Evolutionary interactions
between sex ratio distorters and their hosts

Promotor:	Prof. Dr. Joop C. van Lenteren
	Hoogleraar in de Entomologie
Co-promotor:	Dr. Ir. Richard Stouthamer
	Associate Professor,
	University of California, Riverside, USA
Promotiecommisie:	Prof. Dr. L. W. Beukeboom, Rijksuniversiteit Groningen
	Prof. Dr. M. Dicke, Wageningen Universiteit
	Prof. Dr. R.W. Goldbach, Wageningen Universiteit
	Prof. Dr. R.F. Hoekstra, Wageningen Universiteit

Gilsang Jeong

Evolutionary interactions between sex ratio distorters and their hosts

Proefschrift ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
Prof. Dr. Ir. L. Speelman
in het openbaar te verdedigen
op woensdag 21 januari 2004
des namiddags te vier uur in de Aula

Gilsang Jeong (2004)

Evolutionary interactions between sex ratio distorters and their hosts

Thesis Wageningen University - with references
- with summary in English, Dutch and Korean

ISBN: 90-5808-944-4

Evolutionary interactions between sex ratio distorters and their hosts

Contents

Abstract

There are many sex ratio distorters found in invertebrate taxa. Among them *Wolbachia* and PSR are found in Hymenoptera. The two sex ratio distorters are antagonistic because their pattern of inheritance favors opposite sex to each other. In this thesis interactions between *Wolbachia* and the paternal sex ratio (PSR) chromosome and their hosts genes are described.

In the *Wolbachia*-infected *Telenomus nawai* population unwillingness to mate is genetically based. *Wolbachia* can go to fixation more easily with the mutation, because this mutation drives sexual individuals to extinction in a mixed population. *Wolbachia* infection resulted in the maintenance of the mutation in the population. In the *Trichogramma*- *Wolbachia*- PSR system complex interactions are shown. The interspecific transmission of PSR does appear easy in *Trichogramma*. But the efficiency may be affected by the host genetic background, when the donor is *T. deion*. In *T. kaykai*, the natural host of PSR, *Wolbachia* and the host genes do not have any influence on the transmission of PSR. Consequently PSR is successful and is the factor that keeps the low frequencies of *Wolbachia* infection in the species. In *T. deion*, the novel host, however, the PSR transmission is interrupted by the host genes. Furthermore *Wolbachia* infection enhances the inefficient transmission of PSR in the species. This may imply that there were severe conflicts between the host genes and *Wolbachia*, and PSR in the species. *Wolbachia* may give its host an ability to detect the existence of another sex ratio distorter i.e. PSR in *T. kaykai*. Males carrying PSR unexpectedly mated more with uninfected females in the species. This may be due to the reluctance of infected females to mate with the PSR males.

The results show that there are close interactions among the host genes and *Wolbachia* and PSR and are informative in applying such sex ratio distorters to control pests.

Keywords: Hymenoptera, *Telenomus nawai*, female virginity, *Trichogramma kaykai*, *Trichogramma deion*, sex ratio distorters, *Wolbachia*, PSR, genetic conflicts

Chapter 1

General introduction

Introduction to the thesis

The study on sex determination and sex ratio of sexual organisms has been one of the important topics in evolutionary biology. During the 'Fisherian era' the control of the offspring sex ratio was studied mostly in view of the fitness of the organism's nuclear genes (Fisher, 1930). This trend has been changed by the ground-breaking work of Hamilton who recognized that selfish genetic factors might manipulate the hosts' sex ratio (Hamilton, 1967).

This study centers on the interactions between the host and its two sex ratio distorters, *Wolbachia* and the paternal sex ratio (PSR) chromosome. The introduction comprises of a brief description on sex determination, sex ratio distorters and genetic conflicts.

1. Sex determination systems in Animals

Sex promotes recombination of genes to produce genetically diverse offspring (Stearns and Hoekstra, 2000). In vertebrates and invertebrates diverse sex determination systems can be found (See Hardy (ed) for review, 2002). The common system of sex determination is genetic sex determination (GSD) (Rigaud *et al*., 1997). Mammals have the XY sex determination system in which homogametic XX is female and heterogametic XY is male (Mittwoch, 1996). Avian sex chromosomes show a reversed organization compared with those of mammals, females being heterogametic ZW and males being homogametic ZZ (Ellegren, 2000). In reptiles, amphibia, and fish both the XX/XY and the ZZ/ZW mechanisms exist (Mittwoch, 1996).

In invertebrates haplodiploid sex determination occurs in addition to the two mechanisms mentioned above (Cook, 2002; Stouthamer, 1997). Whereas eggs of most insects normally do not develop unless activated by fertilization, in the haplodiploid system males arise from unfertilized haploid eggs and females from fertilized diploid eggs (Quicke, 1997; Stouthamer, 1997). In Diptera sex determination factors are even more complex; 1) autosomal male determiner (M) in populations with indistinguishable X and Y chromosomes, 2) dominant female determiner (F^d) in populations where the autosomal M is homozygous, and 3) the

X:A ratio in which an individual with 2X:2A (ratio1) is female and an individual with 2X:1A (ratio 0.5) is male (Hodgkin, 1990; Shearman, 2002).

Sex can also be determined by environmental factors, i.e. environmental sex determination (ESD), like temperature, nutritional level, photoperiod, and mate availability, in invertebrate and vertebrate taxa (Cook, 2002; Kraak and Pen, 2002; Roberts and Thompson, 2001). This has mainly been studied in reptiles that have no sex chromosomes, their sex is determined after fertilization (Mittwoch, 1996; Valenzuela *et al*., 2003). Furthermore sex determination is influenced by both GSD and ESD in the turtle, *Emys orbicularis* (Girondot *et al*., 1994) and in the reptile, *Peociliopsis lucida* (Sullivan and Schultz, 1986).

These sex determination systems are not limited to particular taxa, but similar sex determination systems occur in unrelated taxa. This incongruence between the sex determination system and phylogeny implies that particular sex determination systems have independently evolved several times. Sex determination systems appear to have substantial evolutionary plasticity (Marín and Baker, 1998). The view that sex determining mechanisms have been reinvented many times during animal evolution (Koopman and Loffler, 2003) is reinforced by several experiments. The male-determining gene, dmrt1, in medaka fish is not found in closely related fish species (Kondo *et al*., 2003). New stable sex determining systems can be generated by artificial manipulation of sex regulatory genes (Zarkower, 2001). Recent detection of the autosomal sex determination in an isopod species previously having ZW sex determination is also evidence of evolutionary flexibility of sex determination (Rigaud, 1997). Some genes, however, seem to be very conserved across taxa (Marín and Baker, 1998; Raymond *et al*., 1998). The sex determination mechanism is an important constraint on the potential sex ratio evolution of an organism (Antolin, 1999; West *et al*., 2002), and conversely sex ratio evolution plays an important role in the evolution of sex determination mechanisms (Kraak and Pen, 2002).

2. Sex ratio

Sex ratio theory started with Fisher's prediction of the sex ratio of 1:1, where the same amount of resources was invested to both sexes (Fisher, 1930). Consider a

population with female biased sex ratio, then males will gain advantage, on the other hand in a male biased population, the fitness of females will be greater. Consequently, the evolutionary stable strategy (ESS) is to invest equally in male and female offspring by individuals (Godfray and Werren, 1996; West and Herre, 2002).

Hamilton's theory (1967) opened the new era of sex ratio study (Godfray and Shimada, 1999). In a spatially structured population, where mating occurs within a small group before females disperse, a female biased sex ratio is favored (Hamilton, 1967). It is one of the general models to explain the evolution of biased sex ratio (West and Herre, 2002). He proposed three factors as the cause of sex ratio bias i.e. local mate competition (LMC), sib-mating, and inbreeding (Hamilton, 1967). Among them LMC has the largest effect (Taylor, 1981).

The most interesting sex ratio patterns have been found in insects especially the Hymenoptera (ants, bees and wasps), because sex of the offspring can be allocated by the mother (Stouthamer *et al*., 1999; West *et al*., 2000). On the other hand vertebrates do not show sex ratio skews probably due to the complexities of chromosomal sex determination (CSD) and life histories (Pen and Weissing, 2002). But recent studies suggest that even the taxa with CSD, i.e. vertebrates, can adjust offspring sex ratio (West *et al*., 2002). For example the offspring sex ratio of red deer depends on social hierarchy and population density (Clutton-Brock *et al*., 1984; Kruuk *et al*., 1999). In Seychelles warbler the sex ratio changes with the quality of the territory (Komdeur, 1996). In the viviparous lizard, *Eulamprus tympanum*, the sex of its offspring is determined by active regulation of body temperature (Robert and Thompson, 2001).

The offspring sex ratio can also be skewed by unusual nuclear-chromosomal and cytoplasmic factors (Hurst *et al*., 1997; Jaenike, 2001; Kelly *et al*., 2001; Stouthamer, 1997; Stouthamer *et al*., 2002; Werren and Stouthamer, 2003).

3-1 Chromosomal sex ratio distortion factors (XO, X*X) - in Wood lemming and *Drosophila*

Only one case of chromosomal sex ratio distortion is known in vertebrates (Cockburn *et al*., 2002). In wood lemmings, the expression of Y chromosome is

suppressed by a driving X chromosome (X*). Consequently the population carrying X* is heavily female biased, because an X*Y individual becomes a female instead of a male (Fredga *et al*., 1977; Jaenike, 2001; Marín and Baker, 1998).

In meiotic drive the meiosis is manipulated causing one of a pair of heterozygous alleles or heteromorphic chromosomes to be transmitted to progeny in excess of the expected Mendelian proportion of 50% (Lyttle, 1991). Meiotic drive by sex chromosomes is common and mainly known from *Drosophila* (Jaenike, 2001; Stouthamer *et al*., 2002; Werren and Hurst, 2001). Such driving sex chromosomes distort the sex ratio (Hamilton, 1967), modify mating behavior and sexual selection, cause intragenomic conflicts (Hurst *et al*., 1996; Jaenike, 2001; Werren and Beukeboom, 1998) and can eventually lead population to extinction (Presgraves *et al*., 1997). Another extreme example is the paternal sex ratio (PSR) chromosome in the haplodiploid sex determination system (Stouthamer *et al*., 2001; Werren *et al*., 1987; Werren and Stouthamer, 2003).

3-1-1. Paternal sex ratio (PSR) chromosome

PSR is a B-chromosome that is an extremely selfish genetic element and only occurs in males (Beukeboom and Werren, 2000; Nur *et al*., 1988; Stouthamer *et al*., 2001; Werren, 1991; Werren and Stouthamer, 2003). PSR has been found in *Nasonia* and *Trichogramma* (Hymenoptera) (Nur *et al*., 1988; Werren *et al*., 1987; Stouthamer *et al*., 2001). In the hosts a fertilized egg, which would normally develop into a female, develops into a male carrying PSR, because PSR destroys the paternal chromosome set after fertilization (Werren and Stouthamer, 2003) (Table 1a, b). PSR-like factors can only exist in haplodiploid sex determination, even if the paternal chromosomes are destroyed, the fertilized egg can develop into a haploid male carrying PSR. The evolutionary origins of the B-chromosomes in *Nasonia* and *Trichogramma* are independent (Werren and Stouthamer, 2003), but their mode of action is very similar (Reed and Werren, 1995; van Vugt *et al*., 2003). With the mode of action characteristic to PSR, populations carrying PSR can go to extinction if the PSR factor leads to a sufficient suppression of the population growth rate. PSR is considered as a

biological control agent for haplodiploid pests and a vehicle for genetic engineering (Werren and Stouthamer, 2003).

Table 1a. Paternal genome loss (PGL) induced by PSR in *Nasonia*

Crosses	virgin	$Unpsr_{♂}$		$Inpsr_{♂}$	
	U	F	U	F	U
$Un_{♀}$	$Un_{♂}$	$Unpsr_{♂}$	$Un_{♂}$	$Un_{♂}^{*}$	$Un_{♂}$
$In_{♀}$	$In_{♂}$	$Inpsr_{♂}$	$In_{♂}$	$Inpsr_{♂}$	$In_{♂}$

U: unfertilized; F: fertilized; Un: uninfected; In: infected; Unpsr: uninfected PSR; Inpsr: infected PSR; *: normal male offspring production by cytoplasmic incompatibility

Table 1b. General scheme of paternal genome loss (PGL) induced by PSR in *Trichogramma*

Crosses	virgin	$Unpsr_{♂}$		$Inpsr_{♂}$	
	U	F	U	F	U
$Un_{♀}$	$Un_{♂}$	$Unpsr_{♂}$	$Un_{♂}$	$Unpsr_{♂}$	$Un_{♂}$
$In_{♀}$	$In_{♀}$	$Inpsr_{♂}$	$In_{♀}$	$Inpsr_{♂}$	$In_{♀}$

U: unfertilized; F: fertilized; Un: uninfected; In: infected; Unpsr: uninfected PSR; Inpsr: infected PSR

3-2. Extrachromosomal sex ratio distortion factors

A variety of cytoplasmically inherited microorganisms is known (Bourtzis and Miller (eds), 2003; O'Neill *et al*. (eds), 1997; Stouthamer *et al*., 1999). Some of the organisms distort the sex ratio of their hosts in favor of their own transmission by causing parthenogenesis, male killing or feminization (Gherna *et al*., 1991; Hurst *et al*., 1997; Rigaud, 1997; Stouthamer, 1997; Weeks and Breeuwer, 2003). Examples are *Wolbachia* bacteria (Stouthamer *et al*., 1993), Cytophaga-Flexibacterium-Bacteroides (CFB) (Zchori-Fein *et al*., 2001; Weeks and Breeuwer, 2003; Weeks *et al*., 2003), *Entrics* and relatives, γ-Proteobacteria (Werren *et al*., 1986; Gherna *et al*., 1991), *Microsporidia* (Hurst *et al*., 1997), *Nosema granulosis* (Kelly *et al*., 2002), *Rickettsia* (Werren *et al*., 1994), *Rickettsia tsutsugammushi* (Roberts *et al*., 1977) and spirochetes (Poulson and Sakaguchi, 1961). But this list is almost certainly only the tip of the iceberg of symbionts and parasites that affect their hosts' sex ratio. Among them *Wolbachia* bacteria have drawn much attention because of their ability to manipulate the

host's sex ratio in several ways (O'Neill *et al*. (eds), 1997; Stouthamer *et al*., 1999).

3-2-1. *Wolbachia* bacteria and their effects on hosts

Wolbachia bacteria are cytoplasmic α-proteobacterial endosymbionts of a variety of arthropods such as insects, mites and isopods, and also of filarial nematodes (Bandi *et al*., 2001; Stouthamer *et al*., 1999; Weeks and Breeuwer, 2001). They were first described from the ovaries of the mosquito species, *Culex pipiens*, in the 1920's (Hertig and Wolbach, 1924). *Wolbachia* was long considered just another cytoplasmic bacterium until it was discovered that incompatibility between strains of the mosquito, *C. pipiens*, was caused by the bacterium (Yen and Barr, 1971). The discovery of the ability of the bacteria to alter their hosts' sex ratio is one of the landmarks in entomology in the 20th century (Chapman, 2000). *Wolbachia* bacteria are composed of 5 subgroups (Lo *et al*., 2002). Subgroup A and B are found in arthropods, whereas C and D are found in filiarial nematodes (Bandi *et al*., 2001; Lo *et al*., 2002). Recently group E was reported in *Folsomia candida* (Colembola) (Vandekerchove *et al*., 1999). Even though the bacteria are mainly vertically transmitted, the distribution of *Wolbachia* strains and the phylogeny of their hosts are not congruent (Vavre *et al*., 1999). Phylogenetic studies suggest that the bacteria infect their hosts after their hosts diverged (Ishikawa, 2002; Stouthamer *et al*., 1999; Werren *et al*., 1995). Frequent horizontal transmission has occurred in nature on an evolutionary time scale (Schilthuizen and Stouthamer, 1997; Stouthamer *et al*., 1999; Vavre *et al*., 1999; Werren 1997; Werren *et al*., 1995). It has been experimentally confirmed both intraspecifically and interspecifically (Heath *et al*., 1999; Huigens *et al*., 2000; Huigens, 2003). *Wolbachia* infection induces several sex ratio distortions in their hosts (O'Neill *et al*. (eds), 1997; Stouthamer *et al*., 1999).

1) Cytoplasmic incompatibility

The most common effect of the *Wolbachia* infection is cytoplasmic incompatibility (CI). It was first experimentally described in *Culex pipiens* (Yen

and Barr, 1973). The effect is most extensively studied in *Drosophila* (Diptera: Drosophildae) species, in *Nasonia* (Hymenoptera: Pteromalidae) species and to a lesser extent in many other insect species (Hoffmann and Turelli, 1997). When an uninfected female mates with an infected male, the paternal chromosomes are degenerated in the first mitotic division of fertilized eggs. Consequently, the fertilized eggs either die (in diplodiploids and some haplodiploids) or develop into males (in haplodiploids) (Hoffmann and Turelli, 1997; Stouthamer *et al*., 1999; Vavre *et al*., 2000; Werren, 1997) (Table 2a, b). All the other combinations of mating lead to normal development of the fertilized eggs. The phenomenon can also be observed, when the participants of a mating are infected with different strains of the bacteria. This is called bi-directional incompatibility (O'Neill and Karr, 1990; Perrot-Minnot *et al*., 1996) (Table 2a, b).

Table 2a. Cytoplasmic incompatibility induced by *Wolbachia* infection in diplodiploid organisms

Crosses	$Un_{♂}$	$In_{♂}^{a}$	$In_{♂}^{b}$	$In_{♂}^{ab}$
$Un_{♀}$	$Un_{♀}$, $Un_{♂}$	×	×	×
$In_{♀}^{a}$	$In_{♀}^{a}$, $In_{♂}^{a}$	$In_{♀}^{a}$, $In_{♂}^{a}$	×	×
$In_{♀}^{b}$	$In_{♀}^{b}$, $In_{♂}^{b}$	×	$In_{♀}^{b}$, $In_{♂}^{b}$	×
$In_{♀}^{ab}$	$In_{♀}^{ab}$, $In_{♂}^{ab}$	$In_{♀}^{ab}$, $In_{♂}^{ab}$	$In_{♀}^{ab}$, $In_{♂}^{ab}$	$In_{♀}^{ab}$, $In_{♂}^{ab}$

Un: uninfected; In: infected; $^{a, b}$: *Wolbachia* strain; ×: death of offspring

Table 2b. Cytoplasmic incompatibility in haplodiploid organisms

Crosses	Virgin	$Un_{♂}$		$In_{♂}^{a}$		$In_{♂}^{b}$		$In_{♂}^{ab}$	
	U	F	U	F	U	F	U	F	U
$Un_{♀}$	$Un_{♂}$	$Un_{♀}$	$Un_{♂}$	×	$Un_{♂}$	×	$Un_{♂}$	×	$Un_{♂}$
$In_{♀}^{a}$	$In_{♂}^{a}$	$In_{♀}^{a}$	$In_{♂}^{a}$	$In_{♀}^{a}$	$In_{♂}^{a}$	×	$In_{♂}^{a}$	×	$In_{♂}^{a}$
$In_{♀}^{b}$	$In_{♂}^{b}$	$In_{♀}^{b}$	$In_{♂}^{b}$	×	$In_{♂}^{b}$	$In_{♀}^{b}$	$In_{♂}^{b}$	×	$In_{♂}^{b}$
$In_{♀}^{ab}$	$In_{♂}^{ab}$	$In_{♀}^{ab}$	$In_{♂}^{ab}$	$In_{♀}^{ab}$	$In_{♂}^{ab}$	$In_{♀}^{ab}$	$In_{♂}^{ab}$	$In_{♀}^{ab}$	$In_{♂}^{ab}$

U: unfertilized; F: fertilized; Un: uninfected; In: infected; $^{a, b}$: *Wolbachia* strain; ×: death of offspring

CI has been explained with a modification/rescue (mod/res) model (Werren, 1997). According to the model sperm of infected males are modified (mod+), and eggs of infected females rescue (res+) the sperm to participate in the

fertilization. But eggs of uninfected females can not rescue (res-) the *Wolbachia* imprinted sperm. Mod+/res+, mod-/res+ and mod-/res- types have been found (Bourtzis *et al*., 1998; Werren, 1997). CI is induced when *Wolbachia* delay the nuclear envelop breakdown of sperm in a fertilized egg in *Nasonia* (Tram and Sullivan, 2001). The two models are theoretical and mechanistic ways of explaining CI. The bidirectional CI is not explained by the delayed nuclear envelope breakdown. Future study should address why two individuals infected with different strains of CI inducing *Wolbachia* are not compatible in mating. Cytoplasmic incompatibility (CI) is thought to be the primitive form of manipulation, and the other sex ratio distortions, i.e. feminization, male killing and parthenogenesis, seem to be the derived forms (Hurst *et al*., 2002; Stouthamer *et al*., 1999; Vavre *et al*., 2000).

2) Feminization

This has been discovered in taxa having a female heterogametic sex determination system e.g. terrestrial isopods of the genus *Armadillidium* (Rigaud, 1997), the lepidopteran insects *Eurema hecabe* (Hiroki *et al*., 2002) and *Ostrinia furnacalis* (Kageyama *et al*., 2002). In the *Armadillidium* model system the genetic male (ZZ) develops into a functional female. The infected ZW or ZZ genotype female can mate with a normal uninfected ZZ male and produce infected female offspring (Table 3).

Table 3. Feminization induced by *Wolbachia* infection in Crustacea

Crosses	$Un_{♂}ZZ$
$Un_{♀}ZW$	$Un_{♀}ZW$, $Un_{♂}ZZ$
$In_{♀}ZW$	$In_{♀}ZW$, $In_{♀}ZZ$
$In_{♀}ZZ$	$In_{♀}ZZ$

Un: uninfected; In: infected; zw, zz: sex chromosome

Female transsexuality is achieved by blocking the target organ of the androgenic hormone or by reducing the production of this hormone (Le Grand *et al*., 1987). Recent evolution of autosomal sex determination was detected as a consequence

of the Feminizing *Wolbachia* in the terrestrial isopod, *Armadillidium vulgare* (Juchault and Mocquard, 1993).

3) Male killing

Infected eggs destined to be males die during their development in several species of Coleoptera, Lepidoptera (Hurst *et al*., 1999) (Table 4). *Wolbachia* infecting females gains benefit by the altruistic suicide effect of *Wolbachia* in males. There are two types of male killer i.e. early and late male killer. *Wolbachia* are early male killers (Hurst *et al*., 1997). Their molecular mechanisms remain to be investigated. Hurst *et al*. (1997) summarized several advantages of male killing; 1) female survivorship is higher, 2) inbreeding depression can be avoided, 3) females can get more resources, and 4) females can evade the possible antagonistic interactions between siblings.

Table 4. Male killing induced by *Wolbachia* infection

Crosses	$Un_{♂}$
$Un_{♀}$	$Un_{♀}$, $Un_{♂}$
$In_{♀}$	$In_{♀}$, ×

Un: uninfected; In: infected; ×: death of male offspring

A recent finding of sexual selection in response to a very high incidence of male killers in a butterfly species is interesting. Usually in mating males compete for females, but because of scarcity of males in infected populations, the reversed sex role is detected in the butterfly, *Acraea encedon* (Jiggins *et al*., 2000).

4) Parthenogenesis

In the early 90's it was demonstrated that parthenogenesis in two *Trichogramma* species was caused by cytoplasmic microorganisms (Stouthamer and Luck, 1993; Stouthamer *et al*., 1990). These microorganisms were identified as *Wolbachia* bacteria by using molecular methods (Stouthamer *et al*., 1993). Parthenogenesis inducing (PI) *Wolbachia* are exclusively found in taxa having a haplodiploid sex determination system: i.e. a female develops from a fertilized diploid egg and a male from an unfertilized egg (Arakaki *et al*., 2001; Stouthamer, 1997). Recently

female production of virgin *Bryobia praetiosa* mites, proved also to be caused by *Wolbachia* (Weeks and Breeuwer, 2001).

