be much lower than under laboratory conditions, thereby contributing to low infection levels. *T. deion* might rarely acquire *Wolbachia* from *T. kaykai*, or another infected host species, through interspecific horizontal transfer (Chapter 4). The subsequent vertical transmission could then be inefficient. Such interspecific transfers of *Wolbachia* should result in different host species harbouring the same *Wolbachia* variants. To test this we have to sequence *wsp* genes in many different infected *T. deion* and *T. kaykai*. iv) *Abiotic factors*. High temperatures most likely act the same on the sympatric species *T. kaykai* and *T. deion*. In *T. kaykai* this high temperature effect does not seem to be strong because we can explain low infection levels without taking natural curing into account. High

temperatures therefore are not likely to be a major factor in *T. deion* either. On the other hand *T. deion* and *T. kaykai* might be sympatric but largely temporarily separated and therefore temperatures act different on the two species. *T. deion* might parasitize other lepidopteran hosts at another time of the year. This may also explain why we are not able to find many *T. deion* broods emerging from *A. m. deserti* from April to July. v) *Host density dependency*. This does not explain the coexistence of infected and uninfected forms in *T. kaykai*. A reduced offspring production of infected females would mean that infection rates are higher when hosts are scarce. This is not the case when we plot the infection rate against the number of eggs collected per collection day at different locations in the Mojave Desert; R^2= $3.89E^{-05}$, P= 0.973 (Huigens unpublished). In *T. deion* we cannot perform such an analysis because we have too few broods. The relative offspring production of infected versus uninfected females is however the same as in *T. kaykai* suggesting that density dependence cannot explain the coexistence of the two reproductive forms in *T. deion*. vi) *Failures in gamete duplication.* The failure rate in infected *T. deion* and infected *T. kaykai* eggs were equally high and can therefore not alone explain the coexistence of infected and uninfected forms in *T. deion* (Tagami *et al.* 2001). Moreover, I doubt if these high failures in gamete duplication are really a clear pattern: The mortality rates of unfertilized infected *T. kaykai* eggs that Tagami *et al.* (2001) found in *Ephestia kuehniella* (± 65%) are much higher than the mortality rates we found in *Trichoplusia ni* ± 20%. (Chapter 5).

The fact that we only know of a coexistence of infected and uninfected forms in *Trichogramma* wasps is mostly due to our detection methods (see Chapter 2), in which we establish iso-female lines from *Trichogramma* wasps emerging from field-collected host eggs, and, even more importantly, the fact that no other extensive field surveys have been carried out. With our current knowledge, there are no fundamental reasons why low infection levels with PI *Wolbachia* cannot be found in other host species.

Now that we have started to understand the coexistence of infected and uninfected forms, more work on the *Wolbachia*-host association in fixed populations remains to be done. To unravel the evolutionary pathways leading to fixation, we need to test if “virginity” mutations, that have a selective advantage in a partly infected population,

cause the infection to reach fixation (see Chapter 2). Therefore, the genetics of the "virginity" mutation has to be studied. In addition, studies on genetic variation in fixed populations may also give us information on how *Wolbachia* went to fixation.

8.5 IMPLICATIONS ON OTHER ASPECTS OF PI *WOLBACHIA*

In this thesis I focussed on the dynamics of PI *Wolbachia* in natural *Trichogramma* populations but the results may also have important implications on some other aspects of (PI) *Wolbachia*.

8.5.1 *Horizontal transfer of* Wolbachia *and the evolution of virulence*

A parasite obtains highest fitness through a trade-off between its own reproductive capacity and the virulence it inflicts on its host (Messenger *et al.* 1999). When a parasite is purely vertically transmitted, its reproductive capacity, and consequently virulence, cannot be high because the transmission (or fitness) of the parasite depends on the reproductive capacity of its host. With horizontal transmission this is different. Generally, horizontally transmitted parasites are more virulent than vertically transmitted parasites (Ewald 1994). Although *Wolbachia* is not "a parasite in the true meaning of the word" it is shown to negatively affect host fitness in some cases, e.g. the PI *Wolbachia* in *Trichogramma* wasps (Chapter 5). In this thesis, I provided evidence for a much more frequent horizontal transfer of *Wolbachia* than previously expected (Huigens *et al.* 2000 (=Chapter 3); Chapter 4). At high host densities, this mode of transmission may even occur at such a rate, e.g. in *T. kaykai*, that *Wolbachia* might select for higher virulence. The evolution of virulence in PI *Wolbachia* may therefore be simulated by selecting for either horizontal transfer or vertical transfer of *Wolbachia*; then one could compare the fitness (e.g. measuring fecundity, longevity, immature survival with and without competition) of wasps that are infected with a *Wolbachia* that has been transferred horizontally many times with wasps that are infected with a purely vertically transmitted *Wolbachia.*

8.5.2 *PI* Wolbachia *in biological control: rendering wasps parthenogenetic through horizontal transfer*

Little has been done on applied aspects of PI *Wolbachia.* The use of parthenogenetic or unisexual natural enemies has been viewed as a way to enhance the efficacy biological

control of insect pests. Stouthamer (1993) mentioned the advantages of the use of parthenogenetic parasitoid wasps to be: 1) their high rate of increase, 2) their cheap production as all wasps are female, 3) their easy establishment because they are not required to find a mate, and therefore also 4) their effectiveness at low pest densities. The release of infected *Trichogramma* lines in inundative biocontrol therefore sounds attractive. One greenhouse study by Silva *et al.* (2000) showed that in inundative biocontrol the use of infected *Trichogramma* wasps is more economic than the use of sexual forms. However, more studies are needed to determine if this is a general pattern or only a specific example. Important should be in the future to select infected *Trichogramma* lines that originate from fixed populations because in such populations infected wasps do not have a reduced fitness (van Meer 1999; Silva 1999; Chapter 2 & 5). In addition, similar studies should be done with wasps that are used in classical biological control. The use of PI infected wasps for augmenting native sexual populations should be monitored closely, because it may lead to the replacement of native sexual forms with the released infected form.

Interesting would be to render wasps parthenogenetic. This can be done in two ways: through a) natural horizontal transfer when infected and uninfected wasps share the same food source (Chapter 3 & 4) or b) microinjection of PI *Wolbachia* (Grenier *et al.* 1998). Such transfers may only be effective when the *Wolbachia* originates from mixed populations. *Wolbachia* from fixed populations are expected to have co-evolved to some extent with their hosts which may have led to the loss of genes needed to function in other host species (Chapter 4).

8.5.3 *Future work on PI* Wolbachia

Since the beginning of the last decade, when the association between *Wolbachia* and parthenogenesis was first identified (Stouthamer *et al.* 1990; Stouthamer & Werren Stouthamer *et al.* 1993), our knowledge of this association has grown extensively. After the first evidence of bacterial involvement in parthenogenesis through antibiotic treatment and the identification of *Wolbachia*, the findings of intraspecific (Huigens *et al.* 2000 (=Chapter 3)) and interspecific (Grenier *et al.* 1998; Chapter 4) horizontal transfer delivered the final proof of this symbiont as a causal agent of parthenogenesis. Recently, it has also become clear that PI *Wolbachia* may influence cytogenetic events in different ways to cause parthenogenesis, not only through gamete duplication but also

through meiotic modifications (Weeks & Breeuwer 2001), and obviously more cytogenetic studies are needed to determine if yet unknown mechanisms of PI exist. One of the main questions that remains to be answered is: Which *Wolbachia* genes cause the traits expressed in their hosts? And similarly; how important are host effects in expressing the *Wolbachia* phenotype?

The different genome projects that are under way should result in the sequence of several CI *Wolbachia*, a PI *Wolbachia* and a Feminizing *Wolbachia*. Comparative studies on these sequences may help in solving this problem. We will then hopefully also be able to explain why the parthenogenesis phenotype is scattered throughout the phylogenetic trees based on several *Wolbachia* genes.

The study on the CFB bacterium that causes parthenogenesis in several *Encarsia* wasps showed that the oviposition behavior of the infected females was modified by the infection (Hunter 1999; Zchori-Fein *et al.* 2001). We do not know if PI *Wolbachia* also modify their host's behavior.

The discovery of other symbionts involved in parthenogenesis, i.e. the CFB bacterium in the parasitoid wasp *Encarsia* (Zchori-Fein *et al.* 2001) and the verrucomicrobial species in nematodes (Vandekerckhove *et al.* 2000) opens a whole new field of research that should confirm that *Wolbachia*'s host manipulations have evolved in other bacteria as well. The verrucomicrobial species in nematodes and the fact that parthenogenesis may be caused by meiotic modifications (Weeks & Breeuwer 2001) show that not only haplodiploids should be looked upon as possible hosts for PI microbes; in addition, the fact that PI has evolved outside *Wolbachia* should stimulate the search for additional symbionts causing parthenogenesis.

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Nederlandse inleiding en samenvatting

SEX RATIOS

In de natuur produceren de meeste organismen met twee geslachten evenveel mannetjes als vrouwtjes. Deze 50% sex ratios (% vrouwtjes) vinden we onafhankelijk van het mechanisme van geslachtsbepaling, danwel genetisch, bv. bij zoogdieren (mannelijke heterogametie waarbij mannetjes XY zijn en vrouwtjes XX) danwel omgevingsbepaald, bv. temperatuurafhankelijkheid bij krokodillen. Een gebalanceerde sex ratio wordt ondersteund door vele theoriën waarvan Fisher's werk in 1930 één van de meest invoedrijke is. Zeker niet alle organismen vertonen een gebalanceerde sex ratio: bv. bij sluipwespen met een haplodiploïde geslachtsbepaling, m.a.w. bevruchte (diploïde) eieren ontwikkelen zich tot vrouwtjes terwijl onbevruchte (haploïde) eieren zich tot mannetjes ontwikkelen, vinden we sex ratios met meer vrouwtjes dan mannetjes in overeenstemming met de "locale partner competitie" theorie van Hamilton.

De meeste sex ratio theoriën zijn gebaseerd op een sex ratio kenmerk dat wordt bepaald door genen die gelijkwaardig via vader en moeder op het nageslacht worden overgedragen, m.a.w. door genen met een mendelse overerving. Sex ratios worden echter vaak gemanipuleerd of verstoord door egoïstische genetische elementen die geen mendelse overerving hebben. Deze egoïstische genetische elementen worden daarom ook wel sex ratio verstoorders genoemd.

EGOÏSTISCHE GENETISCHE ELEMENTEN

In principe zijn alle genetische elementen geselecteerd om zoveel mogelijk coöpien van zichzelf over te dragen naar toekomstige generaties. Sommigen verhogen hun kans op overdracht naar volgende generaties ten koste van de rest van het genoom. Zulke genetische elementen zijn gedefinieerd als elementen die zich door populaties verspreiden ondanks de kosten die ze veroorzaken bij hun gastheer. Voorbeelden van zulke elementen zijn cytoplasmatisch (via het celvocht) overgedragen microörganismen, meiotische "drive" chromosomen, "homing" endonucleasen, transposabele elementen en B-chromosomen. Ze kunnen grote delen van het een genoom omvatten, bv. ons humane genoom bestaat voor 45% uit transposabele elementen. Egoïstische genetische elementen kunnen een belangrijke rol spelen bij verschillende evolutionaire processen,

bv. soortsvorming, uitsterven en de evolutie van geslachtsbepalingsmechanismen. Cytoplasmatisch overgedragen bacteriën zorgen ervoor dat hun gastheer meer dochters gaat produceren door a) de mannelijke nakomelingen te doden, of door b) eieren die zich normaal gesproken tot zonen zouden ontwikkelen om te vormen tot functionele vrouwtjes. Zulke bacteriën vertonen dit egoïstische gedrag omdat ze verticaal alleeen maar van moeder op dochter overgedragen kunnen worden; spermacellen bevatten niet genoeg cytoplasma en mannelijke gastheren betekenen dus een dood einde voor de bacteriën. Infectie met sex ratio verstorende bacteriën veroorzaakt een sex ratio die afwijkt van een sex ratio die optimaal is voor de genen in de celkern. De conflicten tussen cytoplasmatische elementen met een maternale (via de moeder) overerving en genen in de celkern worden nucleus(celkern)-cytoplasma conflicten genoemd.

Eén zo'n cytoplasmische sex ratio verstoorder, de bacterie ***Wolbachia***, heeft veel aandacht gekregen van biologen over de gehele wereld, vooral omdat deze bacterie verschillende reproductieve manipulaties veroorzaakt in vele arthropoden (geleedpotigen) en nematoden (aaltjes) om haar eigen maternale overerving te vergroten. Parthenogenese (ongeslachtelijke voortplanting) is zo'n manipulatie veroorzaakt door *Wolbachia*, ondekt door Stouthamer en anderen in 1990, en nu bekend van vele hymenoptere (wespachtige), thrips en mijten soorten. In dit proefschrift heb ik mij gericht op de evolutionaire trajecten van ***Wolbachia*-geïnduceerde parthenogenese** bij *Trichogramma* wespen, minuscule sluipwespen van lepidoptere (vlinder- of motten) eieren. Eerst zal ik hier de egoïstische bacterie *Wolbachia* introduceren.

WOLBACHIA

Intracellulaire α-proteobacteriën van het genus *Wolbachia* infecteren een grote variëteit aan insecten, mijten, isopoden (pissebedachtigen) en nematoden. Geïnfecteerde weefsels van adulte (volwassen) gastheren zijn vooral reproductieweefsels, maar ook haemolymfe-, klier- en zenuwweefsels. Deze bacteriën zij voor het eerst beschreven in de mug *Culex pipiens* door Hertig & Wolbach in 1924 en later genoemd *Wolbachia pipientis*. Een aantal decennia later, ontdekten Yen & Barr in 1971 dat een reproductieve manipulatie veroorzaakt werd door *Wolbachia*. Cytoplasmische incompatibiliteit (CI) vondt plaats wanneer ongeïnfecteerde muggenvrouwtjes paarden met *Wolbachia*-geïnfecteerde mannetjes: bij zulke kruisingen stierven de bevruchte eieren.