PI-*Wolbachia* have evolved several ways to restore diploidy in order to feminize infected eggs in their hosts. This is achieved by gamete duplication in the first mitotic division in *Trichogramma* (Stouthamer and Kazmer, 1994) and in *Leptopilina clavipes* (Pannebakker *et al*., in press). In *Muscidifurax uniraptor* nuclear fusion is found to restore diploidy in which the two haploid nuclei of the first mitotic division fuse to form a single diploid nucleus (Gottlieb *et al*., 2002). It is not yet determined what mechanism acts in the mite, *B. praetiosa*, but the authors hypothesize either doubling before meiosis or gamete duplication as the mechanism (Weeks and Breeuwer, 2001).

PI-*Wolbachia* infected *Trichogramma* are able to make use of sperm when infected females mate (Stouthamer and Kazmer, 1994) (Table 5a). Consequently, a sexual line can be established from an asexual line when treated with antibiotics or high temperature (Stouthamer *et al*., 1990). In contrast to *Trichogramma*, it has not been possible to establish a sexual line from all the other PI-*Wolbachia* infected taxa such as *Apoanagyrus diversicornis* (Pijls *et al*., 1995), *Bryobia praetiosa* (Weeks and Breeuwer, 2001), *Eretmocerus mundus* (de Barro and Hart, 2001), *Franklinothrips vespiformis* (Arakaki *et al*., 2001), *Muscidifurax uniraptor* (Zchori-Fein *et al*., 2000), and *Telenomus nawai* (Arakaki *et al*., 2000) (Table 5b). A recent hypothesis may explain why (Huigens and Stouthamer, 2003). When a population is extremely female biased, a mutation that causes a male biased sex ratio will gain a selective advantage. At individual level male biased sex ratio can be achieved by low fertilization or non-mating. The mutation will paradoxically spread into populations as long as females are willing to mate. This mutation may drive sexual individuals to extinction in a mixed population, resulting in a population fixed for the PI-*Wolbachia* infection.

Table 5a. Parthenogenesis induced by *Wolbachia* infection in *Trichogramma*

Crosses	Virgin	$Un_{♂b}$	
	U	F	U
$Un_{♀a}$	$Un_{♂a}$	$Un_{♀ab}$	$Un_{♂a}$
$In_{♀a}$	$In_{♀a}$	$In_{♀ab}$	$In_{♀a}$

U: unfertilized; F: fertilized; Un: uninfected; In: infected; $_{a, b}$: host genotype

Table 5b. Parthenogenesis in other organisms than *Trichogramma*

Crosses	Virgin	$Un_{♂b}$	
	U	F	U
$Un_{♀a}$	$Un_{♂a}$	$Un_{♀ab}$	$Un_{♂a}$
$In_{♀a}$	$In_{♀a}$		$In_{♀a}$

U: unfertilized; F: fertilized; Un: uninfected; In: infected; $_{a, b}$: host genotype

3-2-2. Effects of *Wolbachia* infection other than sex ratio distortion

Recent studies found several novel effects of *Wolbachia* infection most likely specific to each host species. In *Drosophila melanogaster* the *Wolbachia* infection rescues defects in oogenesis caused by mutation on *Sex-lethal* (*Sxl*) (Starr and Cline, 2002). A strain of *Wolbachia* is required for oogenesis in the host, *Asobara tabida* (Dedeine *et al*., 2001). The *Popcorn* strain of *Wolbachia* caused early death of the infected hosts instead of inducing sex ratio distortion (Min and Benzer, 1997; but see Reynolds *et al*., 2003). In nematodes, *Wolbachia* is essential for oogenesis (Bandi *et al*., 1999; Bandi *et al*., 2001; Hoerauf *et al*., 1999; Langworthy *et al*., 2000). Even more striking is that response of the immune system of humans to the *Wolbachia* mutualists of nematodes is what causes people to go blind in river blindness (André *et al*., 2001). It may mean that the strategy for controlling the disease can be changed to something as simple as treating the patients with antibiotics.

Symbionts increasing the fitness of infected females are selected for (Turelli, 1994). Increase of offspring production is another way to enhance the transmission of the symbionts (Stouthamer *et al*., 1999). Cured *Trichogramma bourarachae* females produced half offspring of infected ones (Girin and Boulétreau, 1995). In a strain of *Nasonia vitripennis*, *Wolbachia* infection increased fecundity (Stolk and Stouthamer, 1996; but see Bordenstein and Werren, 2000). In the coleopteran insect, *Tribolium confusum*, sperm from

infected males have a fertility advantage (Wade and Chang, 1995). *Wolbachia* may maintain male fertility in the Stalk-eyed fly, *Sphyracephala beccarii* (Hariri *et al*., 1998). All of the data indicate that there are close interactions between hosts and the symbionts.

4. Genetic conflict

The idea that factors with uniparental inheritance have a potential to evoke genetic conflict is largely synthesized from the work by Cosmides and Tooby (1981). Those factors are defined as selfish genetic elements that enhance their transmission at the expense of their hosts or hosts' genes (Werren *et al*., 1988). They consist of cytoplasmically inherited bacteria, meiotic drive chromosomes, supernumerary chromosomes, transposons (Werren and Beukeboom, 1998; Cosmides and Tooby, 1981). There are two prerequisites for genetic conflicts. First individual genetic elements are the fundamental unit of selection. Second some genetic elements can be selfish or parasitic (Werren and Beukeboom, 1998).

Genetic conflicts can be subdivided into three major types (Rice and Chippindale, 2001).

1) Intragenomic conflicts occur between genes located in the same individual. For example nuclear-cytoplasmic conflicts arise because cytoplasmic factors are only maternally transmitted whereas nuclear factors are transmitted via both parents (Hurst *et al*., 1996; Werren and Beukeboom, 1998). Selection on cytoplasmically inherited factors favors female biased sex ratio, while nuclear factors favor balanced sex ratio (Hurst *et al*., 1996).
2) Intergenomic conflicts occur between different individuals by genes located at different loci causing competition. For example proteins in the seminal fluids of *Drosophila melanogaster* increasing fertilization success of a male decrease the longevity of the female mated with the male (Chapman *et al*., 1995).
3) Intersexual ontogenetic conflict arises when alleles in both sexes are differentially expressed between the sexes (i.e. sexually antagonistic alleles). For example rapid adaptation of evolved males results in high mortality of

experimentally less evolved females in *Drosophila melanogaster* (Rice, 1996).

Conflicts mainly appear to be the battle between sexes. But because of differential transmission or expression of genes in different sexes, genes are the subjects of the conflict.

The possible consequences of conflicts they cause are: eucaryotic speciation, extinction and the structural change of genetic systems and deviation of social behavior (Hurst and Schilthuizen, 1998; Hurst and Werren, 2001; Keller and Ross, 1998; Rice, 1996).

5. Structure of this thesis

The above mentioned two sex ratio distorters, *Wolbachia* and PSR, and their hosts may individually or collectively be an evolutionary force on each other by evoking conflicts. Therefore, the systems with which sex ratio distorters are associated provide opportunities to test various assumptions.

Telenomus nawai is an egg parasitoid that is infected with PI-*Wolbachia*. The genetic basis of why we cannot establish a sexual line from the infected line is determined. In *Trichogramma kaykai* and *T. deion* both PI-*Wolbachia* infected and uninfected individuals co-occur. Huigens (2003) tried to explain why the infection has not gone to fixation in these species. One of the factors that suppress the fixation of *Wolbachia* in *T. kaykai* is PSR (Huigens, 2003). The research aim of this thesis is to investigate possible interactions between PI-*Wolbachia*, PSR and their hosts.

Chapter 1. I briefly review relevant information on sex determination systems of animals and their sex ratios. Furthermore the factors that distort sex ratios and possible conflicts among the hosts and the sex ratio distorting factors are discussed.

Chapter 2. Up to now, with the exception of some *Trichogramma* species, there is no evidence that female wasps infected with PI-*Wolbachia* use sperm when they mate with uninfected conspecific males. A recent hypothesis of Huigens

and Stouthamer (2003) explains the lack of ability to reproduce sexually of these infected females by assuming that a mutant that interferes with sexual reproduction is not simply a result of neutral accumulation of mutations in these traits in completely infected populations, but that during the process of a spreading PI- *Wolbachia* infection in the population this mutant had adaptive value. I experimentally demonstrate the existence of such mutant genes in a parasitic wasp. I discuss how the *Wolbachia* bacteria become essential to their host to maintain the host population.

Chapter 3. The paternal sex ratio (PSR) chromosome is thought to be limited to haplodiploid organisms. I investigated the effect of the two distinct postzygotic reproductive isolation mechanisms on the interspecific transmission of PSR in *Trichogramma*. I further tested the level of reproductive isolation between *T. kaykai* and *T. deion*, and whether *Wolbachia* infecting the males carrying PSR affects the interspecific transmission.

Chapter 4. The genus *Trichogramma* harbours two sex ratio distorters. PI-*Wolbachia* bacteria and PSR are antagonistic in terms of their different transmission strategies. Different from other nuclear genes favoring balanced sex ratio, PSR drives a population to a male biased sex ratio and *Wolbachia* induce parthenogenesis resulting in all-female broods in *Trichogramma*. In this evolutionary context genetic conflict may occur between the two sex ratio distorters. I tested the assumption by comparing the transmission efficiency of PSR in the natural host, *T. kaykai*, and in the novel host, *T. deion*.

Chapter 5. *Trichogramma deion* and *T. kaykai* are very closely related and sympatric, but PSR has only been found in *T. kaykai* populations in the field. Following the results of chapter 4 I investigated the effect of the host's genetic background and the relationship of *Wolbachia* on the transmission efficiency of PSR in *T. deion*.

Chapter 6. Preference in mating is largely dependent on phenotypes. I tested whether *Wolbachia* infection and harboring PSR had an influence on mating frequencies between males and females with different infection and/or PSR status.

Chapter 7. I discuss the findings of the studies, i.e. genetic basis of sexual bahavior and sex ratio distorter dependent life (Chapter 2), the relationship between two types of reproductive isolation and the interspecific transmission of PSR (Chapter 3), comparative study of the transmission efficiency of PSR in two closely related *Trichogramma* species (Chapter 4), the factor limiting the maintenance of PSR in *T. deion* (Chapter 5), and change of mating preference caused by a sex ratio distorter (Chapter 6). Finally, future studies on *Wolbachia* and PSR are suggested in this chapter.

Acknowledgements

Many thanks are given to Mohammad Ardeh, Ties Huigens, Emmanuel Niyibigira, and Joke van Vugt for discussion on the subjects. I am grateful to Isabel Silva, Richard Stouthamer and Joop van Lenteren for help to improve this chapter.

References

André A.S., Blackwell N.M., Hall L.R., Hoerauf A., Brattig N.W., Volkmann L., Taylor M.J., Ford L., Hise A.G., Lass J.H., Diaconu E., and Pearlman E., 2001, the role of endosymbiotic *Wolbachia* bacteria in the pathogenesis of river blindness. *Science*. **295**:1892-1895.

Antolin M.F., 1999, A genetic perspective on mating systems and sex ratios of parasitoid wasps. *Res. Popul. Ecol*. **41**:29-37.

Arakaki N., Noda H., and Yamagishi K., 2000, *Wolbachia*-induced parthenogenesis in the egg parasitoid *Telenomus nawai*. *Entomol. Exp. Appl*. **96**:177-184.

Arakaki N., Miyoshi T., and Noda H., 2001, *Wolbachia*-mediated parthenogenesis in the predatory thrips *Franklinothrips vespiformis* (Thysanoptera: Insecta). *Proc. R. Soc. Lond. B*. **268**:1011-1016.

Bandi C., Anderson T.J.C., Genchi C., and Blaxter M.L., 2001, The *Wolbachia* endosymbionts of filarial nematodes. In Kennedy M.W., and Harnett W. (eds), '*Parasitic nematodes*'. CAB International: Oxon

Bandi C., McCall J.W., Genchi C., Corona S., Venco L., and Sacchi L., 1999, Effects of tetracycline on the filarial worm *Brugia pahangi* and *Dirofilaria immitis* and their bacterial endosymbionts *Wolbachia*. *Int. J. Parasitol*. **29**:357–364.

Bandi C., Trees A.J., and Brattig N., 2001, *Wolbachia* in filarial nematodes: evolutionary aspects and implications for the pathogenesis and treatment of filarial diseases. *Vet. Parasitol*. **98**:215-238.

Beukeboom L.W., and Werren J.H., 2000, The paternal-sex-ratio (PSR) chromosome in natural populations of *Nasonia* (Hymenoptera: Chalcidoidea). *J. Evol. Biol*. **13**:967-975.

Bordenstein S.R., and Werren J.H., 2000, Do *Wolbachia* influence fecundity in *Nasonia vitripennis*? *Heredity*. **84**:54-62.

Bourtzis K., Dobson S.L., Braig H.R., and O'Neill S.L., 1998, Rescuing *Wolbachia* have been overlooked. *Nature*. **392**:852-853.

Bourtzis K., and Miller T.A. (eds), 2003, '*Insect symbiosis*'. CRC Press: Boca Raton.

Chapman R.F., 2000, Entomology in the twentieth century. *Annu. Rev. Entomol*. **45**:261-285.

Chapman T., Liddle L.F., Kalb J.M., Wolfner M.F., and Partridge L., 1995, Cost of mating in *Drosophila melanogaster* females is mediated by male accessory gland products. *Nature*. **373**:241-244.

Clutton-Brock T.H., Albon S.D., and Guinness F.E., 1984, Maternal dominance, breeding success and birth sex ratios in red deer. *Nature*. **308**:358-360.

Cockburn A., Legge S., and Double M.C., 2002, Sex ratios in birds and mammals. In: Hardy I.C.W. (ed), '*Sex ratios: Concepts and research methods*'. Cambridge University Press: Cambridge. pp266-286.

Cook J.M., 2002, Sex-determination in invertebrates. In Hardy I.C.W. (ed), '*Sex ratios: Concepts and research methods*'. Cambridge University Press: Cambridge. pp178-194.

Cosmides L.M., and Tooby J., 1981, Cytoplasmic inheritance and intragenomic conflict. *J. Theor. Biol*. **89**:83-129.

De Barro P.J., and Hart P.J., 2001, Antibiotic curing of parthenogenesis in *Eretmocerus mundus* (Australian parthenogenetic form). *Entomol. Exp. Appl*. **99**:225-230.

Dedeine F., Vavre F., Fleury F., Loppin B., Hochberg M., and Boulétreau M., 2001, Removing symbiotic *Wolbachia* bacteria specifically inhibits oogenesis in a parasitic wasp. *Proc. Nat. Acad. Sci. USA*. **98**:6247-6252.

Ellegren H., 2000, Evolution of the avian sex chromosomes and their role in sex determination. *Trends Ecol. Evol*. **15**(5):188-192.

Fisher R.A., 1930, *The genetical theory of natural selection*. Oxford University Press. Oxford.

Fredga K., Gropp A., Winking H., and Frank F., 1977, A hypothesis explaining the exceptional sex ratio in the wood lemming (*Myopus schisticolor*). *Hereditas*. **85**:101-104.

Gherna R.L., Werren J.H., Weisburg W., Cote R., Woese C.R., Mandelco L., and Brenner D.J., 1991, *Arsenophonus nasoniae* gen. nov., sp. nov., the causative agent of the son killer trait in the parasitic wasp *Nasonia vitripennis*. *Int. J. Syst. Bacteriol*. **41**(4): 563-565.

Girin C., and Boulétreau M., 1995, Microorganism-associated variation in host infestation efficiency in a parasitoid wasp *Trichogramma bourarachae*. *Experientia*. **52**:398-402.

Girondot M., Zaborski P., Servan J., and Pieau C., 1994, Genetic contribution to sex determination in turtles with environmental sex determination. *Genet. Res*. **63**:117-127.

Godfray H.C.J., and Shimada M., 1999, Parasitoids as model organisms for ecologists. *Res. Popul. Ecol*. **41**:3-10.

Godfray H.C.J., and Werren J.H., 1996, Recent developments in sex ratio studies. *Trends Ecol. Evol*. **11**(2):59-63.

Gottlieb Y., Zchori-Fein E., Werren J.H., and Karr T.L., 2002, Diploidy restoration in *Wolbachia*-infected *Muscidifurax uniraptor* (Hymenoptera: Pteromalidae). *J. Invertebr. Pathol*. **81**:166-174.

Hamilton W.D., 1967, Extraordinary sex ratios. *Science*. **156**:477-488.

Hardy I.C.W., 2002, '*Sex ratios: Concepts and research methods*'. Cambridge University Press: Cambridge.

Hariri A.R., Werren J.H., and Wilkinson G.S., 1998, Distribution and reproductive effects of *Wolbachia* in stalk-eyed flies (Diptera: Diopsidae). *Heredity*. **81**:254-260.

Heath B.D., Butcher R.D.J., Whitfield W.G.F., and Hubbard S.F., 1999, Horizontal transfer of *Wolbachia* between phylogenetically distant insect species by a naturally occurring mechanism. *Curr. Biol*. **9**:313-316.

Hertig M., and Wolbach S.B., 1924, Studies on rickettsia-like microorganisms in insects. *J. Med. Res*. **44**:329-374.

Hiroki M., Kato Y., Kamito T., and Miura K., 2002, Feminization of genetic males by a symbiotic bacterium in a butterfly, *Eurema hacabe* (Lepidoptera: Pieridae). *Naturwissenschaften*. **89**:167-170.

Hodgkin J., 1990, Sex determination compared in *Drosophila* and *Caenorhabditis*. *Nature*. **344**:721-728.

Hoerauf A., Nissen-Paehle K., Schmetz C. Henkle-Dürsen K., Blaxter M.L., Büttner D.W., Gallin M.Y., Al-Qaoud K.M., Lucius R., Fleischer B., 1999, Tetracycline therapy targets intracellular bacteria in the filarial nematode *Litomosoides sigmodontis* and results in filarial infertility. *J. Clin. Invest*. **103**(1):11-17.

Hoffman A.A., and Turelli M., 1997, Cytoplasmic incompatibility in insects. In: O'Neill S.L., Hoffmann A.A., and Werren J.H. (eds), '*Influential Passengers-Inherited microorganisms and arthropod reproduction*'. Oxford University Press: Oxford. pp42-80.

Huigens M.E., 2003, *On the evolution of* Wolbachia*-induced parthenogenesis in* Trichogramma *wasps*. Ph.D. thesis. Wageningen University.

Huigens M.E., Luck R.F., Klaassen R.H.G., Maas M.F.P.M., Timmermans M.J.T.N., and Stouthamer R., 2000, Infectious parthenogenesis. *Nature*. **405**:178-179.

Huigens M.E., and Stouthamer R., 2003, Parthenogenesis associated with *Wolbachia*. In: Bourtzis K., and Miller T.A. (eds), '*Insect symbiosis'*. CRC Press: Boca Raton. pp247-266.

Hurst L.D., Atlan A., and Bengtsson B.O., 1996, Genetic conflict, *Q. Rev. Biol*.**71**:317-364.

Hurst G.D.D., Hurst L.D., and Majerus M.E., 1997, Cytoplasmic sex-ratio distorters. In: O'Neill S.L., Hoffmann A.A., and Werren J.H. (eds), '*Influential Passengers-Inherited microorganisms and arthropod reproduction'*. Oxford University Press: Oxford. pp125-154.

Hurst G.D.D., Jiggins F.M., and Pomiankowski A., 2002, Which way to manipulate host reproduction? *Wolbachia* that cause cytoplasmic incompatibility are easily invaded by sex ratio-distorting mutants. *Am. Nat*. **160**:360-373.

Hurst G.D.D., Jiggins F.M., von der Schulenburg J.H., Bertrand D., West S., Goriacheva I.I., Zakharov I.A., Werren J.H., Stouthamer R., and Majerus M.E.N., 1999, Male-killing *Wolbachia* in two species of insect. *Proc. R. Soc. Lond. B*. **266**:735-740.

Hurst G.D.D., and Schilthuizen M., 1998, Selfish genetic elements and speciation. *Heredity*. **80**:2-8.

Hurst G.D.D., and Werren J.H., 2001, The role of selfish genetic elements in eukaryotic evolution. *Nat. Rev. Genet*. **2**:597-606.

Ishikawa H., 2003, Insect symbiosis: An introduction. In Bourtzis K., and Miller T.A. (eds), '*Insect symbiosis*'. CRC Press: Boca Raton. pp1-22.

Jaenike J., 2001, Sex chromosome meiotic drive. *Annu. Rev. Ecol. Syst*. **32**:25-49.

Jiggins F.M., Hurst G.D.D., and Majerus M.E., 2000, Sex-ratio-distorting *Wolbachia* causes sex-role reversal in its butterfly host. *Proc. R. Soc. Lond. B*. **267**:69-73.

Juchault P. and Mocquard J.P., 1993, Transfer of a parasitic sex factor to the nuclear genome of the host: A hypothesis on the evolution of sex determining mechanisms in the terrestrial Isopod *Armadillidium vulgare* Latr. *J. Evol. Biol.* **6**:511-528.

Keller L., and Ross K.G., 1998, Selfish genes: A green beard in the red fire ant. *Nature*. **394**:573-575.

Kelly A., Dunn A.M., and Hatcher M.J., 2002, Incomplete feminisation by the microsporidian sex ratio distorter, *Nosema granulosis*, and reduced transmission and feminisation efficiency at low temperatures. *Int. J. Parasitol.* **32**(7):825-831.

Kageyama D., Nishimura G., Hoshizaki S., and Ishikawa Y., 2002, Feminizing *Wolbachia* in an insect, *Ostrinia furnacalis* (Lepidoptera: Crambidae). *Heredity*. **88**:444-449.

Komdeur J., 1996, Facultative sex ratio bias in the offspring of Seychelles warblers. *Proc. R. Soc. Lond. B*. **263**:661-666.

Kondo M., Nanda I., Hornung U., Asakawa S., Shimizu N., Mitani H., Schmid m., Shima A., and Schartl M., 2003, Absence of the candidate male sex determining gene dmrt1b (Y) of medaka from other fish. *Curr. Biol*. **13**:416-420.

Koopman P., and Loffler K.A., Sex determination: The fishy tale of Dmrt1. *Curr. Biol*. **13**:R177-R179.

Kraak S.B.M., and Pen I., 2002, Sex-determination mechanisms in vertebrate. In Hardy I.C.W. (ed), '*Sex ratios: Concepts and research methods*'. Cambridge University Press: Cambridge. pp158-177.

Kruuk L.E.B., Clutton-Brock T.H., Albon S.D., Pemberton J.M., and Guinness F.E., 1999, Population density affects sex ratio variation in red deer. *Nature*. **399**:459-461.

Langworthy. N.G., Renz A., Meckenstedt U., Henkle-Dürsen K., de Bronsvoort M.B., Tanya V.N., Donnelly M.J., Trees A.J., 2000, Macrofilaricidal activity of tetracycline against the filarial nematode, *Onchocerca ochengi*: elimination of *Wolbachia* precedes worm death and suggests a dependent relationship. *Proc. R. Soc. Lond.B*. **267**:1063–1069.

LeGrand J.J., LeGrand-Hamelin E., Juchault P., 1987, Sex determination in Crustacea. *Biol. Rev*. **62**:439-470.

Lo N., Casiraghi M., Salati E., Bazzocchi C., and Bandi C., 2002, How many *Wolbachia* supergroups exist? *Mol. Biol. Evol.* **19**(3):341-346.