In het laatste decennium is het aantal studies over *Wolbachia* enorm toegenomen door de ontwikkeling van diverse moleculaire (vooral PCR-gebaseerde) technieken. Recente studies schatten dat *Wolbachia* voorkomt bij 17 % of zelfs 76% van de getestte arthropoden en nematoden. Slechts 1 onderzoek - aan slakken - kon geen enkele infectie aantonen. De interesse van wetenschappers voor deze bacterie komt vooral voort uit het feit dat ze zo algemeen is en de reproductie van hun gastheer op verschillende wijze manipuleren. Ze wordt daarom gezien als één van de belangrijke krachten achter soortsvorming en de rijkdom aan geslachtbepalingsmechanismen bij arthropoden, of zelfs andere dieren. Daarbij, vanuit een toegepassingsgericht perspectief, zijn wetenschappers geïnteresseerd in *Wolbachia*'s mogelijk rol in a) biologische bestrijding, als een vector om genetische modificaties te verspreiden of als een hulpmiddel om de effectiviteit van sluipwespen als natuurlijke vijanden te vergroten, en b) (humane) ziektes veroorzaakt door nematoden.

VERWANTSCHAP VAN *WOLBACHIA*: BEWIJS VOOR HORIZONTAL OVERDRACHT

Wolbachia zijn het meest verwant aan rickettsia-achtige bacteriën: ze vormen een monofyletisch cluster ten opzichte van de andere Rickettsiae. Over het algemeen kan *Wolbachia* verdeeld worden in vijf groepen, A-E. De A- en de B-groep omvatten de arthropoden-*Wolbachia*, terwijl de nematoden-*Wolbachia* groep C en D vormen. Groep E bestaat uit een enkele *Wolbachia* infectie in de springstaart *Folsomida candidada*. Deze *Wolbachia* is meer gerelateerd aan groep A en B dan aan C en D. A-B en C-D lijken ongeveer 100 miljoen jaren geleden gedivergeerd te zijn, waarna A en B ongeveer 60 miljoen jaar geleden zijn gedivergeerd.

Fylogeniën (verwantschappen) van *Wolbachia* zijn gebaseerd op verschillende genen, waaronder 16S rDNA, *ftsZ*, *groEl* en *wsp*. Het laatste gen, *wsp* (*Wolbachia* Specific Protein, een celloppervlak-eiwit), wordt nu algemeen gebruikt voor de classificatie van *Wolbachia*. De fylogeniën van *Wolbachia* en gastheren weerspiegelen elkaar niet in de A- en de B-groep, onafhankelijk van het bestudeerde gen: nauw verwante *Wolbachia* worden gevonden, en veroorzaken verschillende reproductieve afwijkingen, in zeer diverse gastheren. Dit geeft te kennen dat horizontale transmissie van *Wolbachia* plaatsgevonden moet hebben op een evolutionaire tijdschaal. Zo'n overdracht word gezien als een zeldzame gebeurtenis. Alleen in nematoden lijkt er een duidelijke co-

evolutie van *Wolbachia* en gastheer te zijn geweest (*Wolbachia*- en nematoden-fylogeniën weerspiegelen elkaar volledig).

REPRODUCTIEVE AFWIJKINGEN VEROORZAAKT DOOR *WOLBACHIA* ANDERS DAN PARTHENOGENESE

Cytoplasmatische incompatibiliteit

De meest algemeen verspreide reproductieve afwijking die door *Wolbachia* wordt veroorzaakt is cytoplasmatische incompatibiliteit (CI): een incompatibiliteit tussen sperma en eicel geïnduceerd door *Wolbachia* die resulteerd in een eliminatie van de paternale (vaderlijke) chromosomen. In diploïde soorten sterven zulke bevruchte eieren terwijl in haplodiploïde soorten de bevruchte eieren ook sterven (vrouwelijk mortaliteit's CI type) of zich tot mannetjes ontwikkelen (mannelijk ontwikkeling's CI type). Recent hebben Vavre en anderen zelfs een 3^{e} CI type beschreven in de sluipwesp *Leptopillina heterotoma* waar sommige bevruchte eieren van één vrouwtje sterven en anderen zich ontwikkelen tot mannetjes (intermediair CI type). CI vinden we bij vele insectenorden, verschillende mijten en een isopoden soort. Verwantschapsanalyse toonde aan dat CI-*Wolbachia* in groep A en B vallen. De meeste CI is incompatibiliteit in één richting: kruisingen tussen geïnfecteerde mannetjes en ongeïnfecteerde vrouwtjes zijn incompatibel terwijl de reciproke kruising tussen geïnfecteerde vrouwtjes en ongeïnfecteerde mannnetjes compatibel is. Bidirectionele CI vindt plaats wanneer mannetjes en vrouwtjes een verschillende *Wolbachia* variant dragen die wederzijds incompatibel zijn. Deze laatste vorm van CI is bijzonder interessant met betrekking tot soortsvorming omdat het bijdraagd aan reproductieve isolatie. In *Nasonia* is deze vorm van CI waarschijnlijk voorafgegaan aan andere isolatie mechanismen en kan dus een rol hebben gespeeld bij de soortsvorming in dit genus. *Wolbachia* infecties die CI veroorzaken kunnen zich snel door populaties verspreiden door het ongeïnfecteerde deel van de populatie te reduceren: geïnfecteerde eieren zijn compatibel met ongeïnfecteerd en geïnfecteerd sperma terwijl ongeïnfecteerde eieren alleen compatibel zijn met ongeïnfecteerd sperma. Theoretisch en empirisch onderzoek heeft aangetoond dat infecties die CI veroorzaken een selectief voordeel hebben en naar fixatie kunnen gaan, m.a.w. dat op een gegeven moment elke individu in de populatie geïnfecteerd is.

Feminizatie

In amphipoden en terrestrische (land) crustaceën (kreeftachtigen) met vrouwelijke heterogametie (mannetjes zijn ZZ en vrouwtjes ZW), worden genetische mannetjes omgevormd, of gefeminizeerd, tot functionele vrouwtjes als ze geïnfecteerd zijn met *Wolbachia*. Deze vorm van feminizatie komt niet alleen voor bij kreeftachtigen: recentelijk werd *Wolbachia* ook geassocieerd met feminizatie in twee vlindersoorten. Feminizerende (F) *Wolbachia* kunnen een belangrijke rol spelen in de evolutie van geslachtsbepaling in kreeftachtigen omdat feminizatie tot het verlies van vrouwelijke heterogametie kan leiden. Dit wordt ondersteund door het voorkomen van verschillende populaties van de pissebed *Armadillidium vulgare* die geen ZW vrouwtjes bevatten. In één onderzoek werd geschat dat 35% van de landpissenbedden geïnfecteerd zijn met *Wolbachia*. Alle *Wolbachia* in isopoden vallen in de B-groep. Alle, behalve één enkele, *Wolbachia* in de oniscoidae (bepaalde pissenbeddenfamilie) vormen een monofyletische groep maar in andere isopoden zijn de *Wolbachia's* minder aan elkaar verwant. De infectiegraden in populaties variëren behoorlijk maar, met uitzondering van één soort die gefixeerd is voor de infectie, m.a.w. alle vrouwtjes in de populatie zijn geïnfecteerd, over het algemeen tussen 10 en 50%. *A. vulgare* is het best bestudeerd en in deze soort lijkt de aanwezigheid van F *Wolbachia* te hebben geleid tot de evolutie van onderdrukkende (in dit geval masculiniserende) genen die het effect van *Wolbachia* in sommige populaties tegengaan.