Lyttle T.W., 1991, Segregation distorters. *Annu. Rev. Genet.* **25**:511-557.

Marín I., and Baker B.S., 1998, The evolutionary dynamics of sex determination. *Science*. **281**:1990-1994.

Min K.T., and Benzer S., 1997, *Wolbachia*, normally a symbiont of *Drosophila*, can be virulent, causing degeneration and early death. *Proc. Natl., Acad. USA*. **94**:10792-10796.

Mittwoch U., 1996, Sex-determining mechanisms in animals. *Trends Ecol. Evol.* **11**(2):63-67.

Nur U., Werren J.H., Eickbush D.G., Burke W.D., and Eickbush T.H., 1988, A "selfish" B chromosome that enhances its transmission by eliminating the paternal genome. *Science*. **240**:512-514.

O'Neill S.L., and Karr T.L., 1990, Bidirectional incompatibility between conspecific populations of *Drosophila simulans*. *Nature*. **348**:178-180.

Pannebakker B.A., Pijnacker L.P., Zwaan B.J., and Beukeboom L.W., Cytology of *Wolbachia*-induced parthenogenesis in *Leptopilina clavipes* (Hymenoptera: Figitidae). *Genome*. In press.

Pen I., and Weissing F.J., 2002, Optimal sex allocation: steps towards a mechanistic theory. In Hardy I.C.W. (ed), '*Sex ratios: Concepts and research methods*'. Cambridge University Press: Cambridge. pp26-45.

Perrot-Minnot M.J., Guo L.R., and Werren J.H., 1996, Single and double infections with *Wolbachia* in the parasitic wasp *Nasonia vitripennis*: effects on compatibility. *Genetics*. **143**:961-972.

Pijls J.W.A.M., van Steenbergen H.J., and van Alphen J.J.M., 1995, Asexuality cured: The relations and differences between sexual and asexual *Apoanagyrus diversicornis*. *Heredity*. **76**:506-513.

Poulson D.F., and Sakaguchi B., 1961, Nature of "sex-ratio" agent in *Drosophila*. *Science*. **133**:1489-1490.

Presgraves D.C., Severance E., and Wilkinson G.S., 1997, Sex chromosome meiotic drive in Stalk-eyed flies. *Genetics*. **147**:1169-1180.

Quicke L.J., 1997. Sex and genetics. In '*Parasitic wasps*'. Chapman and Hall: London. pp19-36.

Raymond C.S., Shamu C.E., Shen M.M., Seifert K.J., Hirsch B., Hodgkin J., and Zarkower D., 1998, Evidence for evolutionary conservation of sex-determination genes. *Nature*. **391**:691-695.

Reed K.M., and Werren J.H., 1995, Induction of paternal genome loss by the paternal sex ratio chromosome and cytoplasmic incompatibility bacteria (*Wolbachia*): a comparative study of early embryonic events. *Mol. Reprod. Dev*. **40**:408-418.

Reynolds K.T., Thomson L.J., and Hoffmann A.A., 2003, The effect of host age, host nuclear background and temperature on phenotypic effects of the virulent *Wolbachia* strain popcorn in *Drosophila melanogaster*. *Genetics*. **164**:1027-1034.

Rice W.R., 1996, Sexually antagonistic male adaptation triggered by experimental arrest of female evolution. *Nature*. **381**:231-234.

Rice W.R., and Chippindale A.K., 2001, Intersexual ontogenetic conflict. *J. Evol. Biol*. **14**:685-693.

Rigaud T., 1997, Inherited microorganisms and sex determination of arthropod hosts. In: O'Neill S.L., Hoffmann A.A., and Werren J.H. (eds), '*Influential Passengers-Inherited microorganisms and arthropod reproduction*'. Oxford University Press: Oxford. pp81-101.

Rigaud T., Juchault P., and Mocquard J., 1997, The evolution of sex determination in isopod crustaceans. *Bioessays*. **19**(5):409-416.

Roberts L.W., Rapmund G., and Cadigan F.C.J., 1977, Sex ratio in *Rickettsia tsutsugamushi* infected and non-infected colonies of *Leptotrombidium* (Acari: Trombiculidae). *J. Med. Entomol*. **14**:89-92.

Roberts K.A., and Thompson M.B., Viviparous lizard selects sex of embryos. *Nature*. **412**:698-699.

Schilthuizen M., and Stouthamer R., 1997, Horizontal transmission of parthenogenesis-inducing microbes in *Trichogramma* wasps. *Proc. R. Soc. Lond. B*. **264**:361-366.

Shearman D.C.A., 2002, The evolution of sex determination systems in dipteran insects other than *Drosophila*. *Genetica*. **116**:25-43.

Starr D.J., and Cline T.W., 2002, A host-parasite interaction rescues *Drosophila* oogenesis defects. *Nature*. **418**:76-79.

Stearns S.C., and Hoekstra R.F., 2000, *Evolution: An introduction*. Oxford University Press. Oxford.

Stouthamer R., 1997, *Wolbachia*-induced parthenogenesis. In: O'Neill S.L., Hoffmann A.A., and Werren J.H. (eds), '*Influential Passengers-Inherited microorganisms and arthropod reproduction*'. Oxford University Press: Oxford. pp102-124.

Stouthamer R., Breeuwer J.A.J., and Hurst G.D.D., 1999, *Wolbachia pipientis*: Microbial manipulator of arthropod reproduction. *Annu. Rev. Microbiol*. **53**:71-102.

Stouthamer R., Breeuwer J.A.J., Luck R.F., and Werren J.H., 1993, Molecular identification of microorganisms associated with parthenogenesis. *Nature*. **361**:66-68.

Stouthamer R., Hurst G.D.D., and Breeuwer J.A.J., 2002, Sex ratio distorters and their detection. In: Hardy I.C.W. (ed), '*Sex ratios: Concepts and research methods*'. Cambridge University Press: Cambridge. pp195-215.

Stouthamer R., and Kazmer D.J., 1994, Cytogenetics of microbe-associated parthenogenesis and its consequences for gene flow in *Trichogramma* wasps. *Heredity*. **73**:317-327.

Stouthamer R., and Luck R.F., 1993, Influence of microbe-associated parthenogenesis on the fecundity of *Trichogramma deion* and *T. pretiosum*. *Entomol. Exp. Appl*. **67**:183-192.

Stouthamer R., Luck R.F., and Hamilton W.D., 1990, Antibiotics cause parthenogenetic *Trichogramma* to revert to sex. *Proc. Natl. Acad. Sci. USA*. **87**:2424-2427.

Stouthamer R., van Tilborg M., de Jong J.H., Nunney L., and Luck R.F., 2001, Selfish element maintains sex in natural populations of a parasitoid wasp. *Proc. R. Soc. Lond. B*. **268**:617-622.

Sullivan J.A., and Schultz R.J., 1986, Genetic and environmental basis of variable sex ratios in laboratory strains of *Peociliopsis lucida*. *Evolution*. **40**:152-158.

Taylor P.D., Intra-sex and inter-sex sibling interactions as sex ratio determinants. *Nature*. **291**:64-66.

Tram U., and Sullivan W., 2002, Role of delayed nuclear envelope breakdown and mitosis in *Wolbachia*- induced cytoplasmic incompatibility. *Science*. **296**:1124-1126.

Turelli M., 1994, Evolution of incompatibility-inducing microbes and their hosts. *Evolution*. **48**:1500-1513.

Valenzuela N., Adams D.C., and Janzen F.J., 2003, Pattern does not equal process: Exactly when is sex environmentally determined? *Am. Nat*. **161**(4):676-683.

Vandekerchove T.M.T., Watteyne S., Willems A., Swings J.G., Mertens J., and Gillis M., 1999, Phylogenetic analysis of the 16S rDNA of the cytoplasmic bacterium *Wolbachia* from the novel host *Folsomia candida* (Hexapoda, Collembola) and its implications for wolbachial taxonomy. *FEMS Microbiol. Lett*. **180**:279-286.

Vavre F., Fleury F., Lepetit D., Fouillet P., and Boulétreau M., 1999, Phylogenetic evidence for horizontal transmission of *Wolbachia* in host-parasitoid associations. *Mol. Biol. Evol*. **16**:1711-1723.

Vavre F., Fleury F., Varaldi J., Fouillet P., and Boulétreau M., 2000, Evidence for female mortality in *Wolbachia*-mediated cytoplasmic incompatibility in haplodiploid insects: Epidemiologic and evolutionary consequences. *Evolution*. **54**(1):191-200.

Wade M.J., and Chang N.W., 1995, Increased male fertility in *Tribolium confusum* beetles after infection with the intracellular parasite *Wolbachia*. *Nature*. **373**:72-74.

Weeks A.R., and Breeuwer J.A.J., 2001, *Wolbachia*-induced parthenogenesis in a genus of phytophagous mites. *Proc. R. Soc. Lond. B*. **268**:2245-2251.

Weeks A.R., and Breeuwer J.A.J., 2003, A new bacterium from the Cytophaga-flavobacterium-bacteroides phylum that causes sex-ratio distortion. In Bourtzis K., and Miller T. (eds), '*Insect symbiosis*'. CRC Press: Boca Raton. pp165-176.

Weeks A.R., Velten R., and Stouthamer R., 2003, Incidence of a new sex-ratio-distorting endosymbiotic bacterium among arthropods. *Proc. R. Soc. Lond. B*. **270**:1857-1865.

Werren J.H., 1991, The paternal-sex-ratio chromosome of *Nasonia*. *Am. Nat*. **137**:392-402.

Werren J.H., 1997, Biology of *Wolbachia*. *Annu. Rev. Entomol*. **42**:587-609.

Werren J.H., and Beukeboom L.W., 1998, Sex determination, sex ratios, and genetic conflict. *Annu. Rev. Ecol. Syst*. **29**:233-261.

Werren J.H., Hurst G.D.D., Zhang W., Breeuwer J.A.J., Stouthamer R., and Majerus M.E.N., 1994, Rickettsial relative associated with male killing in the ladybird beetle (*Adalia bipunctata*). *J. Bacteriol*. **176**:388-394.

Werren J.H., Nur U., and Eickbush D., 1987, An extrachromosomal factor causing loss of paternal chromosomes. *Nature*. **327**:75-76.

Werren J.H., Nur U., and Wu C.I., 1988, Selfish genetic elements. *Trends Ecol. Evol*. **3**(11):297-302.

Werren J.H., Skinner S.W., and Huger A.M., 1986, Male-killing bacteria in a parasitic wasp. *Science*. **231**:990-992.

Werren J.H., and Stouthamer R., 2003, PSR (paternal sex ratio) chromosomes: the ultimate selfish genetic elements. *Genetica*. **117**:85-101.

Werren J.H., Zhang W., and Guo L.R., 1995, Phylogeny of *Wolbachia* bacteria: Reproductive parasites of arthropods. *Proc. R. Soc. Lond. B*. **250**:91-98.

West S.A., and Herre E.A., 2002, Using sex ratios: Why bother? In: Hardy I.C.W. (ed), '*Sex ratios: Concepts and research methods*'. Cambridge University Press: Cambridge. pp399-413.

West S.A., Herre E.A., and Sheldon B.C., 2000, The benefits of allocating sex. *Science*. **290**:288-290.

West S.A., Reece S.E., and Sheldon B.C., 2002, Sex ratios. *Heredity*. **88**:117-124.

Zarkower D., 2001, Establishing sexual dimorphism: Conservation amidst diversity? *Nat. Rew. Genet*. **2**:175-185.

Yen J.H., and Barr A.R., 1971, New hypothesis of the cause of cytoplasmic incompatibility in *Culex pipiens*. *Nature*. **232**:657-658.

Zchori-Fein E., Gottlieb Y., and Coll M., 2000, *Wolbachia* density and host fitness components in *Muscidifurax uniraptor* (Hymenoptera: Pteromalidae). *J. Invertebr. Pathol.* **75**:267-272.

Zchori-Fein E., Gottlieb Y., Kelly S.E., Brown J.K., Wilson J.M., Karr T.L., and Hunter M.S., 2001, A newly discovered bacterium associated with parthenogenesis and a change in host selection behavior in parasitoid wasps. *Proc. Natl. Acad. USA*. **98**:12555-12560.

Chapter 2

Genetics of female virginity in the *Wolbachia* infected wasp *Telenomus nawai*

Genetics of female virginity in the *Wolbachia*-infected wasp *Telenomus nawai* (Hymenoptera: Scelionidae)

Abstract

A lepidopteran egg parasitoid species *Telenomus nawai* consists of two distinct populations with different reproduction modes. One is a completely parthenogenetic population whereas the other displays sexual reproduction. Complete parthenogenesis in *T. nawai* is caused by a bacterial symbiont the so called parthenogenesis inducing (PI) *Wolbachia*. Recent theoretical studies have shown that when a PI-*Wolbachia* is spreading in a population mutations that allow females to produce more male offspring will spread rapidly in the population. This eventually results in a fixation of this mutant in the infected population. The consequences of such a mutation are that infected females will no longer be able to successfully reproduce sexually. This is a pattern found in most PI-*Wolbachia* infected populations. Here we determine the genetic basis of the inability to reproduce sexually by introgressing the genome of the completely parthenogenetic line into the normal sexual line. Results of this introgression show that the mutation(s) are recessive and inherit either as a single locus major gene with some modifiers, or as a two loci model with some linkage between the loci.

Keywords

Female virginity mutation, parthenogenesis, *Wolbachia*, *Telenomus nawai*

Introduction

Heritable symbionts are prevalent among insects and cause a number of alterations in their host's reproduction (see Bourtzis and Miller, 2003 and O'Neill *et al.,* 1997 for reviews). These alterations range from causing a crossing incompatibility (cytoplasmic incompatibility), killing the male offspring, rendering genetic males into functional females and inducing complete parthenogenetic reproduction (thelytoky) in infected females. Many of these symbiont host interactions have been studied to determine the dynamics of the infection in populations. The infection appears to have gone to fixation in most populations that are infected with symbionts inducing crossing incompatibility or parthenogenesis. Such completely infected populations will come about when 1) the symbiont is transmitted efficiently from mother to her offspring, and 2) infected mothers produce on average more female offspring than uninfected mothers do. These two conditions are sufficient for the fixation of the infection to occur.

Bacteria inducing complete parthenogenesis in their hosts are now found in two groups of bacteria: *Wolbachia* (Huigens and Stouthamer, 2003) and the Cytophaga-like bacteria (Zchori-Fein *et al*., 2001; Weeks and Breeuwer, 2003). In almost all populations infected with such PI-symbionts the whole population is infected, only in wasps of the genus *Trichogramma* many populations exist where the infection has not gone to fixation. Little is known about why the PI-*Wolbachia* infection in *Trichogramma* does not go to fixation, with the exception of the species *Trichogramma kaykai*. In this species the infection is maintained at a low level by the presence of a second sex ratio distorter that causes fertilized eggs to grow out to be males (Stouthamer *et al*., 2001; van Vugt *et al*., 2003).

The speed with which a PI infection in a wasp population will go to fixation is a function of the 1) the sex ratio produced by the uninfected females, 2) the relative cost of being infected, 3) the transmission efficiency of the infection (Stouthamer, 1997), 4) the presence of other sex ratio distorters that counteract the PI infection such as the paternal sex ratio chromosome (Stouthamer *et al*., 2001) and 5) the spread of "virginity" mutants in the population during the spread of the PI infection in the population (Huigens and Stouthamer, 2003). In

this latter case mutations that cause females to produce more male offspring will have a selective advantage when the population sex ratio becomes more and more female biased during the spread of the infection. Infection with PI-*Wolbachia* in itself does not interfere with sexual reproduction, infected mated females can fertilize a fraction of their eggs, in these fertilized eggs the paternal chromosome set participates normally in the formation of the infected female offspring. During the spread of the PI infection in a population those uninfected females that produce male offspring instead of both male and female offspring will have many more grandchildren. Uninfected females can produce more male offspring when they reduce their egg fertilization rate, or if they do not mate at all and therefore remain virgins. We refer to these mutations that reduce the egg fertilization, as "virginity" mutations. They will spread from the uninfected population into the infected population, and from the infected back into the uninfected population when infected females produce some uninfected offspring through inefficient transmission of the *Wolbachia* infection. The end result of the presence of this mutation is that the infection goes to fixation and that all females in the population are homozygous for the "virginity" mutation (Huigens & Stouthamer, 2003).

This is consistent with the finding that from many completely infected populations, it is not possible to derive sexually reproducing lines through antibiotic treatment. In several cases it has been shown that the females are indeed the sex that does not mate or does not fertilize the eggs, while the males are still able to successfully father offspring when they mate with females from a sexual population. For instance, in the wasp *Apoanagyrus diversicornis* Pijls *et al*. (1996) showed that males derived from the infected population were capable of fathering offspring with females from a closely related sexual form, while neither the males derived from the infected line nor those derived from the sexual form were capable of fathering offspring with the infected females. Another case of mating unwillingness or failure of fertilization is found in *Telenomus nawai* (Arakaki *et al*., 2000). *T. nawai*, an egg parasitoid of the army worm genus *Spodoptera*, has two distinctive modes of reproduction. One population originating from the island Okinawa is thelytokous, caused by a

Wolbachia infection (Arakaki et al, 2000), while the population collected on the large island reproduces by arrhenotoky.

Arakaki *et al*. (2000) reported that a cured sexual population could not be established from the *Wolbachia* infected thelytokous population of *T. nawai* by antibiotic treatment. They found no evidence that the infected females used sperm when they mated. Other examples of failure of establishment of a cured sexual line from infected thelytokous lines by high temperature or antibiotic treatment are: Apoanagyrus *diversicornis* (Pijls *et al.*, 1996), *Eretmocerus mundus* (De Barro and Hart, 2001), *Franklinothrips vespiformis* (Arakaki *et al.*, 2001), *Muscidifurax uniraptor* (Gottlieb and Zchori-Fein, 2001) and a *Leptopilina* species (B. Pannebakker and F. Vavre, pers. comm.). Alternatively, in a number of species of *Trichogramma* antibiotic curing resulted in the establishment of sexual populations (Stouthamer *et al.,* 1990). The difference of these populations with those described above is that in *Trichogramma* the infected individuals originated from populations where both infected and uninfected individuals co-existed.

Here we study if in the *Wolbachia*-infected *T. nawai* population a mutation exists that prevents the establishment of a sexual population from the infected females, and how this mutation is inherited. Our results show that the failure of sexual reproduction by females from the infected line is caused by recessive mutations where only a few loci have a major effect.

Materials and methods

Strains

T. nawai (Hymenoptera: Scelionidae) is an egg parasitoid of among others of the army worm (*Spodoptera*) species found in the Far East (Arakaki *et al.*, 2000). The *Wolbachia*-infected thelytokous line originated from Ginowan, the island of Okinawa, Japan, the uninfected arrhenotokous line originated from Tsukuba, Ibaraki, on the large island of Japan. In our experiments we used one infected isofemale line and one uninfected isofemale line. Both lines were reared on *Mamestra brassicae* (Lepidoptera: Noctuidae) eggs in a temperature controlled culture room (Temp. 23±1°C, L:D= 16:8, R.H 60±10%).

Wolbachia *Infection*

To verify that the parthenogenetic *T. nawai* females were infected with *Wolbachia*, PCR with *Wolbachia*-specific primers (*wsp*) was performed using a temperature profile of 94°C for 3 min (1cycle); 94°C for 1min, 50°C for 1 min and 72°for 1 min (40 cycles) on a thermocycler (Progene, Techne Co.) (Braig *et al.*, 1998).

ITS-2 (Internally transcribed spacer-2 region) sequence analysis

The uninfected arrhenotokous females and the infected thelytokous females are morphologically indistinguishable (Arakaki *et al.*, 2000). Sequences of internally transcribed spacer-2 region have been used as a taxonomic tool to identify individuals at the species level (Silva, 1999). We determined the ITS-2 sequences of both lines to establish their relationship using ITS-2 specific primers (Silva, 1999). For the PCR reaction the following temperature profile was used: 94°C for 3 min (1 cycle); 94°C for 40sec, 53°C for 45sec, and 72°C for 45sec (33 cycles). The PCR products from the two lines were isolated using the QIAquick® PCR Purification Kit (Qiagen) and cloned into pGEM®-T Vector System I (Promega). They were sequenced on an Applied Biosystems Automatic Sequencer.

Effect of mating circumstances and female age on the sex ratio of the arrhenotokous line

In nature the *T. nawai* females parasitize egg masses of *Spodoptera* that contain a large number of eggs. Males generally emerge one day before the females, remain on the egg mass and mate with the females upon their emergence. In our experiment we noticed that it was sometimes difficult to obtain female offspring from matings set up with one male and one female of the arrhenotokous line. To optimize the mating success we did a number of experiments. First, to determine if mating in groups or as individual pairs was more successful we divided parasitized eggs into two groups. In one treatment, 19 females and 19 males were put in a glass tube (7.5×1cm) to emerge together and to mate in a group. In

the other treatment we separated eggs containing males from eggs containing females before emergence. This was possible because a parasitized egg containing a wasp pupa was translucent enough to determine the sex of the individual inside the egg using a stereomicroscope (magnification 10×). Two eggs, one from which a female would emerge and another from which a male would emerge were put together in a glass tube and the wasps were allowed to mate. In this way, females could mate immediately upon emergence because males generally emerge earlier than females.

Second, to determine the optimum age for a female to mate, females were categorized into four groups by age and allowed to mate individually over a 24-h period. The age categories were less than 24, 24, 48, and 72h. After the mating period, the males were removed, and the females were offered *M. brassicae* egg cards. When offspring emerged from each age category, they were sexed and counted to determine the sex ratios they produced.

Determination of genetic basis of the female virginity mutant

If the female virginity trait in the thelytokous line is genetically based, the genetic basis of this trait can be detected by introgression. To perform introgression males were obtained from the thelytokous females by feeding them 0.1% (v/W) ripampicin with honey and rearing them in a 30°C chamber. To facilitate high mating success six less than 8-hour-old virgin arrhenotokous females were collected for the group mating with 10 males of the thelytokous line. One day later the males were removed and a *M. brassicae* egg card was given to the females. In F_1 female offspring must be hybrid having one set of maternal chromosomes and one set of paternal chromosomes (i.e. AT). Because of the haplo-diploid sex determination system their brothers had only a set of maternal chromosomes (i.e. A). As the sex of an individual wasp could be determined by observing through the translucent eggshell, we collected 9 parasitized eggs from which female offspring would emerge. 13 males from the thelytokous line were allowed to mate with the hybrid females (AT) to obtain F_2 hybrid female offspring (i.e. ATT). One day later the males were removed and the females were placed individually in glass tubes and an egg card containing 25～30 *M. brassicae* eggs

per day for 5 days in succession was given to the females. When all the parasitized eggs turned black (i.e. the wasps inside the eggs had reached the pupal stage), 5 egg cards parasitized by each female were gathered in a glass tube for future group mating in F_2. In F_2 females on average three quarters of the genome originated from the thelytokous line, while in the F_2 males only half of the genome originated from the thelytokous line. The criteria to collect F_2 females that were used for the final experiment were 1) the number of the sisters must be more than 25, 2) the sex ratio must be around normal (20～25%). When the two criteria were not met, the broods were discarded, because a small brood size would result in biased genetic composition among sisters, and skewed sex ratio could create abnormal mating circumstances especially when males were scarce. Nine broods that met the criteria were selected for the experiment. The F_2 hybrid females (ATT) group-mated with their brothers (AT) (i.e. $ATT_{♀} \times AT_{♂}$) for 24 hrs to foster mating (Figure. 1).

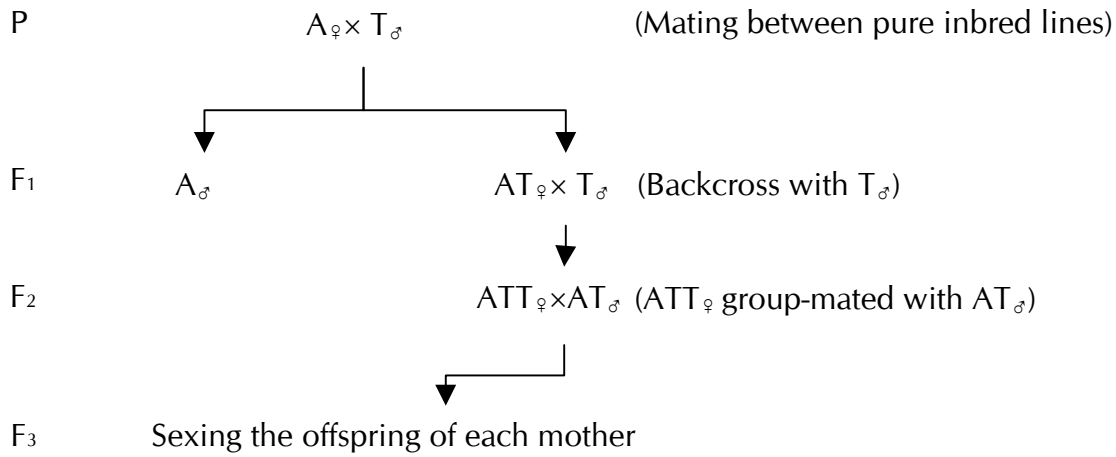

Figure 1. Mating scheme to determine the number of genes involved in the female virginity trait in the thelytokous line.

A = arrhenotokous genome, T = thelytokous genome, AT = hybrid offspring of arrhenotokous mother and thelytokous father, and ATT = hybrid offspring of AT mother and thelytokous father.

304 hybrid female offspring (ATT) group-mated were individually placed in glass tubes. Each female was given an egg card containing 25～30 *M. brassicae* eggs

per day for 5 days in succession and used to determine the sex ratio of their offspring.

92 replicates of arrhenotokous female and male mating ($A_{♀}\times A_{♂}$) were used as a control. 45 replicates of the cross between arrhenotokous females and thelytokous males ($A_{♀}\times T_{♂}$) were done in addition to the ones described above, 27 crosses between arrhenotokous female and hybrid males ($A_{♀}\times AT_{♂}$) were done to determine if AT hybrid males were sexually functional. 54 replicates of the cross between hybrid female and arrhenotokous male ($AT_{♀}\times A_{♂}$) were done to determine if AT hybrid females were sexually functional.

Determination of mating of thelytokous females in a group

To determine if the infected females mate at a lower frequency than arrhenotokous females a mating experiment was done. Nineteen parasitized eggs from which arrhenotokous females would emerge, and 19 eggs from which thelytokous females would emerge were put in a glass tube with 16 arrhenotokous males. After emergence they were left in the tube for about 24 hours to mate. One day later the females were separated and were each given an egg card that contained 25～30 *M. brassicae* eggs to parasitize. Four days later the females were examined for the presence of sperm in their spermathecae under a microscope. Sperm can be seen in the spermatheca when it is removed from the abdomen. The remainder of the wasp was used to determine its *Wolbachia* infection status. They were individually collected into each 0.5ml microtube to extract template DNA. The infection was determined using the PCR protocol described above.

ISSR (Inter simple sequence repeat) analysis on production of hybrid offspring between the two populations

Infected thelytokous females are thought to mate occasionally when confined with males. To determine if they mated under these circumstances and if the mated thelytokous female used sperm resulting in hybrid offspring, thelytokous females were isolated in tubes with ten males each, to allow for mating. When mating was observed, the mated infected female was given a *M. brassicae* egg

card that contained 25～30 eggs to parasitize. After 1 day of oviposition the female was removed from the tube and we determined the presence of sperm in her spermatheca as described above. The genetic make-up of the offspring was determined using ISSR markers to recognize the hybrids. Among the primers of ISSR No. 880 primer (sequences: 5′-GGA GAG GAG AGG AGA) yielded the best distinguishable banding patterns between the thelytokous line and the arrhenotokous line. 0.2μM of the primer was combined with 30ng of template DNA for the reaction. PCR was performed using a temperature profile of 94°C for 2 min, 94°C for 1min, 44.3°C for 1min, and 72°C for 2min (35 cycles).

Statistics

To analyze the difference in offspring sex ratios between group-mated and individually mated females, χ^2 test was applied. For the analyses of offspring sex ratios ANOVAs and Tukey's b post hoc comparison tests were carried out after arcsin transformation of sex ratios of all crosses described in table 2 and 3. All the analyses were performed on SPSS ver. 10.0.

Results

Wolbachia *infection status*

As previously determined by Arakaki *et al*. (2000), the arrhenotokous line is not infected with *Wolbachia* bacteria, and the thelytokous line is infected with *Wolbachia* bacteria.

ITS-2 (Internally transcribed spacer-2 region) sequence analysis

The two sequences from each line show that they are the same species having the same sequence in the ITS-2 region (the arrhenotokous line: GenBank accession number AF467101, and the thelytokous line: GenBank accession number AF467102).

The effect of mating circumstances and female age on the sex ratio of the arrhenotokous line

Arakaki *et al*. (2000) show that the arrhenotokous line they used produced a mean sex ratio 19.4 % males. In our preliminary test the sex ratio was not stable when we allowed individual pairs to mate (data not shown). In this experiment we tested if group mating versus individual pair mating would result in the highest fraction of female offspring. The result shows that in group-mating females produced more female offspring, but the difference is not statistically significant (χ^2 test $P > 0.05$) (Table 1).

Table 1. The sex ratio of the arrhenotokous line in two different mating circumstances

	Mating circumstance	
	Group	Individual
N	19	16
No.♀	349	315
No.♂	76	194
M.O	20.2	24.2
%♂	17.8	38.1
Total	425	509

N: number of replications; M.♀: mean number of female offspring; M.♂: mean number of male offspring; M.O: mean number of offspring; %♂: sex ratio; Total: total number of offspring.

Table 2. The sex ratio of arrhenotokous females as a function of their age at mating

Female age (hr)	N	%♂
Within 24	5	48.7[a]
24	5	61.5[a]
48	5	85.8[b]
72	5	100[c]

N: number of replicates; %♂: sex ratio

%♂ with the same letter does not differ significantly (ANOVA $P < 0.05$, Tukey's b test).

Knowledge on the optimum female age of the arrhenotokous line to mate is important for the introgression experiments, our results show that the percentage female offspring decreased with increasing age of the female when she mated,

females of 5 days old when mated did not produce any female offspring (Table 2).

Determination of genetic basis of the female virginity trait

The mean sex ratio of the cross $AT_{♀}\times A_{♂}$ was not significantly different from those produced by A females when mated with either A, T or AT males (Table 3 and Figure 2 a, b) (ANOVA $P<0.001$, Tukey's b test) indicating that the virginity mutations are recessive. In addition the results of the $A_{♀}\times AT_{♂}$ cross showed that the AT hybrid males are functional resulting in successful mating and female offspring production (Table 3), not different from either A males or T males (ANOVA $P<0.001$, Tukey's b test).

Table 3. The result of crosses among experimental strains

♀×♂	N	M.♀	M.♂	M.O	%♂	Total
$A_{♀}\times A_{♂}$	92	13.2± 9.5	3.7± 4.8	16.9±10.7	22.3[a]	1555
$A_{♀}\times T_{♂}$	45	20.2±10.4	6.6± 4.8	26.9±12.0	24.7[a]	1210
$A_{♀}\times AT_{♂}$	27	16.7±11.7	5.3± 4.2	22.0±13.6	24.0[a]	593
$AT_{♀}\times A_{♂}$	54	16.4± 9.1	8.0± 6.7	24.4±12.2	32.8[b]	1319
$ATT_{♀}\times AT_{♂}$	304	10.9±13.1	19.2±13.1	30.4±12.6	63.7[c]	9251

N: number of replications; M.♀: mean number of female offspring; M.♂: mean number of male offspring; M.O: mean number of offspring; %♂: sex ratio; Total: total number of offspring. A= arrhenotokous line, T= thelytokous line, $AT_{♀}$= offspring of arrhenotokous mother and thelytokous father, $AT_{♂}$= male offspring of $AT_{♀}$, and $ATT_{♀}$= offspring of AT mother and thelytokous father.

%♂ with the same letter does not differ significantly (ANOVA $P<0.001$, Tukey's b test).

We could therefore use the AT males in the cross with their ATT sisters. In the $ATT_{♀}\times AT_{♂}$ cross, 86 out of 304 $ATT_{♀}$ (28.3%) mated with their brothers do not produce female offspring (Table 4 and Figure 2 c). Simply using this value a two loci four alleles model would predict 25% of the females to fall in this class. Consequently, this model would fit reasonable well using only this class of females. Removing the two homozygous classes from the distribution of offspring sex ratios leaves us with a rather broad distribution of values for the two heterozygote types, while a clear bimodal distribution was expected (Table 5). Alternatively, if we assume a single locus model and we use only the sex ratio

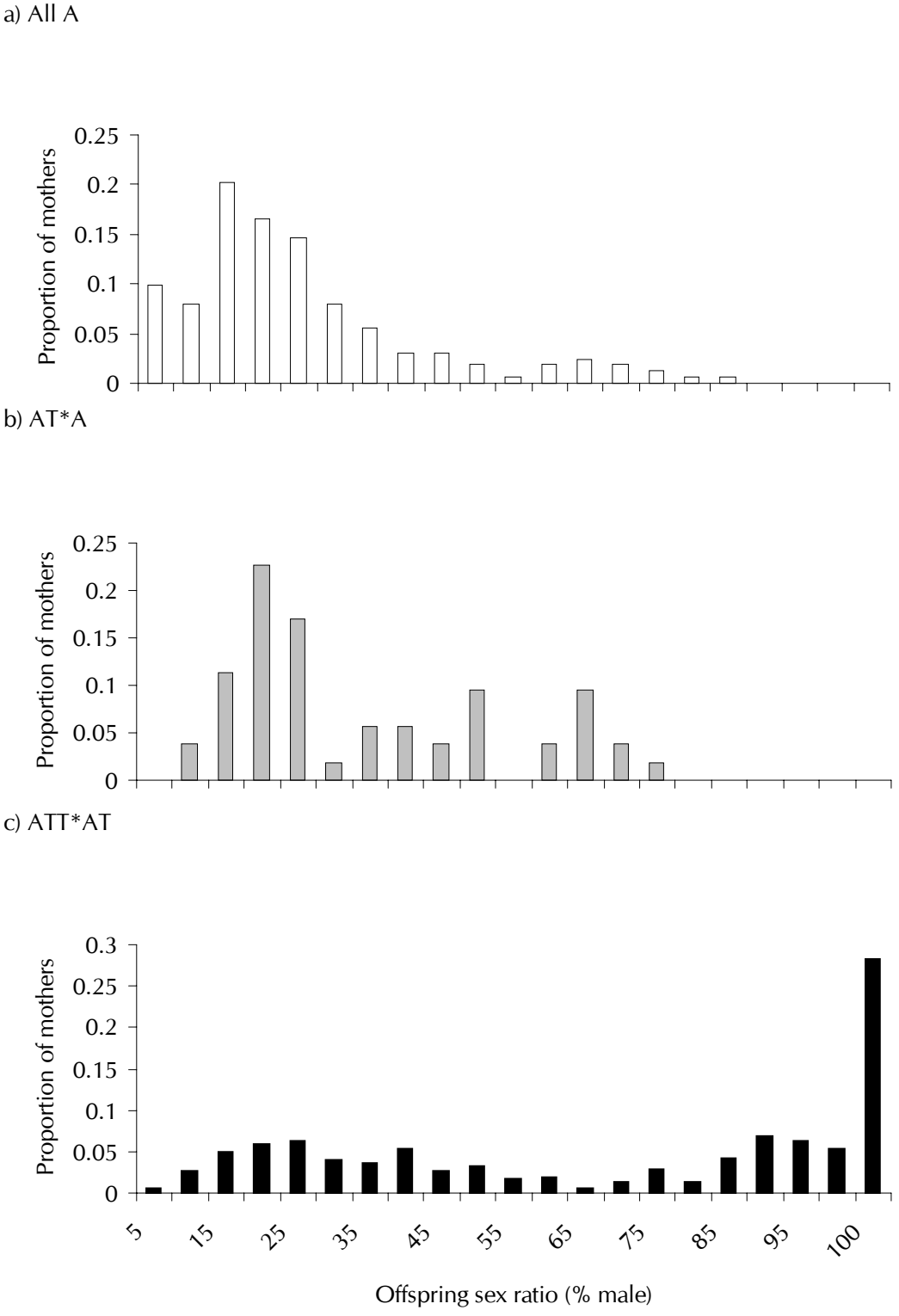

Figure 2. The offspring sex ratio produced by females with different levels of introgression of the thelytokous genome (T) into the arrhenotokous background (A).

a) Sum of all the crosses involving A females ($A_{♀}\times A_{♂}$, $A_{♀}\times T_{♂}$, $A_{♀}\times AT_{♂}$), b) Cross between AT females and A males, c) Cross between ATT females and AT males.

Table 4. Results of the crossing experiments done to determine the genetic basis of the female virginity trait in *Telenomus nawai*

Offspring sex ratio class (%♂)	A♀×A♂	A♀×T♂	A♀×AT♂	AT♀×A♂	ATT♀×AT♂
5	14	2	0	0	2
10	9	4	0	2	8
15	15	8	10	6	15
20	16	7	4	12	18
25	7	9	8	9	19
30	6	5	2	2	12
35	7	0	2	3	11
40	4	1	0	3	16
45	2	2	1	2	8
50	2	1		5	10
55	1	0		0	5
60	1	2		2	6
65	2	2		5	2
70	3	0		2	4
75	2	0		1	9
80	1	0			4
85		1			13
90					21
95					19
99					16
100					86
Total	92	45	27	54	304

classes not frequently found in the normal crosses with A females i.e. those classes with offspring sex ratio's of larger than 85% males as an expression of the homozygous T genotype, then about half of females (155 out of 304) ATT females fall in this class (Table 5). This is consistent with a single locus model, we would also have to assume some other minor modifier of the trait that results in the less than perfect expression of the trait.

Table 5. Comparison of the two models in the $ATT_{♀}\times AT_{♂}$ cross

	Model					
	1 locus 2 alleles		2 loci 4 alleles			
F_1 Genotype of mates	$Aa_{♀}\times a_{♂}$		$AaBb_{♀}\times ab_{♂}$			
F_2 Genotype of female	Aa	Aa	AaBb	Aabb	aaBb	aabb
Proportion	½	½	¼	¼	¼	¼
F_3 Expected sex ratio	Normal	Male biased >80%	Normal or male biased			100% male
Approximate number of mothers	149	155	218			86

The variation in the sex ratios is continuous. The numbers of the mothers allocated in each genotype may not be precise and most likely largely overlap except for the genotype aabb.

The 1 locus 2 alleles model is based on the sex ratio distribution of the sum of all the crosses involving A females ($A_{♀}\times A_{♂}$, $A_{♀}\times T_{♂}$, $A_{♀}\times AT_{♂}$).

The 2 loci 4 alleles model is based on the numbers of the mothers that did not produce female offspring.

Determination of mating of thelytokous females with arrhenotokous males in a groups

Two females were lost during handling (one before giving an egg card and the other after parasitization) and a spermatheca of one female could not be located under a microscope. Sperm was found in the spermatheca of 10 out of 36 females and 17 out of 36 females were infected with *Wolbachia*. 1 out of 10 females that had sperm in their spermathecae was infected with *Wolbachia* (Figure 3). Only nine of the eighteen arrhenotokous females had sperm in their spermathecae which is low compared to the other group matings we set up. However, these results shows that occasionally the thelytokous females will mate and store the sperm in their spermatheca, and that a much lower fraction of the thelytokous females when placed with males will end up with sperm in their spermatheca than arrhenotokous females.

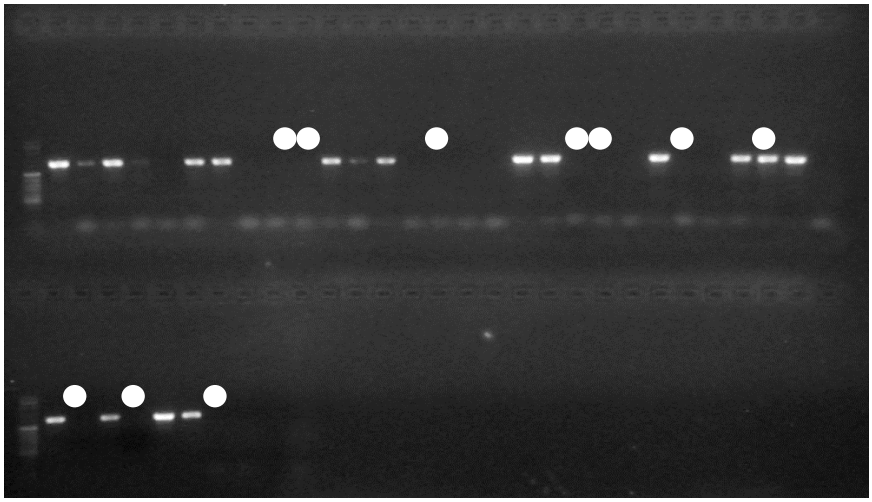

Figure 3. Determination of the existence of sperm in spermathecae of thelytokous females by *wsp* specific primers

The white dots represent the females that had sperm in their spermathecae.

Wolbachia specific band of 28th lane from the top left is from the female that contained sperm in her spermatheca.

The first top left lane: low ladder; second lane: positive control *Muscidifurax uniraptor*; the last bottom right lane: negative control.

ISSR (Inter simple sequence repeat) analysis on production of hybrid offspring between the two populations

All of the fourteen offspring of the thelytokous female that had sperm in her spermatheca had only the banding pattern of the thelytokous line (Figure. 4). Therefore, we conclude that even though the female was inseminated, she did not produce any daughters from fertilized eggs.

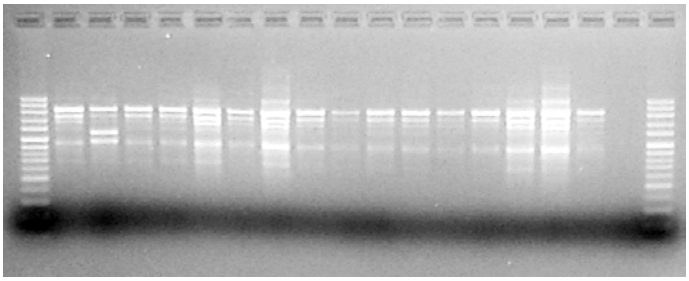

Figure 4. Analysis of ISSR to determine the status of hybrid production

The second lane from the left: thelytokous line specific band pattern

The third lane from the left: arrhenotokous line specific band pattern

The second lane from the right: negative control

All the 14 treatments follow the thelytokous line specific band pattern.

Discussion

The males of the thelytokous line, produced by antibiotic treatment, were capable of fathering offspring with females from the arrhenotokous line. The hybrid female offspring that resulted from the cross between the arrhenotokous females and the thelytokous males were fertile. Sperm of the hybrid males, i.e. male offspring of hybrid females, could also fertilize eggs. Together with the result of ITS-2 sequences we conclude that the two reproductively distinctive lines are indeed conspecific.

The introgression experiment showed that the female virginity trait of the thelytokous females followed the simple Mendelian inheritance, and that it was controlled by a single locus with two alleles and some modifier influencing the complete expression, or by a two loci four alleles model. Many genes are involved in sexual reproduction and mutations in any of them may influence either the mating success, and/or will result in a lower fertilization proportion of the eggs. For instance, Silva and Stouthamer (1997) reported that in *Trichogramma cordubensis* from a *Wolbachia*-fixed population, females were not attractive to conspecific males possibly resulting from a low or a complete lack of pheromone production by the thelytokous females. In *Encarsia formosa* (Hymenoptera: Aphelinidae) females lost the function of spermatheca and males do not generate sperm (Zchori-Fein *et al.*, 1992; Hunter, 1999). In *Muscidifurax uniraptor* (Hymenoptera: Pteromalidae) at least three obstacles act on recovering sexual reproduction (Gottlieb and Zchori-Fein, 2001).

It is generally easier to lose gene function than to gain it. While it is often assumed that populations with haplodiploid sex determination carry a lower genetic load than diplodiploid populations, this only applies to male limited traits and to traits that are expressed in both males and females. In female limited traits the genetic load is similar to that in female limited traits in diplodiploid. Consequently, we expect that in normal sexual populations of these wasps the females will carry some mutations in genes that influence their mating behavior or in genes involved in the chain that eventually results in eggs being fertilized. The relative frequency of such genes compared to other female limited deleterious genes is most likely higher, simply because homozygosity for these

traits will not affect their number of offspring but only the sex ratio of their offspring. In a sexual population that becomes infected with PI-bacteria, these mildly deleterious virginity mutations attain a selective advantage once the infection frequency reaches high frequencies, eventually leading in the fixation of these traits. Those mutant forms that completely suppress sexual reproduction will eventually go to fixation.

Our experiments with *T. nawai* show that at least two behaviors appear to be affected by the mutations, 1) they mate at a much lower rate than the arrhenotokous females, and 2) once inseminated they do not appear to fertilize any of the eggs. This spread of the virginity mutations in these infected populations leads to an irreversible dependence of the reproduction of these lines on the presence of *Wolbachia*. Rare sexual reproduction in these lines is unlikely, apparently rare sex does occur occasionally in other species with a thelytokous reproduction that is not associated with *Wolbachia* infection (Belshaw *et al.*, 1999). The irreversibility of this trait also should lead over time in the accumulation of mutations following Muller's ratchet (Muller, 1949).

As far as we know now, no sexual lines can be established from parthenogenetically reproducing species infected with *Wolbachia* outside *Trichogramma* species (Stouthamer *et al.*, 1990). Why this type of virginity mutations have not spread in many of the *Trichogramma* populations remains to be determined. Possibly, the specific mating structure of *Trichogramma* populations allows for the invasion with male producing sex ratio distorters more readily than the mating structure of some of the species where the infection has gone to fixation.

Acknowledgements

The authors thank Norio Arakaki for providing the experimental species. The authors are grateful to Ties Huigens and Fabrice Vavre for valuable comments on performing the experiment and to Boo-Ja Lee for the sequencing data and to Leo Koopman, Andrea Gidding, and Frans van Aggelen for providing huge amount of *M. brassicae* eggs for the experiment. Isabel Silva and Bart Pannebakker are

recognised for valuable comments on the earlier versions of this manuscript. This study is supported in part by NEXGEN Biotechnologies. Inc. (Korea).

References

Arakaki N., Miyoshi T., and Noda H., 2001, *Wolbachia*-mediated parthenogenesis in the predatory thrips *Franklinothrips vespidormis* (Thysanoptera: Insecta). *Proc. R. Soc. Lond. B*. **268**:1011-1016.

Arakaki N., Noda H., and Yamagishi K., 2000, *Wolbachia*-induced parthenogenesis in the egg parasitoid *Telenomus nawai*. *Ent. Exp. Appl*. **96**:177-184.

Belshaw R., Quicke D.L.J., Völkl W., and Godfray H.C.J., 1999, Molecular markers indicate rare sex in a predominantly asexual parasitoid wasp. *Evolution*. **53**(4):1189-1199.

Bourtzis K., and Miller T.A. (eds), 2003, '*Insect symbiosis'*. CRC Press: Boca Raton.

Braig H.R., Zhou W., Dobson S.L., and O'Neill S.L., 1998, Cloning and characterization of a gene encoding the major surface protein of the bacterial endosymbiont *Wolbachia*. *J. Bacteriol*. **180**:2373-2378.