Mannendoden

Al in 1947 vond Lus maternaal overgeërfde factoren die mannen doodden tijdens de embryogenese in het lieveheersbeestje *Adalia bipunctata*. Momenteel zijn er zes verschillende bacteriën beschreven die geassocieerd zijn met het doden van mannen (MK= male-killing) wat suggereerd dat dit kenmerk makkelijker is geëvolueerd in bacteriën dan het veroorzaken van andere reproductieve afwijkingen. Voordelige effecten van MK zouden kunnen zijn 1) een verlaagde kans op inteelt, 2) een directe toegang tot voedselbronnen door cannibalisme op de dode mannetjes, en 3) een verlaagde concurrentie tussen verwanten (broers-zussen). MK *Wolbachia* zijn op dit moment bekend bij *A. bipunctata* en de vlinder *Acraea encedon*. Omdat het lieveheersbeestje mannelijke heterogametie heeft en de vlinder vrouwelijke heterogametie suggereerd dit dat *Wolbachia* verschillende mechanismen heeft

geëvolueerd om het geslacht van de gastheer te herkennen. Infectiegraden variëren enorm van 20-30% van de vrouwtjes in russische populaties van *A. bipunctata* tot 80% van de vrouwtjes van *A. encedon.* Details over het mannendoden door *Wolbachia* ontbreken nog omdat onderzoek naar dit fenomeen nu aan het ontluiken is.

Verhoging van de nakomelingenproductie en vruchtbaarheid

In de sluipwesp *Trichogramma bourarache* wordt de transmissie van *Wolbachia* verhoogd door een toename in de nakomelingenproductie van geïnfecteerde vrouwtjes. Geïnfecteerde vrouwtjes produceren gemiddeld twee keer zoveel nakomelingen als soortgenoten die genezen zijn van hun infectie door behandeling met antibiotica. Deze *Wolbachia* valt in de A-groep. Helaas is experimenteel and theoretisch onderzoek aan deze nakomelingenproduktie verhoging tot nu toe nog niet gecontinueerd. Eenzelfde soort fenomeen na behandeling met antibiotica is gevonden bij CI-*Wolbachia* in *Drosphila simulans* en *Nasonia vitripennis* maar de resultaten zijn nog wat onduidelijk. Er zijn ook twee gevallen bekend waarbij *Wolbachia* de fitness (fitheid) van haar gastheer verhoogd. In de vlieg *Sphyracephala beccarri* en de kever *Tribolium confusum* is de vruchtbaarheid van geïnfecteerde mannetjes hoger dan die van genezen soortgenoten. *Wolbachia* lijkt in deze gevallen een enigzins mutualistisch gedrag (voordelig effect) geëvolueerd te hebben.

Wolbachia *essentieel voor eiontwikkleing*

Zeer recentelijk vonden Dedeine en anderen een *Wolbachia* variant die essentieel is voor de eiontwikkeling in de sluipwesp *Asobara tabida*. Alle vrouwtjes in franse populaties van *A. tabida* zijn geïnfecteerd met drie *Wolbachia* varianten (gebaseerd op het *wsp* gen), waarvan er twee CI induceren en de andere betrokken is bij de eiontwikkeling. Wespen die van de laatste variant zijn genezen kunnen geen volwassen eieren produceren. Eenzelfde soort fenomeen is beschreven in de nematoden-*Wolbachia*, waar behandeling met antibiotica een nadelig effect had op twee nematoden soorten, *Brugia pahangi* en *Litomosoides sigmodontis*. In dit geval heeft een lange co-evolutie tussen gastheer en symbiont geleidt tot een situatie waar de gastheer afhankelijk is geworden van *Wolbachia,* m.a.w. een obligatorische (bindende) symbiose.

WOLBACHIA-GEÏNDUCEERDE PARTHENOGENESE IN *TRICHOGRAMMA* SP.

Wolbachia veroorzaakt parthenogenese in minstens 14 van ongeveer 180 *Trichogramma* soorten. *Trichogramma* heeft een haplodiploïde voortplanting waarbij dochters (diploïd) ontwikkelen uit bevruchte eieren en zonen (haploïd) uit onbevruchte eieren. Vrouwtjes die geïnfecteerd zijn met parthenogenese-inducerende (PI) *Wolbachia* produceren echter dochters uit zowel de bevruchte als de onbevruchte eieren. Onbevruchte geïnfecteerde eieren ontwikkelen zich tot vrouwtjes omdat er een verdubbeling van de haploïde set van moederlijke chromosomen in de eerste mitotische deling plaatsvindt, een proces dat gameet duplicatie genoemd wordt. Deze vorm van parthenogenese kan worden genezen door antibiotica behandeling.

Wolbachia in *Trichogramma* sp. zijn uniek vergeleken met bijna alle andere *Wolbachia*-gastheer associaties omdat deze *Wolbachia* één cluster vormen in de B-groep in alle verwantschapsbomen gebaseerd op verschillende *Wolbachia* specifieke genen. Een gedetaileerde verwantschapsanalyse van de *Wolbachia-Trichogramma* sp. associatie liet een duidelijk verschil zien tussen de *Trichogramma* fylogenie en de *Wolbachia* fylogenie, waarschijnlijk door horizontale overdracht van *Wolbachia.* Deze *Wolbachia* lijken allemaal één gemeenschappelijke voorouder te hebben en zijn alleen van gastheersoort naar gastheersoort overgedragen binnen het genus *Trichogramma.*