De Barro P.J., and Hart P.J., 2001, Antibiotic curing of parthenogenesis in *Eretmocerus mundus* (Australian parthenogenic form). *Ent. Exp. Appl*. **99**:225-230.

Gottlieb Y., and Zchori-Fein E., 2001, Irreversible thelytokous reproduction in *Muscidifurax uniraptor*. *Entomol. Exp. Appl*. **100**:271-278.

Huigens M.E., and Stouthamer R., 2003, Parthenogenesis associated with *Wolbachia*. In: Bourtzis K., and Miller T.A. (eds), '*Insect symbiosis'*. CRC Press: Boca Raton. pp247-266.

Hunter M.S., 1999, The influence of parthenogenesis-inducing *Wolbachia* on the oviposition behavior and sex-specific developmental requirements of autoparasitoid wasps. *J. Evol. Biol*. **12**:735-741.

Muller H., 1949, The Darwinian and modern conceptions of natural selection. *Proc. Am. Philos. Soc*. **93**:459-470.

Pijls J.W.A.M., Van Steenbergen H.J., and Van Alphen J.J.M., 1996, Asexuality cured: the relations and differences between sexual and asexual *Apoanagyrus diversicornis*. *Heredity*. **76**:505-513.

Silva I.M.M.S., 1999, *Identification and evaluation of* Trichogramma *parasitoids for biological pest control*. Ph.D. thesis. Wageningen University.

Silva I.M.M.S., and Stouthamer R., 1997, To mate or not to mate... Can sex pheromones be used as a taxonomic tool in *Trichogramma* spp.? *Proc. Exper. & Appl. Entomol*. **8**:41-46.

Stouthamer R., 1997, *Wolbachia*-induced parthenogenesis. In: O'Neill S.L., Hoffmann A.A., and Werren J.H. (eds), '*Influential Passengers: Inherited microorganisms and arthropod reproduction*'. Oxford University Press: New York. pp102-124.

Stouthamer R., Luck R.F., and Hamilton W.D., 1990, Antibiotics cause parthenogenetic *Trichogramma* (Hymenoptera/Trichogrammatidae) to revert to sex. *Proc. Natl. Acad. Sci. USA*. **87**:2424-2427.

Stouthamer R., van Tilborg M., de Jong J.H., Nunney L., and Luck R.F., 2001, Selfish element maintains sex in natural populations of a parasitoid wasp. *Proc. R. Soc. Lond. B*. **268**:617-622.

Van Vugt J.J.F.A., Salverda M., de Jong H., and Stouthamer R., 2003, The paternal sex ratio chromosome in the parasitic wasp *Trichogramma kaykai* condenses the paternal chromosomes into a dense chromatin mass. *Genome*. **46**(4):580-587.

Weeks A.R., and Breeuwer J.A.J., 2003, A new bacterium from the Cytophaga-flavobacterium-bacteroides phylum that causes sex-ratio distortion. In Bourtzis K., and Miller T. (eds), '*Insect symbiosis*'. CRC Press: Boca Raton. pp165-176.

Zchori-Fein E., Gottlieb Y., Kelly S.E., Brown J.K., Wilson J.M., Karr T.L., and Hunter M.S., 2001, A newly discovered bacterium associated with parthenogenesis and a change in host selection behavior in parasitoid wasps. *Proc. Natl. Acad. Sci. USA*. **98**:12555-12560.

Zchori-Fein E., Roush R.T., and Hunter M.S., 1992, Male production influenced by antibiotic treatment in *Encarsia formosa*, an asexual species. *Experientia*. **48**:102-105.

Chapter 3

No effect of reproductive isolation on the interspecific transmission of PSR in *Trichogramma*

No effect of postzygotic reproductive isolation on the interspecific transmission of the paternal sex ratio (PSR) chromosome in *Trichogramma*

Abstract

The paternal sex ratio (PSR) chromosome is a supernumerary chromosome that causes the destruction of the paternal chromosomes when sperm fertilizes an egg. In haplodiploids the resulting fertilized egg grows out to be a male that again can transmit the PSR chromosome. It has been found in the genera *Nasonia* and *Trichogramma* (Hymenoptera). Because of its mode of action, the PSR chromosome can easily be transmitted between species. This study determines whether the interspecific transmission of PSR between *Trichogramma kaykai* and *T. deion* is affected by two types of postzygotic reproductive isolation i.e. hybrid sterility and hybrid inviability. The results suggest that PSR can rescue eggs that would normally be inviable. However, the interspecific transmission from *T. kaykai*, the species in which PSR is found in the field, to *T. deion* is much more efficient than the transmission from *T. deion* to *T. kaykai*. The poor transmission efficiency of PSR from *T. deion* to *T. kaykai* implies that the host genetic background has an effect on the efficiency.

Keywords

Trichogramma, PSR, interspecific transmission, reproductive isolation, hybrid sterility, hybrid inviability, host genetic background

Introduction

A species is defined as a group of populations capable of exchanging genes and possessing inherent differences that prevent genetic exchange with other such groups (Wu and Palopoli, 1994). The mechanism of the prevention of the genetic exchange between species is known as reproductive isolation. It can be divided into pre- and postzygotic isolation. Prezygotic reproductive isolation is achieved when mating discrimination is reinforced by natural selection against maladaptive hybridization (Coyne and Orr, 1989). On the other hand postzygotic isolation is caused by genetic incompatibility (but see Bordenstein *et al*., 2001). Postzygotic reproductive isolation imposes a selective disadvantage and cost on the mates. Postzygotic reproductive isolation is composed of hybrid inviability and sterility (Wu and Palopoli, 1994). The two are best described as Haldane's rule in which the heterogametic (i. e. XY and ZW) sex is more prone to be inviable or sterile than the homogametic (i.e. XX and ZZ) sex in the interspecific cross (Haldane, 1922). Several hypotheses have been proposed to explain Haldane's rule (see Orr, 1997 and Wu *et al*., 1996 for review).

Even though Haldane's rule is only applicable to species with diplodiploid heterogametic (XY and ZW) sex determination organisms, inviability of hybrids is observed in haplodiploids in which a female develops from a fertilized diploid egg and a male from an unfertilized haploid egg. For example in *Nasonia* the F_2 hybrid male offspring between *N. vitripennis* and *N. giraulti* suffer from inviability (Breeuwer and Werren, 1995). In the *Trichogramma deion* complex cross-incompatibility between two populations results in inviable female offspring and it is not reciprocal: i.e. the $A_{♀}\times B_{♂}$ cross produces hybrid female offspring while the $B_{♀}\times A_{♂}$ cross does not produce hybrid female offspring (Stouthamer *et al*., 1996).

A selfish genetic element, the paternal sex ratio (PSR) chromosome, is seemingly capable of easily moving beyond the species boundary because of its unique mode of action (Werren and Stouthamer, 2003). When PSR carrying sperm fertilizes an egg, PSR induces loss of the normal paternal chromosome during the first mitotic division. The fertilized egg is converted into a haploid egg and develops into a male harboring PSR (Reed and Werren, 1995; van Vugt *et al*.,

2003). So far it is found in *Nasonia* and *Trichogramma* (Werren and Stouthamer, 2003). In *Nasonia* PSR has been transmitted from its natural host, *Nasonia vitripennis*, to two closely related congeners, *N. giraulti* and *N. longicornis* (Dobson and Tanouye, 1998). In *Trichogramma* the PSR of *T. kaykai* has also been transmitted to congeners (Huigens, 1998; Hulskes, 2002).

The two cases of interspecific transmission of PSR in *Nasonia* and *Trichogramma* reinforce a recent suggestion to use PSR as a potential agent for biological control of haplodiploid pests and as a vehicle for genetic engineering of haplodiploids (Werren and Stouthamer, 2003). The incompatibility between *T. kaykai* and *T. deion* is not caused by bacterial infections as is found in the *Nasonia* complex, where the reproductive isolation is caused by the infection with a cytoplasmic *Wolbachia* bacterium that induces cytoplasmic incompatibility (Bordenstein *et al*., 2001; but see Breeuwer and Werren, 1995),. Currently no detailed study on the effects the two types of postzygotic reproductive isolation between species on the interspecific transmission of PSR has been carried out. Here we report that hybrid sterility and inviability between its donor and recipient do not influence the efficiency of the interspecific transmission of PSR in *Trichogramma*. However the transmission efficiency is not reciprocal and may be influenced by the host genetic background rather than the reproductive isolation.

Materials and methods

Trichogramma *species and lines*

T. deion and *T. kaykai* were originally collected from the Mojave Desert, California, USA. In the laboratory the two species were reared on *Ephestia kuehniella* (Lepidoptera: Pyralidae) eggs provided by Koppert B.V. at 23±1°C, 16:8 LD, and 60±10% RH.

A *Wolbachia*-infected parthenogenetic isofemale line of *T. deion* was collected from the Sidewinder Mountains, San Bernardino County, California, and is abbreviated to SWT. A sexual isofemale line was cured through antibiotic treatment, and is abbreviated to SWA. An infected PSR line is maintained in the SWT genetic background by mating a SWT female with an infected PSR male,

and is abbreviated to Inpsr^{d}. An uninfected PSR is obtained by mating a SWA female with an Inpsr^{d}, and is abbreviated to Unpsr^{d}. A second isofemale *T. deion* line used in these experiments was collected in Last Chance Canyon, Kern County, California, and is abbreviated to TDA. Three additional *T. deion* lines, i.e. SW1 (originally uninfected), SW2, SW3, were used for a limited number of experiments; these originated all from the Sidewinder Mountains, San Bernardino County, California.

A *Wolbachia*-infected parthenogenetic isofemale line of *T. kaykai* was collected in Last Chance Canyon, Kern County, California, and is abbreviated to LCT. A sexual isofemale line is derived from LCT by antibiotic treatment, and is shortened to LCA. An infected PSR line is maintained in the LCT genetic background by mating a LCT female with an infected PSR male, and is abbreviated to Inpsr^{k}. Uninfected PSR males are obtained by mating a LCA female with an Inpsr^{k} male, and are abbreviated to Unpsr^{k}.

Determination of reproductive isolation between the two species

To test if the two species were reproductively isolated, interspecific mating was performed using an originally uninfected isofemale line and two cured sexual isofemale lines of the two species (SWA, TDA: *T. deion* and LCA: *T. kaykai*). One day before the individuals emerged, parasitized *E. kuehniella* eggs were placed individually in glass tubes (7.5×1cm). When they emerged, individual females were confined with heterospecific males for 24 hrs. In case of intraspecific crosses, each couple was confined for 4 hrs. After the mating period, the males were removed from the tubes, and the females were allowed to lay eggs in 120～160 *E. kuehniella* eggs for about 72 hrs. Due to their haplo-diploid sex determination system, female offspring production is evidence of hybrid formation, because female offspring develop from fertilized diploid eggs. When the offspring emerged, their number and sex were determined under a stereomicroscope (magnification 10×). When females confined with heterospecific males produced normal numbers of male offspring compared to that of virgin conspecific females, they were assumed to have remained a virgin and consequently the offspring number and sex ratio of these broods were put in

prezygotic reproductive isolation. Three additional crosses between *T. deion* females and *T. kaykai* males were carried out as described above using three *T. deion* lines from Sidewinder Mountains.

Determination of hybrid status of female offspring in interspecific crosses

When an uninfected female mated with a heterospecific normal male produced female offspring, we assumed that the female offspring were hybrids. To determine if the females were indeed hybrids we used *Trichogramma*-specific microsatellites (TTG49: Forward 5′-GTAGTCTGGTTTTCGATTCCCA-3′, Reverse 5′-TCCCCGACCTATCGATTTTCC-3′). The temperature profile of the PCR reaction was 94ºC for 5 min (1 cycle); 94ºC for 1 min, 58ºC for 1 min and 72ºC for 1min (33 cycles) on a thermocycler (Mastercycler® gradient, eppendorf).

Determination of the interspecific transmission of PSR

To determine if the interspecific transmission of PSR took place efficiently, interspecific mating of females of the three isofemale lines with heterospecific infected and uninfected PSR males were performed. One day before the individuals of the three isofemale lines emerged, parasitized *E. kuehniella* eggs were placed individually in glass tubes (7.5×1cm). When they emerged, individual females were confined with heterospecific infected and uninfected PSR males for about 24 hrs. After the mating period, the males were removed from the tubes, and the females were allowed to lay eggs in 120～160 *E. kuehniella* eggs for about 72 hrs.

When an infected female mated with a PSR male produced male offspring, the male offspring were assumed to harbor PSR. When a SWT or a LCT female confined with a heterospecific PSR male produced all female offspring, the infected female was assumed to have remained unmated. Consequently, the offspring number and sex ratio of the broods of these females were not taken into account in the analysis of the transmission efficiency, but were seen as a case of prezygotic isolation.

For the uninfected line TDA, a different procedure was followed. Here we could not rely on the offspring sex ratio to distinguish between females that have mated

with a PSR male and those that remained virgin, because both of them would produce only male offspring. Only those females were used where we observed mating with PSR males and subsequently their offspring was tested on the presence of PSR using PCR with PSR specific primers (Van Vugt *et al.,* in prep). The temperature profile was 94°C for 2 min (1cycle); 94°C for 1min, 61°C for 1 min and 72°C for 1 min (35 cycles) on a thermocycler (Mastercycler® gradient, eppendorf).

In addition, we tested if infection with *Wolbachia* of the PSR males had an effect on the interspecific transmission by comparing the transmission efficiency of PSR via infected PSR males with that via uninfected ones.

Statistics

To analyze the number of offspring, T tests or ANOVAs were used. Tukey's b post hoc comparison tests were done to determine if there was variation in the number of offspring in the same maternal group. Mann Whitney U tests and Kruskall Wallis tests were applied for the analysis of sex ratio and fertilization rate. The hierarchical cluster analysis, a method to find relatively homogeneous clusters of cases, based on the variation of the number of offspring was applied to identify the coexistence of pre- and postzygotic isolation in interspecific crosses. All the analyses were performed on SPSS ver. 10.0.

Results

Hybrid formation in interspecific crosses

The results to test reproductive isolation between *T. kaykai* and *T. deion* are shown in Table 1. All the interspecific crosses are divided into two, i.e. prezygotic and postzygotic reproductive isolation. Females that did not mate or did not fertilize any eggs were identified as those having offspring numbers similar to those produced by virgin females. In prezygotic reproductive isolation females of SW2 mated with LCA males produced male biased sex ratios. It may be caused by the small sample sizes (Table 1). With exception of the line TDA all *T. deion* lines produced some "hybrid" female offspring which were produced in respectively 15 out of 20, 4 out of 20 and 8 out of 19 crosses (Table

1). But it seems that more SW2 and SW3 females mated with the heterospecific males based on the reduced number of offspring (Hierarchical cluster analysis) (Table1).

1) SWA female group

In the interspecific cross between SWA♀ and LCA♂ some of the fertilized eggs developed into female offspring (Table 1). The females carried the specific microsatellite of their father but the microsatellite marker of their mother was not present (Figure 1). Their phenotype reflected their paternal origin, in resembling *T. kaykai* females, which are yellow, while *T. deion* females, are brown. These females were sterile and carried a very low egg complement 1～7 eggs (N＝5) in their ovaries. All the introduced males of both species were immediately attracted to, and performed mating with these "hybrid" females within 30 minutes (N of hybrid♀×SWA♂＝8, and N of hybrid♀×LCA♂＝11). Even though they were sterile and contained few eggs, they showed normal egg-laying behavior (N＝7). "Hybrid" female production only was found when the mother was *T. deion*.

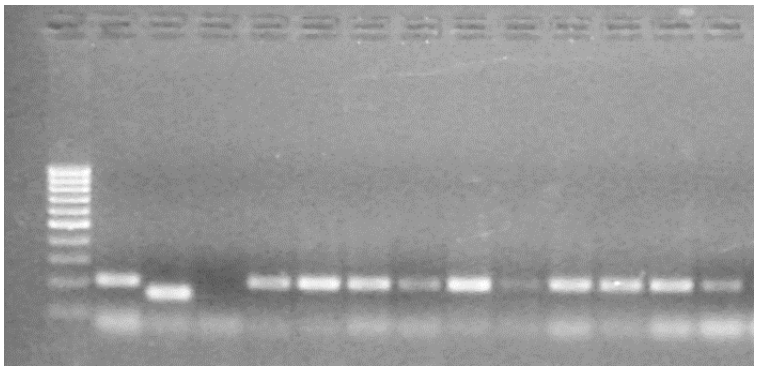

Figure 1. Photo of agarose gel showing the size of the species specific microsatellite markers.

T. kaykai (lane 2), *T. deion* (lane3) and females that were the offspring of a cross between *T. deion* females and *T. kaykai* males (SWA×LCA) (lane 5～14) First lane from the left: size ladder; Fourth: negative control

Table 1. Reproductive isolation between the two species

	♀×♂	N	M.♀	M.♂	M.O	%♂	Total
Control	SWA	19	0	31.0± 6.6	31.0± 6.6[a]	100	589
	SWA×SWA	18	24.2± 8.2	9.2± 2.7	33.4± 8.9[a]	27.6	602
	TDA	20	0	23.5±13.5	23.5±13.5[c]	100	470
	TDA×TDA	11	18.3± 7.1	9.4± 3.8	27.6±10.3[c]	33.9	304
	LCA	19	0	33.8±11.5	33.8±11.5[e]	100	642
	LCA×LCA	18	22.1± 8.9	6.9± 5.9	29.1± 9.6[e]	23.9	523
	SW1	19	0	45.7± 6.8	45.7± 6.8[g]	100	868
	SW1×SW1	19	33.0± 4.7	11.0± 4.7	44.0± 5.3[g]	24.9	835
	SW2	18	11.1± 4.8	4.7± 3.5	15.8± 5.2[i]	29.9	284
	SW3	20	14.0± 6.2	0	14.0± 6.2[k]	0	280
Prezygotic reproductive isolation	SWA×LCA	8	0	29.4± 8.3	29.4± 8.3[a]	100	235
	TDA×LCA	13	0	24.9± 5.3	24.9± 5.3[c]	100	323
	LCA×SWA	6	0	32.0± 6.0	32.0± 6.0[e]	100	192
	LCA×TDA	2	0	30.5±10.6	30.5±10.6[e]	100	61
	SW1×LCA	5	0	46.2± 4.0	46.2± 4.0[g]	100	231
	SW2×LCA	4	5.5± 6.6	8.5± 4.4	14.0± 4.6[i]	60.7	56
	SW3×LCA	6	14.7± 3.9	0	14.7± 3.9[k]	0	88
Postzygotic reproductive isolation	SWA×LCA	12	3.8± 2.5	8.8± 1.8	12.6± 3.3[b]	69.5	151
	TDA×LCA	9	0	8.7± 2.9	8.7± 2.9[d]	100	78
	LCA×SWA	10	0	4.4± 1.8	4.4± 1.8[f]	100	44
	LCA×TDA	8	0	4.4± 1.7	4.4± 1.7[f]	100	35
	SW1×LCA	15	10.7± 6.6	12.8± 7.8	23.6± 9.5[h]	54.5	354
	SW2×LCA	16(4)	a) 3.5± 2.6 b) 4.3± 3.8	1.9± 1.7	5.8± 1.9[j]	33.7	92
	SW3×LCA	13(8)	a) 7.5± 4.0 b) 3.6± 2.1	0	8.2± 5.9[l]	0	106

N: number of replications; M.♀: mean number of female offspring; M.♂: mean number of male offspring; M.O: mean number of offspring; %♂: sex ratio; Total: total number of offspring

In postzygotic reproductive isolation a) is non-hybrid female offspring, and b) is hybrid female offspring.

M.O followed by the same letter does not differ significantly from each other.

In SWA: ANOVA $P < 0.001$, Tukey's b test a, and b

In TDA: ANOVA $P < 0.001$, Tukey's b test c, and d

In LCA: ANOVA $P < 0.001$, Tukey's b test e, and f

In SW1: ANOVA $P < 0.001$, Tukey's b test g, and h.

In SW2: ANOVA $P < 0.001$, Tukey's b test i, and j

In SW3: ANOVA $P < 0.01$, Tukey's b test k, and l

In the SWA♀×LCA♂ cross, eight females that produced a normal brood size compared with the reference brood size of virgin SWA♀ and that of the SWA♀×SWA♂ cross (ANOVA $P>0.1$, Tukey's b test) appear not to have mated, or not to have used sperm if they had mated (Hierarchical cluster analysis).

In the postzygotic reproductive isolation cases we assume that the fertilization rate in the SWA♀×LCA♂ cross is the same as in the control SWA♀×SWA♂ cross. The mean number of male offspring 8.8 in the SWA♀×LCA♂ cross is similar to the number of males found in the SWA♀×SWA♂ cross (T test $P>0.1$). The number of female offspring, however, differs substantially with 24.2 in the SWA♀×SWA♂ cross and only 3.8 in the SWA♀×LCA♂ cross, indicating that a substantial number of the fertilized eggs in the interspecies cross died (Table 1). So, among the fertilized eggs the other about 84% of them died during development (Figure 2). The relative compatibility of an interculture cross (A♀×B♂) is expressed as (mean proportion of female offspring of A♀×B♂)×100/(mean proportion of female offspring of A♀×A♂) (Pinto *et al.*, 1997). In the SWA♀×LCA♂ cross the relative compatibility is 30.5×100/72.4 = 42.1.

2) TDA female group

TDA females mated with LCA males did not produce female offspring (Table 1). Like SWA females some of the TDA females appear to mate with LCA males.

In the TDA♀×LCA♂ cross, 13 out of 22 females produced a normal brood size. It indicates that they were produced by females that remained virgin (Hierarchical cluster analysis) (Table 1)

In the postzygotic reproductive isolation case all the fertilized eggs by heterospecific sperm are thought to die (Hierarchical cluster analysis). This is supported by the fact that there is no difference in the number of male offspring in the TDA♀×TDA♂ cross and that of the TDA♀×LCA♂ cross (T test $P>0.1$). In the TDA♀×LCA♂ cross the relative compatibility is 0.

3) LCA female group

In the LCA♀×SWA♂ cross and in the LCA♀×TDA♂ cross all the fertilized eggs are thought to die (Figure 2). In these crosses 6 females and 2 females, respectively,

produced normal brood sizes compared with the reference brood size of the LCA♀×LCA♂ cross, and are thought to have been cases where the females did not mate (Hierarchical cluster analysis) (Table 1).

The fertilization rate of the LCA♀×SWA♂ cross and the LCA♀×TDA♂ cross in the postzygotic reproductive isolation appear not to be different from that of the LCA♀×LCA♂ cross (Table 1, and Table 2). This is supported by the fact that there is no difference in the number of male offspring in the LCA♀×LCA♂ cross and those of the LCA♀×SWA♂ and the LCA♀×TDA♂ crosses (ANOVA $P>0.1$) (Table 1). In the LCA♀×SWA♂ and the LCA♀×TDA♂ crosses the relative compatibility is 0 respectively.

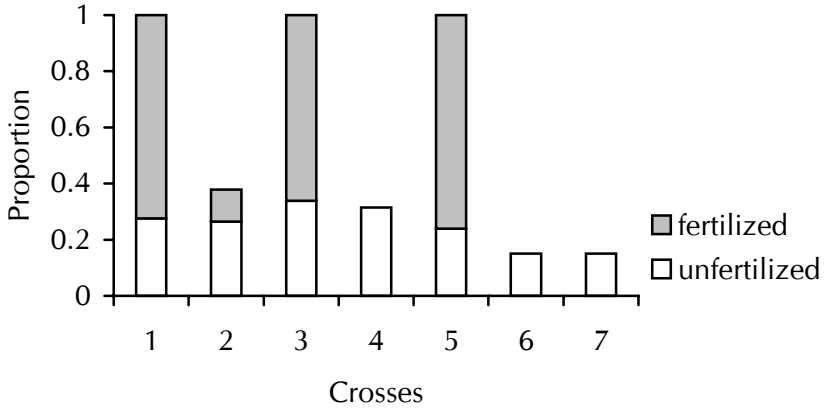

Figure 2. Estimated fertilization rate and hybrid survival rate of the heterospecific crosses (♀×♂).