PI *Wolbachia* in *Trichogramma* verschillen van diegenen in andere gastheersoorten omdat infecties niet naar fixatie zijn gegaan, m.a.w. slechts een deel van de populatie is geïnfecteerd. In alle *Trichogramma* soorten, behalve twee, vinden we lage infectie percentages wat uniek lijkt te zijn voor PI *Wolbachia*. Alleen in *Trichogramma cordubensis* and *T. oleae* populations zijn populaties gefixeerd voor de infectie. Theoretisch onderzoek heeft aangetoond dat stabiele lage infectiegraden alleen verklaard kunnen worden door de aanwezigheid van een bepaalde vorm van *Wolbachia* onderdrukkend element als geïnfecteerde vrouwtjes tenminste meer dochters produceren dan ongeïnfecteerde vrouwtjes en de transmissie efficiëntie van *Wolbachia* van moeder op dochter hoog is. In *Trichogramma kaykai*, waar 6-26% van de vrouwtjes geïnfecteerd zijn, heeft een extensive zoektocht naar onderdrukkende factoren de aanwezigheid van een ander egoïstisch genetisch element aan het licht gebracht. Ongeveer 10% van de mannetjes dragen een *PSR* (Paternale Sex Ratio) chromosoom. Tot dan toe was zo'n *PSR* chromosoom alleen bekend van de sluipwesp *Nasonia*

vitripennis. Als een vrouwtje paart met een *PSR*-dragend mannetje worden de vaderlijke chromsomen in bevruchte eieren vernietigd en blijven alleen de moederlijke chromosomen en de *PSR* factor zelf over. Zulke bevruchte eieren ontwikkelen zich tot mannetjes met alleen de moederlijke chromosomen en het *PSR* chromosoom van hun vader. *PSR* zorgt er daarom voor dat ongeïnfecteerde en geïnfecteerde vrouwtjes zonen produceren uit hun bevruchte eieren. In ongeïnfecteerde *T. kaykai* paart een groot deel van de ongeïnfecteerde vrouwtjes met hun broers op de ouderlijke "patch" en zijn daarbij beschermt tegen het vernietigende effect van de *PSR* factor. Dit in tegenstelling tot de geïnfecteerde vrouwtjes die uit een broedsel komen met alleen maar zussen en daarom zullen paren met mannetjes uit de populatie, van wie 10% dragers van *PSR* zijn. De consequentie van deze asymmetrie is dat de hoge dochterproductie van de geïnfecteerde populatie meer onderdrukt wordt dan die van de ongeïnfecteerde populatie. Zo'n paringstructuur en de aanwezigheid van de *PSR* factor zorgt voor een stabiele co-existentie van beide vormen in de populatie.

In zulke populaties waar geïnfecteerde en ongeïnfecteerde individuen samen voorkomen verwachten we een nucleus-cytoplasma conflict tussen *Wolbachia* en het genoom in de celkern van de gastheer. *Wolbachia* begunstigd 100% vrouwtjes terwijl de genen in de celkern een sex ratio begunstigen met in ieder geval een paar mannetjes. In deze situaties kan een wedloop tussen de genen in de celkern en die van *Wolbachia* resulteren in nulceaire (celkern) genen die *Wolbachia* of haar effect onderdrukken. Vervolgens verwachten we hoge fysiologische kosten van het geïnfecteerd-zijn in gemixte opulaties. De gereduceerde nakomelingenproductie van geïnfecteerde *Trichogramma* vrouwtjes uit zulke gemixte populaties kan een gevolg zijn van het nucleus-cytoplasma conflict.

In hoofdstuk 2 heb ik de discussie over het onderzoek aan parthenogenese geïnduceerd door *Wolbachia*, dat in ongeveer de laatste 12 jaar uitgevoerd is na de eerste ontdekking door Stouthamer en anderen in 1990, uitgebreid.

BIOLOGIE VAN *TRICHOGRAMMA* SP. IN DE MOJAVE WOESTIJN

Trichogramma kaykai, T. deion en *T. pratti* komen sympatrisch (op dezelfde locaties) voor in de Mojave woestijn van zuid-west Amerika. *T. kaykai* (zie figuur 1.1a, hoofdstuk 1) en *T. deion* zijn beiden geïnfecteerd met PI *Wolbachia* en bestaan uit gemixte populaties. Deze drie soorten parasiteren eieren van de "mormon metalmark"

vlinder *Apodemia mormo deserti* (Lepidoptera, Lycaenidae) (zie figuur 1.1c, hoofdstuk 1).

Volgens de literatuur, heeft de ondersoort *A. m. deserti* twee generaties per jaar, één vliegt van Maart tot April en de ander van September tot November. Wij hebben echter ook eind Juli eieren gevonden wat duidt op een tweede generatie in de lente. *A. m. deserti* gebruikt *Eriogonum inflatum* (Polygonacae), ook wel de "desert trumpet" genoemd, als haar larvale gastheerplant (zie figuur 1.1b, hoofdstuk 1). Deze - in patches verspreide - meerjarige plant wordt gevonden door de gehele Mojave woestijn maar vooral in de buurt van wegkanten en op hellingen. *E. inflatum* heeft grijs-groene tot groene stengels die uit een basale rozet omhoog groeien. De omhoogstaande stengels hebben een groot aantal dubbele of drie-dubbele vertakkingen. Sommige delen van de 10 tot 120 cm hoge stengels zijn verdikt. The bloemen zijn geel en de bloeiperiode is vooral in de lente. Het is op de vertakkingen van de plant waar *A. m. deserti* haar eieren legd (zie figuur 1.1c, hoofdstuk 1).

Van 1998-2001 hebben we jaarlijks de natuurlijke *Trichogramma* populaties op *A. m. deserti* bestudeerd om de PI *Wolbachia*-infectiegraad en de aanwezigheid van andere factoren, zoals het *PSR* chromosoom, die bij kunnen dragen aan het verklaren van de co-existentie van geïnfecteerde en ongeïnfecteerde individuen, te bepalen. *T. kaykai* was duidelijk meer algemeen op *A. m. deserti* dan de twee andere soorten. Soms vonden we twee soorten gezamelijk in een vlinderei.

DOEL, OPZET EN SAMENVATTING VAN DIT PROEFSCHRIFT

Het grote doel van dit promotieonderzoek is onze kennis over de evolutionaire trajecten van *Wolbachia*-geïnduceerde parthenogenese in *Trichogramma* wespen vergroten. Of meer specifiek: we willen verklaren waarom geïnfecteerde en ongeïnfecteerde individuen naast elkaar voorkomen in deze sluipwespen. Stouthamer en anderen hebben in 2001 in een meer theoretische studie laten zien dat de aanwezigheid van een *PSR* factor bij mannetjes hoofdzakelijk bijdraagd aan stabiele lage PI *Wolbachia*-infectiegraden in *Trichogramma kaykai,* waarbij ze rekening hielden met het bevruchtingspercentage van de eieren en de frequentie van broer-zus paring tussen ongeïnfecteerde individuen. Twee andere hypotheses van a) nucleaire onderdrukkers genen en b) bacteriële transmissie konden de lage infectiegraad in deze soort niet verklaren. Om die laatste hypothese te modelleren hadden zij de evenwichtsfrequentie

van PI *Wolbachia* berekend als een functie van de vertikale (van moeder op dochter) transmissie efficiëntie van *Wolbachia* en de fitness kosten die geassocieerd zijn met *Wolbachia* infectie in termen van nakomelingenproductie.
Het evolutionaire traject van een PI *Wolbachia*-gastheer interactie wordt echter waarschijnlijk bepaald door meerdere elementen die elk hun eigen rol spelen in het traject dat leidt naar een verlies van de infectie, fixatie of stabiele lage infectiegraden.
In dit proefschrift heb ik verschillende nieuwe elementen getest en bediscussieerd die van belang kunnen zijn voor de evolutie van *Wolbachia*-geïnduceerde parthenogenese (en ook *Wolbachia*-gastheer interacties in het algemeen) door extensief veldwerk, moleculaire technieken, gedrags- en kruisingsexperimenten te combineren met model studies.

In **hoofdstuk 2** wordt eerst een overzicht van de literatuur van de laatste twaalf jaar over *Wolbachia*-geïnduceerde parthenogenese gegeven. Onderzoek heeft aangetoond dat vele gevallen van parthenogenese bij arthropoden opgelegd worden door intracellulaire *Wolbachia* bacteriën en niet worden gereguleerd door de genen van de gastheer zelf. Recentelijk bewijs voor een CFB (Cytophaga-Flexibacter-Bacteroides) bacterie, die parthenogenese veroorzaakt bij een aantal soorten van de sluipwesp *Encarsia,* en een andere groep van bacteriën, de *Verrumicrobia*, die geassocieerd zijn met parthenogenese in de nematode *Xiphinema americanum*, maakt duidelijk dat parthenogenese-inductie dus ook in andere bacteriën buiten *Wolbachia* is geëvolueerd. Het PI *Wolbachia* onderzoek dat op verschillende niveaus wordt uitgevoerd, van de cytogenetische mechanismen van PI tot de evolutie en dynamica van infecties in gastheer populaties is in dit hoofdstuk bediscussieerd.
Vertikale transmissie van *Wolbachia* van moeder op dochter werd gezien als de belangrijkste wijze van transmissie maar in **hoofdstuk 3 & 4** laten we een onverwacht frequente natuurlijke inter- en intraspecifieke horizontale transmissie zien tussen *Trichogramma kaykai* en *T. deion* larven die een algemene voedselbron delen, een vlinderei. Oorspronkelijk ongeïnfecteerde onvolwassen vrowtjes konden in het gastheerei geïnfecteerd raken maar niet alle nieuw geïnfecteerde wespen vertoonden parthenogenese. In *T. kaykai,* werd intraspecifieke horizontale transmissie gevolgd door complete parthenogenese in toekomstige generaties maar wanneer *T. kaykai* vrouwtjes *Wolbachia* van *T. deion* ontvingen leek de *Wolbachia* infectie niet meer aanwezig in een

aantal generaties na de interspecifieke overdracht. Onze resultaten verklaren het verschil tussen *Wolbachia*- en (Trichogrammatide) gastheer verwantschappen. Frequente horizontale transmissie kan voor hoge virulentie in deze bacteriën selecteren.

Vanwege een nucleus-cytoplasma conflict tussen *Wolbachia* en de genen in celkern van *Trichogramma* en de zojuist beschreven horizontale transmissie van *Wolbachia*, is de infectie in populaties waar geïnfecteerde en ongeïnfecteerde individuen naast elkaar voorkomen waarschijnlijk geassocieerd met fitness kosten. In **hoofdstuk 5** laten we zien dat geïnfecteerde *T. kaykai* vrouwtjes een gereduceerde overleving hebben in vergelijking met ongeïnfecteerde soortgenoten als ze hetzelfde gastheerei delen. De overleving van onvolwassen geïnfecteerde wespen was hoger als ze concurreerden met andere onvolwassen geïnfecteerde wespen van een andere moeder dan in competitie met onvolwassen ongeïnfecteerde wespen. Hieruit blijkt dat PI *Wolbachia*-geïnfecteerde *Trichogramma* wespen een behoorlijke fitness kost hebben. Dankzij deze gereduceerde concurrentiekracht van geïnfecteerde larven kan horizontale transmissie van *Wolbachia*, dat plaatsvindt onder dezelfde superparasiteringsomstandigheden, niet in grote mate bijdragen aan een verhoging van de infectiegraad in de populatie.

Eerder onderzoek had aangetoond dat de aanwezigheid van een andere sex ratio verstoorder in mannetjes, een B-chromosoom genaamd *PSR* (Paternale Sex Ratio), dat de paternale chromosomen vernietigd na bevruchting en daarmee een volledig mannelijke of een voornamelijk mannnelijke sex ratio van het nageslacht veroorzaakt, bijdraagd aan een lage infectiegraad in *T. kaykai*. In **hoofdstuk 6** hebben we bepaald of een *PSR* factor ook lage infectiegraden veroorzaakt in een andere soort. We hebben natuurlijke populaties van drie *Trichogramma* soorten - *T. kaykai*, *T. deion* en *T. pratti* - uit the Mojave woestijn bestudeerd. Onze data laten zien dat alle *Trichogramma* broedsels, verzameld op *Apodemia mormo deserti*, die mannetjes bevatten met het *PSR* fenotype (uiterlijke kenmerk) tot *T. kaykai* behoorden. In laboratorium experimenten droeg 71.4% van de *T. kaykai PSR* mannetjes het *PSR* fenotype horizontaal over naar *T. deion*. Dit percentage is vergelijkbaar met het transmissiepercentage van *PSR* naar *T. kaykai* vrouwtjes, namelijk 81.6%. *PSR* kan derhalve overgedragen worden naar *T. deion* en we verwachten dat dit in het veld ook gebeurd aangezien *T. kaykai* en *T. deion* soms tegelijk uit hetzelfde vlinderei komen. Ondanks dit, kunnen we *PSR* niet in *T. deion* vinden. Als we de situatie in *T. deion* modelleren laat dit zien dat lage infectiegraden alleen vastgehouden kunnen worden als de *PSR* graad heel hoog is.

Daarom moeten andere factoren de infectie ervan weerhouden om naar fixatie te gaan, bv. nucleaire onderdrukker genen.
De paringsstructuur in de gastheerpopulatie speelt een belangrijke rol in de dynamiek van PI *Wolbachia* en *PSR*. Een *PSR* factor weerhoudt de *Wolbachia* infectie ervan om naar fixatie te gaan in *T. kaykai* omdat ongeïnfecteerde *T. kaykai* vrouwtjes vaak met een broertje paren. Broer-zus paring is een barrière tegen het vernietigende effect van het paren met een *PSR*-dragend mannetje. Geïnfecteerde vrouwtjes hebben dit voordeel niet. In **hoofdstuk 7** hebben we een populatie genetisch model gebruikt met microsatellieten als genetische merkers en hebben we een broer-zus paring van 70% en een buiten-de-ouderlijke patch paring van 15% gevonden. Vijfendertig procent van de vlindereieren waren geparasiteerd door twee *T. kaykai* vrouwtjes. Als we deze hoge percentages van broer-zus paring in een model stoppen dat de dynamiek van PI *Wolbachia* en *PSR* in een *Trichogramma* populatie beschrijft, resulteerd dit in een stabiele lage infectiegraad, m.a.w. een co-existentie tussen geïnfecteerde en ongeïnfecteerde individuen, en een laag percentage *PSR*-dragende mannetjes. Onze resultaten laten zien hoe de paringstructuur bijdraagd aan het samen voorkomen van de twee sex ratio verstoorders in een populatie.