1: SWA×SWA; 2: SWA×LCA; 3: TDA×TDA; 4: TDA×LCA; 5: LCA×LCA; 6: LCA×SWA; 7: LCA×TDA

Interspecific transmission of PSR

1) SWT female group

In SWT♀×Inpsr^{k}♂ and SWT♀×Unpsr^{k}♂ almost all eggs inseminated by sperm carrying PSR are thought to survive because the total numbers of offspring produced in these crosses are not different from that produced by SWT♀×SWA♂ (ANOVA $P>0.1$) (Table 2, and Figure 3). There is no difference in the sex ratio and the number of offspring between the SWT♀×Inpsr^{k}♂ cross and the SWT♀×Unpsr^{k}♂ cross (Mann Whitney U test $P>0.1$, and T test $P>0.1$

respectively). This implies that there is no influence of the *Wolbachia* infection in the Inpsr^{k} males on their ability to transmit the PSR factor to their offspring.

2) TDA female group

The brood sizes of the TDA♀×Inpsr^{k}♂ and the TDA♀×Unpsr^{k}♂ crosses are not different from that of TDA♀×TDA♂ implying that all the fertilized eggs survived (ANOVA $P>0.1$) (Table 2). In the TDA♀×Inpsr^{k}♂ cross, 117 male offspring (5 broods) and in the TDA♀×Unpsr^{k}♂ cross, 123 male offspring (5 broods) were used to determine the proportion of PSR among them. Using the PSR specific PCR, 78 out of 117 (67.5%) in the TDA♀×Inpsr^{k}♂ cross and 84 out of 123 (68.3%) in the TDA♀×Unpsr^{k}♂ cross proved to carry PSR. The fertilization rates of the two crosses are not different from that of TDA♀×TDA♂ (Kruskall Wallis test $P>0.1$).

3) LCT female group

Regardless of the infection status of the heterospecific PSR males, most eggs fertilized by heterospecific PSR sperm are thought to die (ANOVA $P<0.01$) (Table 2 and Figure 3). There is no difference in the sex ratio and the number of offspring between the LCT♀×Inpsr^{d}♂ cross and the LCT♀×Unpsr^{d}♂ cross (Mann Whitney U test $P>0.1$, and T test $P>0.1$ respectively). The similarity of the transmission rate of PSR between the LCT♀×Inpsr^{d}♂ cross and the LCT♀×Unpsr^{d}♂ cross implies that there is no effect of *Wolbachia* infection on the ability of the PSR males to transmit PSR, and that there is no interaction effect of the different PI-*Wolbachia* of the father (i.e. the *deion-Wolbachia*) and *Wolbachia* of the mother (i.e. the *kaykai-Wolbachia*).

Table 2. Crosses between *T. deion* and *T. kaykai* to determine the level of intra- and interspecific transmission of PSR.

♀×♂	N	M.♀	M.♂	M.O	%♂	Total
SWT	19	19.5± 4.6	0	19.5± 4.6[b]	0	370
SWT×SWA	19	24.4± 5.4	0	24.4± 5.4[a]	0	464
SWT×LCA	15	6.8± 2.3	0	6.8± 2.3[c]	0	102
SWT×Inpsr[k]	17	4.9± 2.2	18.8± 4.3	23.7± 4.3[a]	79.4	402
SWT×Unpsr[k]	15	5.1± 3.3	17.1± 4.7	22.2± 5.4[ab]	76.9	333
TDA	18	0	27.9± 7.3	27.9± 7.3[d]	100	503
TDA×TDA	18	16.7± 4.9	8.5± 2.7	25.2± 5.4[d]	33.8	453
TDA×Inpsr[k]	21	0	27.1± 5.4	27.1± 5.4[d]	100	569
TDA×Unpsr[k]	17	0	27.7± 9.7	27.7± 9.7[d]	100	470
LCT	19	17.6± 3.2	0	17.6± 3.2[f]	0	334
LCT×LCA	19	26.2± 5.1	0	26.2± 5.1[e]	0	498
LCT×SWA	12	5.8± 4.0	0	5.8± 4.0[g]	0	70
LCT×Inpsr[d]	7	4.0± 6.3	1.6± 0.8	5.6± 6.0[g]	28.2	39
LCT×Unpsr[d]	7	4.3± 5.1	1.9± 1.1	6.1± 4.5[g]	30.2	43

N: number of replications; M.♀: mean number of female offspring; M.♂: mean number of male offspring; M.O: mean number of offspring; %♂: sex ratio; Total: total number of offspring

M.O followed by the same letter does not differ significantly from each other.

In SWT: ANOVA $P < 0.001$, Tukey's b test a, b and c

In TDA: ANOVA $P > 0.1$, Tukey's b test d

In LCT: ANOVA $P < 0.001$, Tukey's b test e, f and g

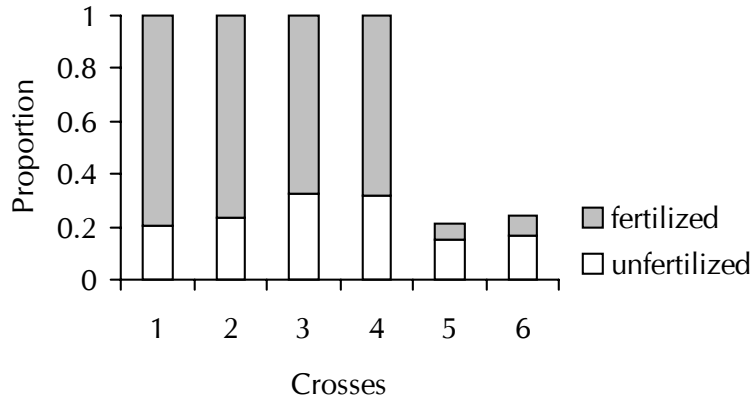

Figure 3. Transmission efficiency of PSR and mortality of fertilized eggs in the two *T. deion* female groups (♀×♂).

1: SWT× Inpsr[k]; 2: SWT× Unpsr[k]; 3: TDA× Inpsr[k]; 4: TDA×Unpsr[k]; 5: LCT×Inpsr[d]; 6: LCT×Unpsr[d]

In the figure the bars of TDA×Inpsr[k] and TDA×Unpsr[k] are based on the PCR results. The others bars are based on the sex ratio in table 3

Discussion

Cross-incompatibilities between *T. deion* and *T. kaykai* are complex; first prezygotic reproductive isolation; second hybrid sterility; and third hybrid inviability. There is a substantial level of prezygotic reproductive isolation between these two species. For *T. deion* females, a total of 8/20 SWA females and 13/20 TDA females appeared not to have mated or at least did not use the sperm of the LCA (*T. kaykai*) male. In SW2, 4 out 16 females mated with LCA males produced hybrid offspring. In the other 12 females all the fertilized eggs appear to have died. In SW3 8 females produced some hybrid daughters. It implies that "hybrid" offspring production depends on the individual level in the isofemale lines. In the reverse cross between *T. kaykai* females and *T. deion* males the numbers were 6/16 when exposed to SWA males and 2/10 for TDA males. The postzygotic isolation generally manifested itself in the almost complete inviability of the fertilized eggs. The exception was the production of hybrid female offspring in the *T. deion* female×*T. kaykai* male crosses, because in 4 out of 5 isofemale lines tested, some eggs inseminated by *T. kaykai* sperm developed into females. All these "hybrid" females were sterile. The lack of the maternal marker in the female offspring of these crosses is remarkable. We tested to determine if the maternal marker would be less amplified in the PCR reaction by mixing DNA extracted from both lines in equal amounts. The results showed that the maternal marker was equally well amplified in the mixture as the paternal marker. Therefore, it seems unlikely that in the "hybrid" female offspring the maternal marker was present. An alternative hypothesis is that for some reason 1) the maternal genome does not participate in the formation of the zygote and 2) subsequently the paternal genome undergoes a diploidization leading to diploid female offspring. In this latter hypothesis the fact that these paternally derived females are sterile would then be caused by the incompatibility of the *kaykai* genome with the *deion* mitochondria. It is suggested that in *Nasonia* hybrid breakdown may be due to negative epistatic interactions between mitochondria and nuclear genes, because nuclear genes encode mitochondrial proteins (Breeuwer and Werren, 1995). It may apply to the *Trichogramma* case. The nuclear genes of *T. kaykai* and the mitochondria of

T. deion may be compatible enough to rescue some fertilized eggs. The mechanism proposed here for the explanation of the "hybrid" females is speculative and cytogenetic studies need to be done to understand the exact nature of these paternally derived females in the interspecific cross.

We tested if the "hybrid" production of *T. deion* females in the interspecific crosses was related with the fact that the line was originally infected with *Wolbachia*. But two originally uninfected *T. deion* lines also produced females when mated with *T. kaykai* males. Therefore, the hybrid formation may occur between many lines of these species, although no hybrids were found in an earlier study (Pinto *et al.*, 1997). Crossing patterns in *T. deion* show a large amount of interspecific variation (Stouthamer *et al.*, 1996). Geographic variation may exist in the expression of these "hybrid" females because all of the cases of "hybrid" female formation stem from the same collection site. The line TDA was collected from the same site where the *kaykai* line was collected. This indicates that there may be selection on mate discrimination or isolation mechanisms between the two species in the same area. Quantitative data are needed to determine the level of isolation between the two species.

Our results show that the postzygotic reproductive isolation does not appear to have an effect on the interspecific transmission of PSR. We find a more or less equal postzygotic isolation between the lines with a somewhat lower isolation when *T. deion* females mate with *T. kaykai* males. However, in the transmission of PSR the asymmetry between these species becomes much more pronounced. The transmission of PSR from *T. kaykai* males to *T. deion* females is much more efficient than the transmission in the opposite direction. In the cross between *T. deion* PSR males and *T. kaykai* females, many of the fertilized eggs die, but in the reciprocal cross between *T. kaykai* PSR males and *T. deion* females, many of the eggs fertilized by *T. kaykai* PSR sperm are rescued. This difference may be caused by the inefficient transmission of PSR in *T. deion*. In *T. deion* PSR males produce normal PSR-free sperm as well as sperm harboring PSR (Chapter 4 and 5). Even so in this study about 90% of the fertilized eggs by $Inpsr^d$ and $Unpsr^d$ seem to die compared with the brood size of $LCT_{♀}\times LCA_{♂}$. This is very high and is most likely caused by an additional effect of the cross incompatibility between

T. kaykai females and *T. deion* males which even overcomes the rescuing ability of PSR.

In *Nasonia,* PSR was successfully transmitted from *N. vitripennis* to *N. giraulti* and subsequently to *N. longicornis* (Dobson and Tanouye, 1998). In that system the donor species and recipient species produced hybrid offspring, although there is haploid hybrid inviability in the F_2 between *N. vitripennis* and *N. giraulti* (Breeuwer and Werren, 1995). The reason why PSR was not transmitted directly from *N. vitripennis* to *N. longicornis* was prezygotic reproductive isolation between the two species (Dobson and Tanouye, 1998; van den Assem and Werren, 1994). However, a recent study showed that hybrid inviability between *N. giraulti* and *N. longicornis* was caused by cytoplasmic bacterial infections, not by their genetic backgrounds. The two species are infected with different strains of *Wolbachia* causing bidirectional cytoplasmic incompatibility. When the bacteria are removed, the two species are compatible (Bordenstein *et al.*, 2001).

The study on the PSR in *Nasonia* and this study show that PSR can easily be transmitted interspecifically when there is limited prezygotic reproductive isolation between the donor and the recipient species at least in *Nasonia* and *Trichogramma*. The ability of PSR of moving across species boundaries is attractive and PSR is considered as a potential biological control agent or a vehicle for genetic modification for this reason (Werren and Stouthamer, 2003). PSR is expected to be more common in haplodiploid organisms than the two cases recognized so far (Werren and Stouthamer, 2003). At present, we can conclude that both PSR chromosomes known so far seem to be easily transmitted to closely related taxa.

Acknowledgements

Peter de Jong is appreciated for his careful review. We thank Ties Huigens for his useful comments on earlier versions of the manuscript, Leo Koopman and Koppert B.V. for providing *E. kuehniella* eggs. Peter de Jong and Leo Koopman are acknowledged for sharing experimental space. This study is supported in part by NEXGEN Biotechnologies. Inc. (Korea).

References

Bordenstein S.R., O'Hara F.P., and Werren J.H., 2000, *Wolbachia*-induced incompatibility precedes other hybrid incompatibilities in *Nasonia*. *Nature*. **409**:707-710.

Breeuwer J.A.J., and Werren J.H., 1995, Hybrid breakdown between two haplodiploid species: The role of nuclear and cytoplasmic genes. *Genetics*. **49**(4):705-717.

Coyne J.A., and Orr H.A., 1989, Patterns of speciation in *Drosophila*. *Evolution*. **43**(2):362-381.

Dobson S.L., and Tanouye M.A., 1998, Interspecific movement of the paternal sex ratio chromosome. *Heredity*. **81**:261-269.

Haldane J.B.S., 1922, Sex ratio and unisexual sterility in hybrid animals. *J. Genet*. **12**:101-109.

Orr H.A., 1997, Haldane's rule. *Annu. Rev. Ecol. Syst*. **28**:195-218.

Huigens M.M.E., 1998, *Identification of PSR-carrying* Trichogramma *species*. M.Sc. thesis. Wageningen University.

Huigens M.E., 2003, *On the evolution of* Wolbachia*-induced parthenogenesis in* Trichogramma *wasps*. Ph.D. thesis. Wageningen University.

Hulskes A., 2002, *The origin of the paternal sex ratio chromosome in* Trichogramma kaykai. M.Sc. thesis. Wageningen University.

Pinto J.D., Stouthamer R., and Platner G.R., 1997, A new cryptic species of *Trichogramma* (Hymenoptera: Trichogrammatidae) from the Mojave Desert of California as determined by morphological, reproductive and molecular data. *Proc. Entomol. Soc. Wash*. **99**(2):238-247.

Reed K.M., and Werren J.H., 1995, Induction of paternal genome loss by the paternal sex ratio chromosome and cytoplasmic incompatibility bacteria (*Wolbachia*): a comparative study of early embryonic events. *Mol. Reprod. Dev*. **40**:408-418.

Stouthamer R., Luck R.F., Pinto J.D., Platner G.R., and Stephen B., 1996, Non–reciprocal cross-incompatibility in *Trichogramma deion*. *Entomol. Exp. Appl*. **80**:481-489.

Van den Assem J., and Werren J.H., 1994, A comparison of the courtship and mating behavior of three species of *Nasonia* (Hymenoptera, Pteromalidae). *J. Insect Behav*. **7**:53-66.

Werren J.H., Nur U., and Eickbush D., 1987, An extrachromosomal factor causing loss of paternal chromosomes. *Nature*. **327**:75-76.

Werren J.H., and Stouthamer R., 2003, PSR (paternal sex ratio) chromosomes: the ultimate selfish genetic elements. *Genetica*. **117**:85-101.

Wu C.I., 1992, A note on Haldane's rule: Hybrid inviability versus hybrid sterility. *Evolution*. **46**(5):1584-1587.

Wu C.I., Johnson N.A., and Palopoli M.F., 1996, Haldane's rule and its legacy: why are there so many sterile males? *Trends Ecol. Evol*. **11**(7):281-284.

Wu C.I., and Palopoli M.F., 1994, Genetics of postmating reproductive isolation in animals. *Annu. Rev. Genet*. **27**:283-308.

Chapter 4

Inefficient transmission of the paternal sex ratio chromosome of *Trichogramma kaykai* in the novel host *T. deion*

Inefficient transmission of the paternal sex ratio chromosome of *Trichogramma kaykai* in the novel host *T. deion*

Abstract

Evolutionary interests of the two sex ratio distorters, the *Wolbachia* bacterium and the paternal sex ratio chromosome (PSR), found in *Trichogramma* are antagonistic due to their different transmission strategies. *Wolbachia* infection induces parthenogenesis in this genus and results in all-female broods, and PSR is only transmitted via male and results in male biased broods. The transmission efficiency of PSR in *T. kaykai*, a natural host, and in *T. deion*, a novel host, was investigated. In *T. kaykai*, PSR is efficiently transmitted regardless of the *Wolbachia* infection status of the male. In *T. deion*, however, PSR males also father female offspring. The female production by PSR fathers strikingly increases when they are infected with *Wolbachia*. The results show that the transmission efficiency of PSR is dependent on the host genetic background and that the sex ratio distorter -*Wolbachia*- plays a role as a repressor of another sex ratio distorter- PSR- in the novel host.

Keywords

Trichogramma, Parthenogenesis inducing *Wolbachia*, PSR, host genetic background, genetic conflict

Introduction

In many taxa uninvited passengers share the cellular space with the genome of their host. Among such passengers are selfish genetic elements that disrupt the Mendelian inheritance to their own advantage and are either neutral or detrimental to the host. They have been recognized for over 75 years (Werren *et al.,* 1988; Hurst and Werren, 2001) and include: cytoplasmically inherited bacteria, meiotic drive chromosomes, homing endonuclease genes, transposable elements and B-chromosomes (Werren and Beukeboom, 1998; Gimble, 2000). When these elements are uniparentally inherited, they can provoke genetic conflict between genetic elements of maternal and paternal origin (Cosmides and Tooby, 1981; Werren *et al.,* 1988). Many very basic processes may be the outcome of such conflicts. Genetic systems, from patterns of gene expression, through variation in copy number of multicopy genes, to non-Mendelian patterns of inheritance and the amount of DNA and recombination are proposed as evolutionary consequences of the conflict (Hurst *et al.,* 1992). Selfish genetic elements have been implicated as contributing to eukaryotic speciation, extinction, and the structure of genetic systems (Hurst and Schilthuizen, 1998; Hurst and Werren, 2001). In addition to the structural and functional aspects of gene evolution, recent studies expand genetic conflicts as a factor that affects social behavior of insects (Keller and Ross, 1998; Abbot *et al.,* 2001). Amid selfish genetic elements, sex ratio distorters are the factors that promote their own transmission through changing the offspring sex ratio of their hosts. They can reside either on the nuclear chromosomes or in the cytoplasm of their hosts (Stouthamer *et al.,* 2002).

Trichogramma (Hymenoptera: Trichogrammatidae) is a parasitoid genus, worldwide in distribution and consisting of more than 190 described species (Pinto, 1999; Querino and Zucchi, 2003a; Querino and Zucchi, 2003b). Parasitism by *Trichogramma* species is known from eggs of Lepidoptera, Coleoptera, Diptera, Hemiptera, Homoptera, Hymenoptera and Neuroptera (Pinto and Stouthamer, 1994). In *Trichogramma* species two sex ratio distorters are known, the *Wolbachia* bacterium and the paternal sex ratio chromosome (PSR) (Stouthamer, 1997; Stouthamer *et al.*, 2001). *Wolbachia* is a

cytoplasmically inherited endosymbiont that manipulates their host's reproduction in several ways, but in *Trichogramma* it causes complete parthenogenetic reproduction (thelytoky) where virgin females produce daughters (Stouthamer, 1997). PSR is a B-chromosome that is an extremely selfish genetic element (Beukeboom and Werren, 1993; Camacho *et al.,* 2002; Werren *et al.,* 1988; Werren and Stouthamer, 2003). PSR is only found in hosts with a haplodiploid sex determination system. PSR destroys the paternal chromosome set in fertilized eggs and PSR itself survives this paternal chromosome destruction. This process results in the production of males that have the haploid maternal chromosome set with PSR from the father (Werren *et al.,* 1987; van Vugt *et al.,* 2003).

Trichogramma is so far the only known taxon infected with parthenogenesis inducing (PI)-*Wolbachia* where we find a coexistence of *Wolbachia* infected and uninfected individuals. In such mixed populations infected females mate with males and use their sperm to fertilize their eggs at a similar rate as uninfected females do (Huigens, 2003). Infected fertilized eggs develop into infected heterozygous females, infected unfertilized eggs however develop into infected homozygous females (Stouthamer and Kazmer, 1994). When *Wolbachia* infected eggs are fertilized by sperm harboring PSR, the eggs develop into *Wolbachia* infected PSR males (Stouthamer *et al.,* 2001).

While *Wolbachia* are transmitted only through female offspring, PSR is exclusively inherited through males. The presence of the PSR element in the species *Trichogramma kaykai* is thought to keep the *Wolbachia* infection from going to fixation in the population (Stouthamer *et al*., 2001). *T. kaykai* coexists in field populations with a taxonomically closely related species *T. deion* (Pinto *et al.,* 1997) and both species parasitize the same host species. So far PSR has only been detected in *T. kaykai* populations (Huigens *et al*., 2003), despite the fact that it can be readily transmitted to *T. deion* in interspecific crosses (Hulskes, 2002; Chapter 3). Under laboratory conditions PSR can be maintained in *T. deion*. The frequency that PSR can attain in field populations depends on the population structure and fertilization rate (Werren and Beukeboom, 1993; Stouthamer *et al*., 2001). PSR can attain higher frequencies in field populations

when it occurs in populations where female biasing sex ratio distorters are also found. In case of the *Nasonia* PSR the sex ratio distorting factor MSR (maternal sex ratio) plays an important role in maintaining PSR in the populations (Werren and Beukeboom, 1993), similarly the presence of PI-*Wolbachia* allows PSR to attain higher frequencies in *T. kaykai* (Stouthamer *et al.*, 2001).

With all the requisites for success, the fact that we do not find PSR in *T. deion* field populations needs an explanation. We investigated the interactions of the two sex ratio distorters in the natural host, *T. kaykai*, and the novel host, *T. deion*. Here we report that the transmission of PSR in *T. kaykai* is efficient, while in *T. deion*, the transmission efficiency is much lower in addition there is a significant difference in the transmission of PSR through *Wolbachia* infected males versus uninfected PSR males.

Materials and methods

Trichogramma *species and lines*

T. kaykai and *T. deion* were originally collected in the Mojave Desert, California, USA, from parasitized eggs of the butterfly, *Apodemia mormo deserti* (Lepidoptera; Lycaenidae). In the laboratory the two species have been reared on *Ephestia kuehniella* (Lepidoptera: Pyralidae) eggs provided by Koppert B.V. at 23±1°C, 16:8 LD, and 60±10 % RH.

In our experiments we used one *T. kaykai* and one *T. deion* isofemale line each originally collected as *Wolbachia* infected (thelytokous) females. Each of these *T. kaykai* and *T. deion* lines was cured of their *Wolbachia* infection through antibiotic treatment and subsequently these lines reproduce by the normal mode of reproduction in Hymenoptera: arrhenotoky.

In *T. kaykai* the thelytokous (unisexual) isofemale line infected with *Wolbachia* collected at Last Chance Canyon, Kern County, California, is abbreviated to LCT. The arrhenotokous (bisexual) isofemale line is cured through the antibiotic treatment is abbreviated to LCA. The infected PSR line is maintained on the LCT genetic background by mating a LCT female with an infected PSR male is shortened to Inpsr^k. The uninfected PSR males are obtained by mating a LCA female with an Inpsr^k male and are abbreviated to Unpsr^k.

In *T. deion* the thelytokous isofemale line infected with *Wolbachia* collected at Sidewinder Mountains, San Bernardino County, California, is abbreviated to SWT. The arrhenotokous isofemale line is cured through the antibiotic treatment is abbreviated to SWA. The infected PSR line is maintained on the SWT genetic background by mating a SWT female with an infected PSR male and is shortened to Inpsr^{d}. The uninfected PSR males are obtained by mating a SWA female with an Inpsr^{d} male and are abbreviated to Unpsr^{d}.

Detection of* Wolbachia *and PSR

Genomic DNA of wasps was extracted with 50µl of 5% Chelex-100 solution and 2µl of proteinase K and incubated for 3 hours at 56°C and for 10 min at 95°C (Mastercycler® gradient, eppendorf).

To determine infection with *Wolbachia*, PCR with a *Wolbachia*-specific primer (*wsp*) was performed. The temperature profile was 94°C for 3 min (1cycle); 94°C for 1min, 50°C for 1 min and 72°C for 1 min (40 cycles) on a thermocycler (Mastercycler® gradient, eppendorf) (Braig *et al.,* 1998).

The PCR with a PSR-specific primer set (Van Vugt *et al*., in prep) was performed in the experiment of sex ratio shift by day and the effect of male age, while in all the other experiments the mating method was used for determining the presence of PSR. The temperature profile was 94°C for 2 min (1cycle); 94°C for 1min, 61°C for 1 min and 72°C for 1 min (35 cycles) on a thermocycler (Mastercycler® gradient, eppendorf).

***Transmission efficiency of PSR in* T. kaykai *and* T. deion**

T. kaykai. One day before the wasps of each group emerged, parasitized *E. kuehniella* eggs were taken off of the egg cards and were placed individually in glass tubes (7.5×1cm). Freshly emerged wasps, less than 24 hour old, were used for the crossing experiment (Table 1a). Each mating pair was confined for about 4 hours in a tube so that mating could take place. After the mating period, male wasps were taken out of the tubes and were frozen to later extract DNA to determine the *Wolbachia* infection status and whether a male carried PSR. After 1 day each female was given an egg card containing 120～160 *E. kuehniella*

eggs to allow oviposition for about 72 hours. After egg-laying all the females were removed from the tubes and genomic DNA was extracted from the females to determine their *Wolbachia* infection status.

Table 1a. Mating scheme and the possible outcome of the crosses

Crosses (*T. kaykai*)	Expected outcome	
	Fertilized egg	Unfertilized egg
♀ Un× ♂ Un	♀ Un	♂ Un
♀ In × ♂ Un	♀ In	♀ In
♀ Un× ♂ Inpsr	♂ Unpsr	♂ Un
♀ In × ♂ Inpsr	♂ Inpsr	♀ In
♀ Un× ♂ Unpsr	♂ Unpsr	♂ Un
♀ In × ♂ Unpsr	♂ Inpsr	♀ In

T. deion. The experimental set up in *T. deion* was the same as for *T. kaykai* but, instead of a single mating we allowed each infected and uninfected PSR male to mate twice, first with an uninfected female and then an infected female (Table 1 b).

Table 1b. Mating scheme and the possible outcome of the crosses

Crosses (*T. deion*)	Expected outcome	
	Fertilized egg	Unfertilized egg
♀ Un× ♂ Un	♀ Un	♂ Un
♀ In × ♂ Un	♀ In	♀ In
♀ Un× ♂ Inpsr	♂ Unpsr	♂ Un
♀ In × ♂ Inpsr	♂ Inpsr	♀ In
♀ Un× ♂ Unpsr	♂ Unpsr	♂ Un
♀ In × ♂ Unpsr	♂ Inpsr	♀ In

Proportion of PSR among offspring

Because there are no morphological differences between non-PSR males and PSR males, the proportion of PSR among offspring was determined as follows.

1) When an infected mother (LCT or SWT) mated with a PSR father (Inpsr or Unpsr) produced male offspring, we assumed that the father and the male offspring carried PSR.
2) Broods produced by uninfected mothers mated with PSR fathers should consist of males only because unfertilized eggs develop into normal (non-

PSR) males, and fertilized eggs should develop into PSR males. To determine the proportion of male offspring that carried PSR we crossed the male offspring with infected females. One day after mating, the females were given host eggs to parasitize. When a female produced male offspring, we assumed that her male mate carried PSR.

***Relationship between* Wolbachia *infection status of PSR males and the sex ratio in* T. deion**

To determine the effect of *Wolbachia* infection on the sex ratio and on PSR transmission in *T. deion*, each PSR male was allowed to mate first with an uninfected female. As soon as we observed that the males mated with uninfected females, they were transferred into another tube containing an infected female, and confined for about 4 hours to mate. When their offspring emerged, the offspring sex ratios produced by the uninfected female and the infected female mated with an identical PSR male were paired and plotted. We selected sex ratio pairs based on the male offspring production by infected females mated with PSR males.

Sex ratio shift by day and the effect of male age

Some incompatibility effects caused by the cytoplasmic incompatibility inducing (CI) *Wolbachia* in *Drosophila melanogaster* are dependent on the age of the father, while young fathers inducing a high level of incompatibility, and older fathers having hardly any effect (Reynolds *et al.*, 2003). We examined if a possible effect of the *Wolbachia* infection on the PSR transmission in *T. deion* was age dependent. In this test each Inpsr^{d} male and Unpsr^{d} male was given a mating opportunity with a SWA female and after two hours interval with a SWT female. The females in each experimental set up were allowed to parasitize *E. kuehniella* eggs immediately after mating between SWT females and PSR males. The mean sex ratio of the offspring of each cross was determined for the three days that the wasps were allowed to produce offspring.

The Inpsr^{d} males were allowed to mate at the age of 1 day with an SWA female and again at the age of 7 days to check the effect of male age on the sex ratio of

their offspring. After mating the males were removed and each female was given an egg card containing 120～160 *E. kuehniella* eggs to allow oviposition for about 72 hours. Genomic DNA was extracted from the males to confirm that they carried PSR. The offspring sex ratios of the one day old fathers were compared with those when they were seven days old.

In these two tests all the matings were observed under a stereomicroscope (magnification 10×)

Statistics

To analyze the number of offspring, T tests and ANOVAs were used. Tukey's b post hoc comparison tests were done to determine if there was variation in the number of offspring in the same maternal group. Wilcoxon signed ranks test and Friedman's test were applied for the analysis of sex ratio. Spearman's nonparametric correlation analyses were performed for determining correlation in sex ratios produced by infected and uninfected PSR males and sex ratio variation by aging of infected PSR males. All the analyses were performed on SPSS ver. 10.0.

Results

Detection of* Wolbachia *and PSR among the individuals used

The groups of LCT, SWT, Inpsr^{k} and Inpsr^{d} were infected with *Wolbachia,* while the groups of LCA, SWA, Unpsr^{k} and Unpsr^{d} were uninfected. The group of virgin LCA and SWA females produced only male offspring. The group of virgin LCT and SWT females produced female offspring, but virgin LCT females also produced some male offspring most probably due to inefficient transmission of the bacteria (Table 2).

Table 2. Mean values with standard deviation of female and male and total offspring of the crosses

♀×♂	N	M.♀	M.♂	M. O	%♂	Total
LCA	22	0	33.0±11.8	33.0±11.8^{ab}	100	726
LCA×LCA	19	31.5± 6.5	6.6± 1.8	38.1± 7.5^{a}	17.3	723
LCA×Inpsr^{k}	23	0	35.8±11.7	35.8±11.7^{ab}	100	824
LCA×Unpsr^{k}	17	0	26.9±12.5	26.9±12.5^{b}	100	457
LCT	17	14.4± 6.1	1.7± 4.8	16.2± 5.1^{c}	16.2	275
LCT×LCA	22	15.6± 7.5	0.3± 1.1	15.9± 7.3^{c}	15.9	350
LCT×Inpsr^{k}	20	4.1± 3.1	17.4± 8.3	21.4± 7.8^{d}	81.1	428
LCT×Unpsr^{k}	11	4.8± 2.2	21.0±10.5	25.8±10.1^{d}	81.3	284
SWA	20	0	50.8± 5.9	50.8± 5.9^{e}	100	1016
SWA×SWA	21	31.6± 5.9	10.9± 5.7	42.5± 8.3^{f}	25.6	892
SWA×Inpsr^{d}	19	15.4±11.5	29.2±13.5	43.3± 5.3^{f}	65.4	981
SWA×Unpsr^{d}	11	4.1± 3.8	34.1± 8.1	38.2±10.2^{f}	89.3	420
SWT	20	26.5± 8.9	0	26.5± 8.9^{g}	0	530
SWT×SWA	17	31.6±10.9	0	31.6±10.9^{g}	0	537
SWT×Inpsr^{d}	22	19.6±11.0	9.1± 8.1	29.7± 8.9^{g}	31.5	631
SWT×Unpsr^{d}	11	13.1± 5.2	19.7± 5.4	32.8± 5.2^{g}	60.1	361

N: number of replications; M.♀: mean number of female offspring ± standard deviation; M.♂: mean number of male offspring ± standard deviation; M. O: mean number of offspring ± standard deviation; %♂: percent of male offspring among Total; Total: total number of offspring;

LCA: uninfected *T. kaykai*; LCT: infected *T. kaykai*; SWA: uninfected *T. deion*; SWT: infected *T. deion*; Inpsr^{k}: infected *T. kaykai* PSR male; Unpsr^{k}: uninfected *T. kaykai* PSR male; Inpsr^{d}: infected *T. deion* PSR male; Unpsr^{d}: uninfected *T. deion* PSR male

M.O followed by the same letter does not differ significantly from each other.

In LCA: ANOVA $P<0.05$, Tukey's b test a, and b

In LCT: ANOVA $P<0.001$, Tukey's b test c, and d

In SWA: ANOVA $P<0.001$, Tukey's b test e, and f

In SWT: ANOVA $P>0.1$, Tukey's b test g

Uninfected PSR males emerge in broods with normal males, and had to be identified later using the sex ratio of their offspring when mated with an infected female. In table 1 only the confirmed uninfected PSR male matings are shown. In the cross of an uninfected female with an infected PSR male, no offspring were infected with *Wolbachia* confirming the lack of paternal transmission of the bacteria.

***Proportion of PSR among offspring of* T. kaykai *and* T. deion**

The transmission of PSR is efficient in *T. kaykai* resulting in 100% male offspring. However, in *T. deion*, PSR males produced daughters as well as PSR sons (Table 3). This causes the relatively low transmission efficiencies of PSR via infected and uninfected PSR males in *T. deion* compared to *T. kaykai*. In addition, more daughters are produced when the PSR father is infected with *Wolbachia* (Table 3).

Table 3. The number and the proportion of PSR of the crosses

♀×♂	N	No.♀	No.♂	♂ tested	No. PSR	% PSR	eP	eP+No.♀	Te
LCA×Inpsr^{k}	13	0	594	447	311	69.6	413.4	413.4	100
LCA×Unpsr^{k}	12	0	402	329	222	67.5	271.4	271.4	100
SWA×Inpsr^{d}	13	157	411	317	186	58.7	241.2	398.2	60.6
SWA×Unpsr^{d}	11	45	375	302	177	58.6	219.8	264.8	83.0

N: number of replications; No.♀, No.♂: Number of female and male offspring; ♂ tested: Number of male offspring used to determine the proportion of PSR among them; No. PSR: Number of PSR males among ♂ tested; % PSR: The percentage of PSR among the male offspring; eP: Estimated number of PSR among the male offspring = No.♂×% PSR×0.01; eP+No.♀: Estimated number of PSR + number of female offspring; Te: Transmission efficiency of PSR =eP/eP+No.♀×100

Fertilization rate and the proportion of PSR in offspring

Due to the development of fertilized eggs into PSR males, the proportion of PSR can be referred to as the fertilization rate if all fertilized eggs develop into PSR males. To calculate the fertilization rates the following abbreviations are used: No.♀, No.♂: Number of female and male offspring; ♂ tested: Number of male offspring used to determine the proportion of PSR among them; No. PSR: Number of PSR males among ♂ tested; % PSR: The percentage of PSR among ♂ tested; eP: Estimated number of PSR among the male offspring = No.♂×%PSR×0.01; eP+No.♀: Estimated number of PSR + number of female offspring. (1) When the father is a normal male, the fertilization rate is calculated as fertilization rate = No.♀/(No.♀+No.♂)×100; (2) when the mother is uninfected and the father is PSR regardless of the infection status of the father = (eP+No.♀)/(No.♀+No.♂)×100; (3) when the mother is infected and the father is

PSR regardless of the infection status of the father = eP/(No.♀ + No.♂)×100 (Figure 1).

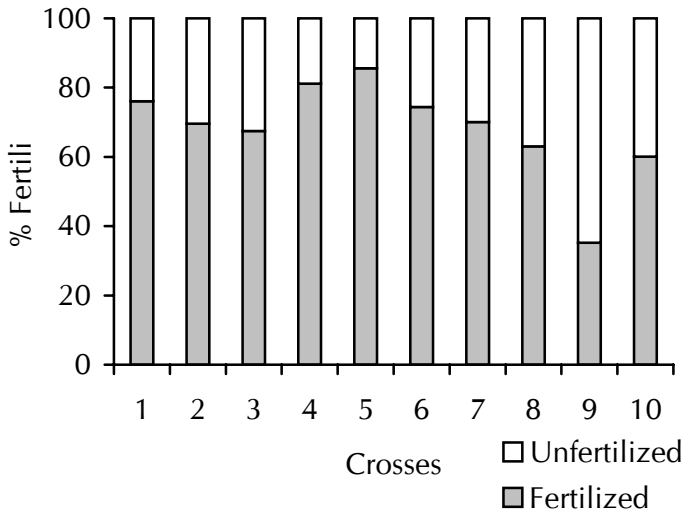

Figure 1. Fertilization rate by the proportion of PSR

% Fertili: Fertilization rate =formula 1 – in case of 1, 6; = formula 2 – in case of 2, 3, 7, 8; = formula 3 – in case of 4, 5, 9, 10. The 3 formulae can be found in the text.

1. LCA♀×LCA♂ (76.1%), 2. LCA♀×Inpsr^{k}♂ (69.6%), 3. LCA♀×Unpsr^{k}♂ (67.5%), 4. LCT♀×Inpsr^{k}♂ (84.2%), 5. LCT♀×Unpsr^{k}♂ (86.5%), 6. SWA♀×SWA♂ (74.4%), 7. SWA♀×Inpsr^{d}♂ (70.1%), 8. SWA♀×Unpsr^{d}♂ (63%), 9. SWT♀×Inpsr^{d}♂ (35.2%), 10. SWT♀×Unpsr^{d}♂ (60.1%). In 9 and 10 the rates will likely be larger than the actual values, because some of the fertilized eggs develop into female offspring, and all the females were counted as from unfertilized eggs.

In *T. kaykai* when PSR males mate with infected females, fertilization rate increases compared to that of uninfected females, this is most likely caused by the higher mortality rate of the unfertilized infected eggs (Tagami *et al.*, 2001). This is corroborated by the fact that the absolute number of the fertilized eggs is the same in uninfected and infected females (T test $P > 0.1$). In *T. deion*, the SWT♀×Inpsr^{d}♂ cross shows a low fertilization rate likely caused by the fact that some of the fertilized eggs developed into female offspring rather than PSR male offspring (Figure 1 and Table 4).

Female development following fertilization with sperm from a PSR male

In *T. kaykai* no LCA females mated with PSR males produced female offspring confirming that PSR was efficiently transmitted in the species (Table 4).

In *T. deion,* however, female offspring were also fathered by Inpsr[d] and by Unpsr[d] males. Infected PSR males produced more daughters than uninfected PSR males (ANOVA $P<0.01$); 45.8% of the uninfected eggs fertilized by infected PSR sperm developed into female offspring (Table 4). This shows that PSR males produce normal sperm as well as PSR sperm, probably because of abnormal mitotic division of PSR in spermatogenesis in *T. deion*.

Table 4. Estimated values of PSR males and females developed from fertilized eggs

♀×♂	N	No.♀	No.♂	Total	%♂	E PSR	% E PSR	% F af
LCA×Inpsr[k]	18	0	744	744	100	517.8	69.6	0
LCA×Unpsr[k]	16	0	534	534	100	360.5	67.5	0
LCT×Inpsr[k]	19	66	347	413	84.2	347	84.2	0
LCT×Unpsr[k]	15	53	286	339	86.5	286	86.5	0
SWA×Inpsr[d]	19	264	559	823	67.9	312.9	38	45.8
SWA×Unpsr[d]	11	45	375	420	89.3	219.6	52.4	17
SWT×Inpsr[d]	19	366[a]	199	565	35.2	199	35.2	64.8[b]
SWT×Unpsr[d]	11	144[a]	217	361	60.1	217	60.1	39.9[b]

N: number of replications; No.♀: number of female offspring; No.♂: number of male offspring; Total: total number of offspring; %♂: sex ratio; E PSR: Estimated number of PSR males among total = (Total×% Fertili×0.01)-No.♀; in case where their mother is uninfected, = No.♂: in case where their mother is infected; % E PSR: Estimated % of PSR males among total = E PSR/Total×100; % F af: % of females among fertilized eggs = No.♀/(No.♀+E PSR)×100; [a]: Sum of female offspring from unfertilized and fertilized eggs; [b]:the actual values will most likely be lower, because the percents shown in the table also include the proportion of female offspring from unfertilized eggs.

Transmission efficiency of PSR

The overall proportion of PSR is significantly higher in *T. kaykai* than in *T. deion* (Table 5). There is no difference in the percentage of PSR males among the offspring of LCA versus LCT females and SWA versus SWT females, suggesting that the transmission efficiency of PSR is not related with the infection status of mothers. The PSR frequencies in the offspring in the SWA and SWT subgroups

are reduced probably because part of the sperm of *T. deion* PSR males lacks the PSR chromosome.

Table 5. The transmission efficiency of PSR in each group

Mother	N	E PSR	No.♀ af	Total	% PSR	Te
LCA	34	878.3	0	1278	68.7	100
LCT	34	633	0	752	84.2	100
SWA	30	532.5	309	1243	42.8	67.7
SWT	30	416	210.9*	926	44.9	66.4

N: number of replications; E PSR: Estimated number of PSR males among total; % PSR: % of PSR males among total =E PSR/Total×100; No.♀ af: Number of female offspring from fertilized eggs; *: estimated number of female offspring from fertilized eggs; Te: transmission efficiency of PSR =E PSR/(E PSR+No.♀)×100

***Relationship between* Wolbachia *infection status and the sex ratio in* T. deion**

The offspring sex ratios produced by SWA and SWT females are more strongly correlated with each other, when the PSR fathers are infected with *Wolbachia* than when uninfected (Table 6).

Table 6. Correlation between the sex ratios produced by SWA and SWT females

♀×♂		N	% M	Spearman's rho Correlation Coefficient
SWA×	Inpsr[d]	24	63.5±27.3	0.926*
SWT×			36.2±26.7	
SWA×	Unpsr[d]	41	89.0±14.6	0.522*
SWT×			61.3±18.6	

%M: Mean sex ratio±standard deviation

Spearman's nonparametric correlation (*): Correlation is significant at the 0.01 level (2-tailed).

Even though the value of R^2 of Unpsr is relatively low (Figure 2), SWA and SWT females mated with uninfected PSR males also showed strong correlation in the offspring sex ratio (Table 6). This suggests that the offspring sex ratio is dependent on the fathers not on the infection status of the mothers. A recent finding shows that the frequency of PSR sperm varies individually among infected and uninfected PSR males (Chapter 5). The sex ratio pairs produced by

SWA and SWT females mated with Inpsr^{d} males are widely distributed along the regression line (Figure 2).

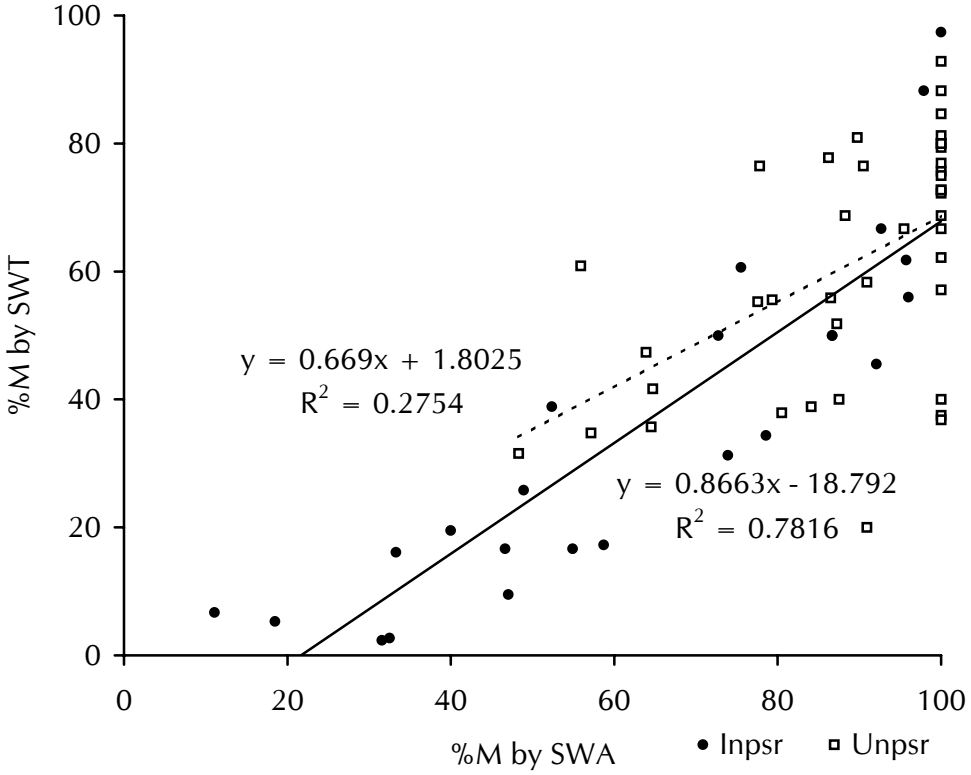

Figure 2. Correlation of the sex ratios produced by uninfected females and infected females mated with infected PSR males and uninfected PSR males

% M by SWA: the percentage of male offspring produced by uninfected females; % M by SWT: the percentage of male offspring produced by infected females; ●: the sex ratios fathered by infected PSR males (N=24); □: the sex ratios fathered by uninfected PSR males (N=41); solid line: regression line of the sex ratio by infected PSR males; dotted line: regression line of the sex ratio by uninfected PSR males

Among the SWA females mated with Inpsr^{d} males only one female produced the sex ratio of 100% males. The sex ratios produced by SWA females mated with Unpsr^{d} males range from male biased to 100% males. This is most likely due to the host genetic background effect and a lack of a *Wolbachia* effect in uninfected PSR males; 20 out of 41 SWA females produced only male offspring. The other 21 SWA females produced not only male offspring but also female offspring.

Sex ratio shift by day

There is no consistent shift in sex ratio among the 5 subgroups by day. In the SWA♀×Inpsr^{d}♂ and SWA♀×Unpsr^{d}♂ crosses, male production increased in the 3rd day (Table 7). It might be caused either by sperm depletion or more PSR male production. In the SWT♀×Inpsr^{d}♂ and SWT♀×Unpsr^{d}♂ crosses, male production decreased in the 3rd day (Table 7). It may also result from sperm depletion.

Table 7. Sex ratio shift by day

♀×♂	N	%♂ (mean rank for Friedman test)			Friedman test
		Day 1	Day 2	Day 3	P-value (χ^2)
SWA×SWA	20	19.0 (1.40)	28.5 (2.42)	23.5 (2.17)	0.002 (12.026)
SWA×Inpsr^{d}	30	70.5 (1.88)	69.6 (2.12)	72.3 (2.00)	0.448 (1.607)
SWT×Inpsr^{d}	30	36.3 (2.23)	36.4 (2.10)	31.2 (1.67)	0.030 (7.022)
SWA×Unpsr^{d}	19	93.7 (2.24)	85.6 (1.58)	94.0 (2.18)	0.005 (10.722)
SWT×Unpsr^{d}	19	61.3 (2.16)	68.2 (2.08)	55.4 (1.76)	0.422 (1.726)

%♂: sex ratio

Effect of male age on the sex ratio

The sex ratios produced by each Inpsr^{d} are not correlated (Table 8).