De hoofdconclusie van dit proefschrift is dat, ondanks de hoge vertikale en de regelmatige horizontale transmissie van *Wolbachia*, een PI *Wolbachia*-infectie op een laag niveau gehouden kan worden in *Trichogramma*, dankzij de aanwezigheid van een niet-mendels overgerfde onderdrukker, zoals de *PSR* factor, maar ook dankzij andere factoren. In *T. deion*, bijvoorbeeld, houdt *PSR* de infectiegraad niet op een laag niveau maar kan een nucleaire mendelse onderdrukker tegen de PI *Wolbachia* geëvolueerd zijn. Het feit dat we alleen van een co-existentie van geïnfecteerde en ongeïnfecteerde vormen in *Trichogramma* wespen weten komt vooral door onze detectiemethoden waarin we vrouwtjes, die uit in-het-veld-verzamelde vlindereieren komen, apart opzetten en kunnen bepalen of ze geïnfecteerd zijn, en misschien nog belangrijker, het feit dat zulke extensieve zoektochten verder nog niet zijn uitgevoerd. Met onze huidige kennis zijn er geen duidelijke redenen om aan te nemen dat lage infectiegraden met PI *Wolbachia* niet in andere gastheersoorten voor kunnen komen.

IMPLICATIES VOOR ANDERE ASPECTEN VAN PI *WOLBACHIA*

In dit proefschrift heb ik mij gericht op de dynamiek van PI *Wolbachia* in natuurlijke *Trichogramma* populaties maar de resultaten kunnen ook belangrijk zijn voor andere aspecten van (PI) *Wolbachia*.

Horizontale transmissie van **Wolbachia** *en de evolutie van virulentie*

Een parasiet vergrijgt de hoogste fitness via een "trade-off" tussen reproductiecapaciteit en de virulentie die het toebrengt aan de gastheer. Als een parasiet puur vertikaal wordt overgedragen kan z'n reproductiecapaciteit, en vervolgens virulentie, niet hoog zijn omdat de transmissie (of fitness) van de parasiet afhangt van de reproductiecapaciteit van de gastheer. Bij horizontale transmissie ligt dit anders. Over het algemeen zijn horizontaal overdraagbare parasitieten meer virulent dan vertikaal overdraagbare parasitieten. Ondanks dat *Wolbachia* niet "een parasiet in de juiste betekenis van het woord" is kan ze de fitness van de gastheer in een aantal gevallen negatief beïnvloeden, bv. de PI *Wolbachia* in *Trichogramma* wespen. In dit proefschrift, heb ik bewijs aangevoerd voor een meer frequente horizontale transmissie van *Wolbachia* dan vooraf gedacht. Bij hoge gastheerdichtheden, kan deze manier van transmissie zelfs zo vaak voorkomen, bv. in *T. kaykai*, dat *Wolbachia* voor hogere virulentie kan selecteren. De evolutie van virulentie in PI *Wolbachia* kan daardoor worden gesimuleerd door vóór horizontale of vertikale transmissie van *Wolbachia* te selecteren; op een gegeven moment kan de fitness (bv. het meten van nakomelingenproduktie, levensduur, overleving van onvolwassen wespen met en zonder competitie) van wespen die geïnfecteerd zijn met een bacterie die vaak horizontaal is overgedragen vergeleken worden met wespen die geïnfecteerd zijn met een puur vertikaal overgedragen *Wolbachia.*

PI **Wolbachia** *in biologische bestrijding: wespen parthenogenetisch maken door horizontale overdracht*

Er is weinig onderzoek gedaan aan de toegepaste aspecten van PI *Wolbachia.* Het gebruik van parthenogenetische of unisexuele natuurlijke vijanden wordt gezien als een manier om de effectiviteit van de biologische bestrijding van pestinseckten te verhogen. Stouthamer noemde in 1993 de voordelen van het gebruik van parthenogenetische sluipwespen als zijnde: 1) hun snelle populatiegroei, 2) hun

goedkope produktie omdat alle wespen vrouwelijk zijn, 3) hun snelle vestiging omdat ze geen partners hoeven te vinden, en daarom ook 4) hun effectiviteit bij lage pestdichtheden. Het loslaten van geïnfecteerde *Trichogramma* lijnen in de biologische bestrijding klinkt dan ook aantrekkelijk. Eén kasstudie uitgevoerd door Silva en anderen in 2000 toonde aan dat het massaal loslaten van geïnfecteerde *Trichogramma* wespen economischer is dan het gebruik van sexuele vormen. Er zijn echter meer studies nodig om te bepalen of dit een algemeen patroon is of slechts een specifiek voorbeeld. Het is belangrijk om in de toekomst geïnfecteerde *Trichogramma* lijnen te nemen die afkomstig zijn uit gefixeerde populaties omdat deze (waarschijnlijk) geen gereduceerde fitness hebben. Daarbij moeten vergelijkbare studies gedaan worden met wespen die veel in de biologische bestrijding gebruikt worden. Het gebruik van PI *Wolbachia*-geïnfecteerde wespen moet nauwkeurig worden gevolgd omdat het kan leiden tot het vervangen van de locale sexuele vorm door de losgelaten geïnfecteerde vorm.

Interessant zou het parthenogenetisch maken van wespen kunnen zijn. Dit zou op twee manieren kunnen gebeuren: door a) natuurlijke horizontale transmissie als geïnfecteerde en ongeïnfecteerde wespen dezelfde voedselbron delen, of b) microinjectie van PI *Wolbachia*. Zulke overdrachten kunnen misschien alleen effectief zijn als de *Wolbachia* afkomstig is uit een gemixte populatie. *Wolbachia* uit gefixeerde populaties zijn waarschijnlijk al zo lang samen met hun gastheer geëvolueerd dat ze geen genen meer hebben die nodig zijn om te kunnen functioneren in andere gastheersoorten.

Toekomstig onderzoek aan PI Wolbachia

Sinds het begin van het laatste decennium, toen de associatie tussen *Wolbachia* en parthenogenese voor het eerst was geïdentificeerd is onze kennis van deze associatie enorm gegroeid. Na het eerste bewijs voor de rol van bacteriën in parthenogenese door antibiotica behandeling en de identificatie van *Wolbachia*, zijn de vondsten van intra- en interspecifieke horizontale transmissie het definitieve bewijs dat deze symbiont een veroorzaker van parthenogenese is. Recentelijk is het duidelijk geworden dat PI *Wolbachia* cytogenetische gebeurtenissen op verschillende manieren kunnen beïnvloeden om parthenogenese te bewerkstelligen, niet alleen via gameet duplicatie in de eerste mitotische deling maar ook via meiotische modificaties. Er zijn meer cytogenetische studies nodig om te bepalen of er nog onbekende mechanismen van PI bestaan. Eén van de belangrijkste vragen die nog beantwoord moet worden is: Welke

Wolbachia genen zijn betrokken bij de effecten die zichtbaar zijn bij de verschillende gastheren? En daarmee ook; hoe belangrijk zijn gastheereffecten voor het *Wolbachia*-geïnduceerde fenotype?

De verschillende genoomprojecten die nu onderweg zijn moet resulteren in de complete basepaarvolgorde van een aantal CI *Wolbachia*, een PI *Wolbachia* en een F *Wolbachia*. Vergelijkende studies naar deze basepaarvolgorden kunnen helpen om dit probleem op te lossen. Dan kunnen we hopelijk ook verklaren waarom het parthenogenese fenotype verspreid is door alle fylogenetische bomen gebaseerd op verschillende *Wolbachia* genen.

Het onderzoek aan de CFB bacterie die parthenogenese veroorzaakt bij een aantal *Encarsia* wespen toonde aan dat het eileggedrag van geïnfecteerde vrouwtjes was gemodificeerd door de infectie. We weten nog niet of PI *Wolbachia* ook het gedrag van een gastheer kunnen beïnvloeden.

De ontdekkingen van andere symbionten die betrokken zijn bij parthenogenese, bv. de CFB bacterie in *Encarsia* en, misschien, de *Verrucomicrobia* in nematoden openen een geheel nieuw onderzoeksgebied dat moet bevestigen of *Wolbachia*'s gastheermanipulaties ook in andere bacteriën zijn geëvolueerd. De *Verrucomicrobia* in nematoden en het feit dat parthenogenese ook veroorzaakt kan worden door een meiotische modificatie tonen aan dat niet alleen haplodiploïden moeten worden gezien als mogelijke gastheren voor PI bacteriën; daarnaast zal het feit dat PI is geëvolueerd buiten *Wolbachia* de zoektocht naar andere PI symbionten moeten stimuleren.

Dankwoord

Vier-en-een-half jaar lang mensen uitleggen dat je OIO bent, ik geef het je te doen. Het omschrijven van die functie van **O**nderzoeker **I**n **O**pleiding was soms lastiger dan het uitvoeren ervan dankzij de hulp van een flink aantal mensen. Waarschijnlijk ga ik in het hieronderstaande enkele namen vergeten en daarom alvast voor iedereen met wie ik in de afgelopen periode meer dan een handvol, min of meer, betekenisvolle zinnen heb uitgewisseld: bedankt! Een aantal mogen echter wel wat nadrukkelijker genoemd worden.

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Daarnaast wil ik Joop van Lenteren (promotor) bedanken voor alle hulp die hij tijdens het onderzoek geboden heeft. Veel van die hulp was op het vaak onderschatte organisatorisch/financieel vlak. Ik was erg onder de indruk hoe je de boel regelde. Sinds Richard's vertrek naar de VS hebben we wat intensiever contact gekregen en wat me daarbij opviel is je bijzonder heldere kijk op, en grote kennis van, het doen van onderzoek. Ik ben vooral bijzonder dankbaar voor je commentaar op mijn hoofdstukken gedurende de laatste maanden van het onderzoek.

In totaal heb ik 4 keer een aantal maanden in Riverside Californië doorgebracht om het veldwerk in de Mojave woestijn uit te voeren. Bob Luck heeft me in die periodes bijzonder veel geholpen en dan niet alleen uit praktisch oogpunt. Bob, our long discussions on many different topics -from sex determination in parasitoids to different issues of life in general– have really inspired me a lot. Your enthousiasm for, and knowledge of, entomological research have helped me tremendously and, maybe even more important, from a social point of view: it was really great to get to know you. Many thanks!

De leden van onze '*Wolbachia-PSR-Trichogramma*' groep waarin vooral Bertha, Astrid, Isabel, Marnix, Emmanuel, Raul, Gilsang, Fabrice, Patrick, Peter en natuurlijk Joke me echt heel veel geholpen hebben met van alles; van het verlenen van potloden tot en met het opzetten en uitvoeren van experimenten. Het was ontzettend fijn om met jullie te kunnen werken. Mijn grote dank daarvoor; many thanks et merci (sorry Fabrice, my knowledge of the beautiful French language doesn't go much further than that yet!). Gerrit, bedankt voor je statistische adviezen en het feit dat je zoveel geduld met me had. Tony en Marijke, dank voor het sequencen van diverse bacteriele- of wespengenen en de mogelijkheid om diverse microsatelliet analyses uit te voeren. Voor dat laatste was de Apple computer van Eddy ook onmisbaar. Eddy bedankt!

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Ties Huigens Wageningen, 27-03-2003

Curriculum vitae

Op 26 September 1975 werd ik, Martinus Erris Huigens, geboren in Scherpenzeel (gld). Na het behalen van mijn VWO diploma in 1994 aan het Cristelijk Lyceum te Zeist ben ik Biologie gaan studeren aan de toenmalige Landbouwuniversiteit Wageningen. Nadat ik in 1995 mijn propedeuse behaalde, koos ik voor de specialisatie Populatiebiologie. Tijdens mijn doctoraalperiode heb ik mij gericht op de evolutionaire ecologie van geslachtsmanipulerende factoren, die zeer algemeen zijn in het dierenrijk. In deze fase heb ik twee afstudeervakken en een stage bij Richard Stouthamer van het Laboratorium voor Entomologie afgerond. In mijn eerste afstudeervak keek ik samen met Mariska te Beest naar transsexualiteit bij verschillende nederlandse pissebeddensoorten dat veroorzaakt wordt door een *Wolbachia* bacterie. Hierna heb ik -samen met Bart Pannebakker, Elmer van Baal en Jeroen Witteveldt- veldonderzoek in de Mojave woestijn uitgevoerd dat mede begeleidt werd door Bob Luck van het Department of Entomology, UCR, Riverside (VS). Tijdens deze stage hebben we het -al dan niet- voorkomen bepaald van twee zgn. egoïstische genetische elementen, wederom de *Wolbachia* bacterie en een *PSR* (Paternal Sex Ratio) chromosoom, die beiden de voortplanting van hun gastheer op verschillende wijze beïnvloeden. Deze gastheren waren minuscule sluipwespen van het genus *Trichogramma*. Na mijn terugkomst in Wageningen heb ik in een volgend afstudeervak m.b.v. moleculaire technieken en kruisingsexperimenten aangetoond welke *Trichogramma* soorten het *PSR* chromosoom bevatten dat ervoor zorgt dat vrouwtjeswespen alleen nog maar zonen produceren en hoe dat chromosoom van wespensoort op wespensoort overgedragen kan worden. In November 1998 studeerde ik af.

Van januari 1999 t/m april 2003 was ik aangesteld als onderzoeker in opleiding (OIO) aan de Wageningen Universiteit bij het Laboratorium voor Entomologie. Dit project werd in de eerste vier jaar gefinancierd door NWO-ALW en in de laatste drie maanden door Wageningen Universiteit. In deze periode werkte ik onder begeleiding van Richard Stouthamer en Joop van Lenteren aan diverse evolutionaire aspecten van *Wolbachia*-geïnduceerde ongeslachtelijke voortplanting bij *Trichogramma* wespen. Tijdens dit promotieonderzoek heb ik drie keer een aantal maanden gewerkt bij het Department of Entomology, UCR, Riverside (VS) onder begeleiding van Bob Luck, om veldonderzoek te doen aan *Trichogramma* wespen in de Mojave woestijn. De resultaten van het onderzoek heeft u kunnen lezen in de hier aan voorafgaande pagina's.

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