Table 8. Correlation of the sex ratios produced by SWA females mated with identical males when the males are 1-day old and 7-day old

♂	N	%M	Spearman's rho Correlation Coefficient
1-day old Inpsr^{d}	33	61.5±22.9	0.156
7-day old Inpsr^{d}		61.0±25.5	

%M: Mean sex ratio±standard deviation

The value of R^2 is also very low (Figure 3). Overall sex ratios did not change in a specific direction with the Inpsr^{d} male age (Wilcoxon signed ranks test $P>0.1$), and but some showed substantial change in their offspring sex ratios (Figure 3).

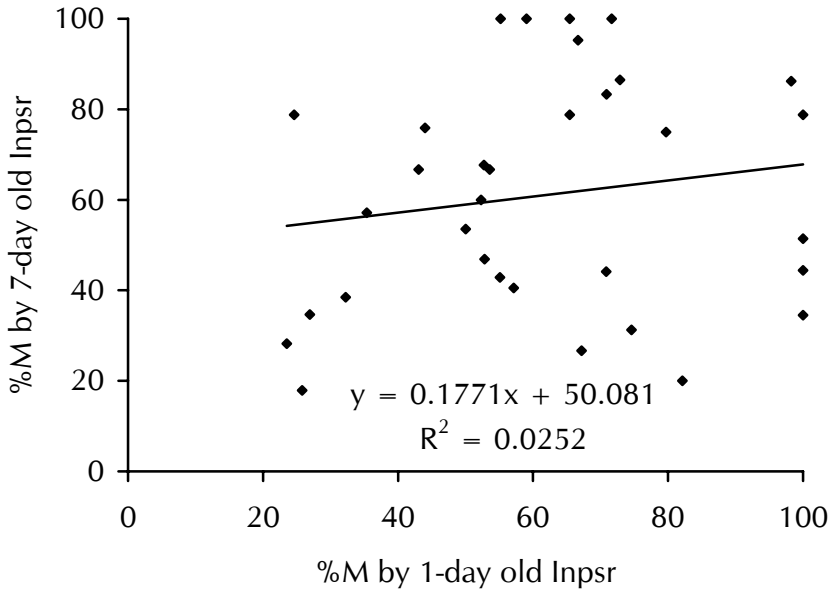

Figure 3. Sex ratio shift as a function of male

% M by 1-day old Inpsr: the percentage of male offspring produced by SWA females mated with 1-day old Inpsr^{d}; % M by 7-day old Inpsr: the percentage of male offspring produced by SWA females mated with 7-day old Inpsr^{d}; ♦: the sex ratios produced by Inpsr^{d} (N = 33)

Discussion

Our results show that the transmission efficiency of PSR depends on the host species and that in *T. deion* also *Wolbachia* plays a role in repressing the normal transmission of PSR through *Wolbachia*-infected PSR males. In *T. kaykai, Wolbachia* infection of PSR males has no effect on the PSR transmission resulting in all male broods via uninfected females and male biased broods via infected females.

It is very interesting that in *T. deion* there is no correlation between the sex ratios produced by identical males when they were 1 day old and 7 days old. At least in some males composition of PSR and non-PSR sperm might change in the interval of 7 days or the effect of the *Wolbachia* infection on sperm production may be age specific. The fact that the overall sex ratios did not change may imply a limit in the fluctuation of sex ratio. So the temporal frequency of PSR in the species may be dependent on the chance and timing of mating.

Theoretical work appears to indicate that CI is the primitive phenotype induced by *Wolbachia* and that the other sex ratio distorting phenotypes - feminization, male killing and parthenogenesis - are evolved forms (Hurst *et al*., 2002; Stouthamer *et al*., 1999; Vavre *et al*., 2000). The CI form would spread first through a population and subsequently a sex ratio distortion mutant *Wolbachia* strains that could induce parthenogenesis swept the ancestral CI inducing strain out of *Trichogramma* in their evolutionary history. Using the infected PSR males we have the ability to test if the parthenogenesis *Wolbachia* of *Trichogramma* also induces a measurable level of cytoplasmic incompatibility. In *Nasonia vitripennis* the effects of the PSR appear to be overruled by the *Wolbachia* crossing incompatibility effects resulting in the destruction of PSR in a cross between an infected PSR male and an uninfected female (Reed and Werren, 1995).

There are two CI crosses in our experimental setup, i.e. Inpsr males mated with uninfected females. If incompatibility is indeed induced by the *Wolbachia,* we would expect the infected PSR males to have a lower transmission when mated with uninfected females than when mated to infected females. Table 3 shows this not to be the case for *T. deion* where the transmission frequencies of PSR are similar, however in case of *T kaykai* there appears to be a lower transmission when an infected PSR male mates with an uninfected female. This would be consistent with a cytoplasmic incompatibility effect. However, when we compare the numbers of offspring produced from fertilized eggs in these two crosses we find that they are very similar, indicating that there is no CI induced mortality. The difference in the transmission frequency is due to a lower survival of the unfertilized eggs from infected females compared to that of the uninfected mothers. The high egg mortality of unfertilized infected eggs is known in *Trichogramma* species (Tagami *et al*., 2001; Huigens, 2003). Consequently, we have no strong evidence for CI induction by the PI-*Wolbachia*.

In *Nasonia* PSR was successfully transmitted to closely related species and their maintenance in the novel hosts was stable (Dobson and Tanouye, 1998). PSR has only been found in natural *T. kaykai* populations in the field even though *T. deion* occurs in the same area and even sometimes emerges together with *T.*

kaykai from the same butterfly egg (Stouthamer *et al.,* 2001; Huigens 2003). In the laboratory the PSR of *T. kaykai* was successfully transmitted to few other *Trichogramma* species including *T. deion* (Hulskes, 2002; Chapter 3). *T. kaykai* and *T. deion* are infected with different strains of *Wolbachia* (Schilthuizen *et al.,* 1998). A question still remaining is how does the *deion-Wolbachia* specifically cancel out the PSR chromosome? Is it a characteristic of the *Wolbachia* strain? Or, is the ability of *Wolbachia* to intervene the PSR transmission influenced by the host genetic background as well? At present the first question can not be answered until we are able to horizontally transfer the *kaykai Wolbachia* to *T. deion* (Huigens, 2003). The second question implicates that the host genetic background has an effect on the intervention of *Wolbachia* and complex interactions between bacteria and host genetic background alter the transmission of PSR in *T. deion*. A recent study on a *Wolbachia* strain may provide an explanation. The *Popcorn* strain of *Wolbachia,* that does not induce CI in *Drosophila melanogaster*, the natural host, induces CI in *D. simulans*, the novel host. (McGraw *et al.*, 2002). The authors postulated that the ability of the *Wolbachia* strain to induce CI in *D. simulans* might rely on the contact of *Wolbachia* with developing sperm in the testes (McGraw *et al.*, 2001). It is possible that the contact of *Wolbachia* with sperm in testes or bacterial density may have an influence on the transmission of PSR in *T. deion* genetic background. But recently Reynolds *et al*. (2003) found that the *Popcorn* strain in young males also induced CI in the natural host. So the ability of *Wolbachia* to induce CI may not be related with the contact with sperm bundles (Reynolds *et al*., 2003). It is, therefore, also possible that the *Wolbachia* infection influences the transmission of PSR in other ways in *T. deion*.

The reduced transmission efficiency of PSR in *T. deion*, in addition to the low infection frequency with *Wolbachia* in this species, most likely causes it not to be present in field populations at measurable frequencies (Huigens, 2003). Why is the transmission efficiency of PSR so low in *T. deion*? A speculative hypothesis could be that this is the result of a suppressor being present in *T. deion* that has been able to suppress the PSR in *T. deion* evolutionary past. It has been proposed that a modifier that restrains the expression of a self-promoting element

will be neutral or even be costly when the element is extinct by the modifier (Hurst *et al.,* 1996). Eventually the modifier will be under selection or drift by which the host mitigates the burden keeping the useless modifier. If PSR indeed were in *T. deion*, it would have been expelled recently in evolutionary time scale. The reason why is that the traits responsible for the suppression of PSR is not yet under selection or drift considering that newly introduced PSR is still affected by the host genetic background and the *Wolbachia* infection.

Acknowledgements

Hans Breeuwer, Evert Jan Bakker, Ties Huigens and Joke van Vugt are appreciated for useful comments in doing the experiment and in interpreting data. Many thanks are given to Ties Huigens and Isabel Silva for careful review on an earlier version of the manuscript. The Ph.D. discussion group in the laboratory also made useful suggestion for the improvement of the manuscript. The authors thank Peter de Jong and Leo Koopman for sharing experimental space. This study is supported in part by Nexgen Biotechnologies Inc. (Korea).

References

Abbot P., Withgott J.H., and Moran N.A., 2001, Genetic conflict and conditional altruism in social aphid colonies. *Proc. Natl. Acad. Sci. USA*. **98**(21):12068-12071.

Braig H.R., Zhou W., Dobson S.L., and O'Neill S.L., 1998, Cloning and characterization of a gene encoding the major surface protein of the bacterial endosymbiont *Wolbachia*. *J. Bacteriol*. **180**:2373-2378.

Cosmides L.M., and Tooby J., 1981, Cytoplasmic inheritance and intragenomic conflict. *J.Theor. Biol*. **89**:83-129.

Camacho J.P., Sharbel T.F., and Beukeboom L.W., 2002, B-chromosome evolution. *Phil. Trans. R. Soc. Lond. B*. **355**:163-178

Dobson S.L., and Tanouye M.A., 1998, Interspecific movement of the paternal sex ratio chromosome. *Heredity*. **81**:261-269.

Gimble F.S., 2000, Invasion of a multitude of genetic niches by homing endonuclease genes. *FEMS Microbiol. Lett*. **185**:99-107.

Huigens M. E., 2003, *On the evolution of* Wolbachia*-induced parthenogenesis in* Trichogramma *wasps*. Ph.D. thesis. Wageningen University.

Hulskes A., 2002, *The origin of the paternal sex ratio chromosome in* Trichogramma kaykai. M.Sc. thesis. Wageningen University.

Hurst G.D.D., Hurst L.D., and Johnstone R.A., 1992, Intranuclear conflict and its role in evolution. *Trends Ecol. Evol*. **7**(11):373-378

Hurst G.D.D., Jiggins F.M., and Pomiankowski A.P., 2002, Which way to manipulate host reproduction? *Wolbachia* that cause cytoplasmic incompatibility are easily invaded by sex ratio-distorting mutants. *Am. Nat*. **160**(3):360-373.

Hurst G.D.D., and Schilthuizen M., 1998, Selfish genetic elements and speciation. *Heredity*. **80**:2-8.

Hurst G.D.D., and Werren J.H., 2001, The role of selfish genetic elements in eukaryotic evolution. *Nat. Rev. Genet*. **2**:597-606.

Hurst L.D., 1992, Intragenomic conflict as an evolutionary force. *Proc. R. Soc. Lond. B*. **248**:135-140

Hurst L.D., Atlan A., and Bengtsson B.O., 1996, Genetic conflict. *Q. Rev. Biol*. **71**(3):317-364.

Keller L., and Ross K.G., 1998, Selfish genes: A green beard in the red fire ant. *Nature*. **394**:573-575.

McGraw E.A., Merritt D.J., Droller J.N., and O'Neill S.L., 2001, *Wolbachia*-mediated sperm modification is dependent on the host genotype in *Drosophila*. *Proc. R. Soc. Lond. B*. **268**:2565-2570.

McGraw E.A., Merritt D.J., Droller J.N., and O'Neill S.L., 2002, *Wolbachia* density and virulence attenuation after transfer into a novel host. *Proc. Natl. Acad. Sci. USA*. **99**(5):2918-2923.

Pinto J.D., 1999, Systematics of the North American species of *Trichogramma* (Hymenoptera: Trichogrammatidae). *Mem. Entomol. Soc. Wash*. **22**:1-287.

Pinto J.D., and Stouthamer R., 1994, Systematics of the Trichogrammatidae with emphasis on *Trichogramma*. In: Wajnberg E., and Hassan S. (eds), '*Biological control with egg parasitoids*'. CAB International: pp1-36.

Pinto J.D., Stouthamer R., and Platner G.R., 1997, A new cryptic species of *Trichogramma* (Hymenoptera: Trichogrammatidae) from the Mojave Desert of

California as determined by morphological, reproductive and molecular data. *Proc. Entomol. Soc. Wash.* **99**(2):238-247.

Querino R.B., and Zucchi R.A., 2003a, Six new species of *Trichogramma* Westwood (Hymenoptera: Trichogrammatidae) from a Brazilian forest reserve. *Zootaxa*. **134**:1-11.

Querino R.B., and Zucchi R.A., 2003b, New species of *Trichogramma* Westwood (Hymenoptera: Trichogrammatidae) associated with lepidopterous eggs in Brazil. *Zootaxa*. **163**:1-10.

Reed K.M., and Werren J.H., 1995, Induction of paternal genome loss by the paternal sex ratio chromosome and cytoplasmic incompatibility bacteria (*Wolbachia*): a comparative study of early embryonic events. *Mol. Reprod. Dev.* **40**:408-418.

Reynolds K.T., Thomson L.J., and Hoffmann A.A., 2003, The effects of host age, host nuclear background and temperature on phenotypic effects of the virulent *Wolbachia* strain popcorn in *Drosophila melanogaster*. *Genetics*. **164**:1027-1034.

Schilthuizen M., Honda J., and Stouthamer R., 1998, Parthenogenesis-inducing *Wolbachia* in *Trichogramma kaykai* (Hymenoptera: Trichogrammatidae) originates from a single infection. *Ann. Entomol. Soc. Am.* **91**(4):410-414.

Stouthamer R., 1997, *Wolbachia*-induced parthenogenesis. In: O'Neill S.L., Hoffmann A.A., and Werren J.H. (eds), '*Influential Passengers: Inherited microorganisms and arthropod reproduction*'. Oxford University Press: Oxford. pp102-124.

Stouthamer R., Breeuwer J.A.J., and Hurst G.D.D., 1999, *Wolbachia pipientis*: microbial manipulator of arthropod reproduction. *Annu. Rev. Microbiol.* **53**:71-102.

Stouthamer R., Hurst G.D.D., and Breeuwer J.A.J., 2002, Sex ratio distorters and their detection. In: Hardy I.C.W. (eds), *Sex ratios: Concepts and research methods*. Cambridge University Press: Cambridge. pp195-215.

Stouthamer R., and Kazmer D.J., 1994, Cytogenetics of microbe-associated parthenogenesis and its consequences for gene flow in *Trichogramma* wasps. *Heredity*. **73**:317-327.

Stouthamer R., van Tilborg M., de Jong J.H., Nunney L., and Luck R.F., 2001, Selfish element maintains sex in natural populations of a parasitoid wasp. *Proc. R. Soc. Lond. B*. **268**:617-622.

Tagami Y., Miura K., and Stouthamer R., 2001, How does infection with parthenogenesis-inducing *Wolbachia* reduce the fitness of *Trichogramma*? *J. Invertebr. Pathol*. **78**:267-271.

Van Vugt J.J.F.A., Salverda M., de Jong H., and Stouthamer R., 2003, The paternal sex ratio chromosome in the parasitic wasp *Trichogramma kaykai* condenses the paternal chromosomes into a dense chromatin mass. *Genome*. **46**(4):580-587.

Vavre F., Fleury F., Varaldi J., Fouillet P., and Boulétreau M., 2000, Evidence for female mortality in *Wolbachia*-mediated cytoplasmic incompatibility in haplodiploid insects: Epidemiologic and evolutionary consequences. *Evolution*. **54**(1):191-200.

Werren J.H., and Beukeboom L.W., 1993, Population genetics of a parasitic chromosome: theoretical analysis of PSR in subdivided populations. *Am. Nat*. **142**:224-241.

Werren J.H., and Beukeboom L.W., 1998, Sex determination, sex ratios, and genetic conflict. *Annu. Rev. Ecol. Syst*. **29**:233-261.

Werren J.H., Nur U., and Eickbush D., 1987, An extrachromosomal factor causing loss of paternal chromosomes. *Nature*. **327**:75-76.

Werren J.H., Nur U., and Wu C.I., 1988, Selfish genetic elements. *Trends Ecol. Evol*. **3**(11):297-302

Werren J.H., and Stouthamer R., 2003, PSR (paternal sex ratio) chromosomes: the ultimate selfish genetic elements. *Genetica*. **117**:85-101.

Chapter 5

Interaction between *Wolbachia* infection and transmission of the paternal sex ratio (PSR) chromosome in *Trichogramma deion*

Interaction between *Wolbachia* infection and transmission of the paternal sex ratio (PSR) chromosome in *Trichogramma deion*

Abstract

In *Trichogramma* two sex ratio distorters, *Wolbachia* and PSR (paternal sex ratio) are found. *Wolbachia* is a cytoplasmically inherited bacterium that induces parthenogenesis in this genus and results in all-female broods. PSR is only transmitted via males and produces all-male broods. PSR can be successfully transferred from its natural host *Trichogramma kaykai* to *T. deion*. The transmission efficiency of PSR in *T. deion* is lower than in *T. kaykai* and is negatively influenced by the *Wolbachia* infection in their male hosts. The results show that *Wolbachia* infection in males significantly increases the proportion of PSR males that produce non-PSR sperm and that the level of PSR transmission by *T. deion* males ranges from; a) efficient PSR transmission, b) partial transmission resulting in female and PSR male offspring c) non-functional sperm production or low sperm production, and d) complete lack of transmission. While the first two transmission types are found in uninfected males that carry the PSR chromosome, all four patterns are found in infected males that carry the PSR chromosome. The results imply complex interactions between the bacterium, the PSR chromosome and the species specific genetic background.

Keywords

Trichogramma, *Wolbachia*, PSR, host genetic background, variation, genetic conflict

Introduction

Many species in the egg parasitoid genus *Trichogramma* are infected with *Wolbachia* bacteria that allow infected virgin females to produce daughters (Stouthamer, 1997). The normal reproduction in this genus and in Hymenoptera in general is arrhenotoky or haplodiploidy, where unfertilized eggs become males and fertilized eggs become females. The infection with the Parthenogenesis-inducing (PI) *Wolbachia* causes the abortion of the first mitotic anaphase in unfertilized infected eggs resulting in the retention of the two identical chromosome sets in a single nucleus (Stouthamer and Kazmer, 1993). The subsequent divisions are normal and the individual grows out to be a homozygous infected female. In many *Trichogramma* populations a low level of infection with PI-*Wolbachia* is found, and in these populations infected females mate and will fertilize some of their eggs. The fertilization of the egg by sperm somehow precludes the action of the *Wolbachia*, and the fertilized infected eggs grow out to be infected heterozygous females (Stouthamer and Kazmer, 1993).

In most known cases of PI-*Wolbachia* the infection has gone to fixation in the population. In contrast in many species of *Trichogramma* the PI-*Wolbachia* infection remains at a low level, and less than 20% of the females are infected. This low infection frequency in these populations can be caused by a number of factors such as the inefficient transmission of the infection, high fitness cost of being infected or by suppressor genes that either kill the bacteria or negate their effect. Only in the species *Trichogramma kaykai* has this low infection frequency been studied extensively. The low PI-*Wolbachia* infection can be explained in this species by the presence of a second sex ratio distorter, the paternal sex ratio (PSR) chromosome (Stouthamer *et al.*, 2001). The PSR chromosome is a supernumerary chromosome that is not part of the normal chromosome complement and is not required for the normal functioning of the wasp. The PSR chromosome is transmitted exclusively through males and it causes eggs that are fertilized with sperm harboring PSR to develop into PSR males. While normally fertilized eggs develop into diploid females, in eggs fertilized with the PSR sperm the PSR chromosome causes the destruction of the paternal set of normal chromosomes. Consequently these fertilized eggs grow out to be males that carry

the maternal set of chromosomes plus the PSR chromosome (van Vugt *et al*., 2003). The PSR chromosome of *T. kaykai* is only the second known PSR chromosome (Stouthamer *et al*., 2001), the first one was discovered in the parasitoid wasp *Nasonia vitripennis* (Werren *et al*., 1987). The PSR chromosomes in the two species are thought to have originated from other species via interspecific transmission or to be a remnant of the A-chromosome from an interspecific hybridization (Werren and Stouthamer, 2003).

T. deion is a species of *Trichogramma* with a wide distribution throughout North America, and it overlaps with *T. kaykai* in the Mojave Desert, where both species are found to parasitize eggs of the lycaenid butterfly *Apodemia mormo* (Pinto, 1999). In *T. deion,* the *Wolbachia* infection frequency is lower than in *T. kaykai* (Huigens, 2003). The PSR chromosome of *T. kaykai* can be transferred to *T. deion*, simply through interspecific crosses (Hulskes, 2002; Chapter 3). In the laboratory the interspecific (*kaykai* to *deion*) and intraspecific (*kaykai* to *kaykai*) transfers are equally successful (Huigens, 2003; Chapter 3, 4). Yet the PSR factor has not been found in field populations of *T. deion*. It is thought that PSR is not the factor that maintains the low *Wolbachia* infection frequency in *T. deion*, instead suppressor genes in the host may play a role (Huigens, 2003).

Detailed studies of PSR transmission in the parasitoid wasp *Nasonia vitripennis* have shown that within this species some males are better capable of transmitting PSR to their offspring than others (Beukeboom and Werren, 1993). While 90 percent of the males transmitted the PSR chromosome perfectly, up to 10 percent of the males also produced some female offspring when they mated with normal sexual females. In those families where PSR fathers also had some female offspring the transmission of PSR to the males varied from 0 to 94 percent. The authors state that the incomplete transmission of PSR is most likely caused by a mitotic instability of PSR in spermatogonial cells, resulting in males producing both PSR sperm and normal sperm.

Here we study in detail the interaction between PSR and PI-*Wolbachia* in *T. deion*. We show that while in *T. kaykai* the infection does not appear to influence the transmission and expression of PSR, in *T. deion* there is a clear interaction between PSR and the PI-*Wolbachia*. In *T. deion* PI-*Wolbachia*

infected PSR males transmit the PSR with a lower frequency to their offspring than uninfected PSR males. We show that the *Wolbachia* interaction with PSR results in four different classes of males that can be recognized by the sex ratio of the offspring they father when mated with PI-*Wolbachia* infected and with uninfected females.

Materials and methods

Trichogramma *species and lines*

T. deion was originally collected from the Mojave Desert, California, USA, by collecting parasitized eggs of its host, the butterfly, *Apodemia mormo deserti* (Lepidoptera: Lycaenidae). In the laboratory *T. deion* has been reared on *Ephestia kuehniella* (Lepidoptera: Pyralidae) eggs provided by Koppert B.V. at 23±1°C, 16:8 LD, and 60±10% RH. All experiments were performed under the same conditions.

The *Wolbachia* infected parthenogenetic (thelytokous) isofemale line has been collected at Sidewinder Mountains, San Bernardino County, California, and is abbreviated as SWT. The sexual isofemale line was derived from SWT by antibiotic treatment, and abbreviated as SWA. PSR was interspecifically transmitted from *T. kaykai* to *T. deion*. The infected PSR line is maintained on the SWT genetic background by mating a SWT female with an infected PSR male, and is abbreviated as Inpsr. Inpsr males used in the experiments originated from eggs obtained from this mass culture. The uninfected PSR males are obtained by mating a SWA female with an Inpsr male, and are abbreviated as Unpsr.

Detection of Wolbachia *and PSR*

Wolbachia infection and the presence of PSR were determined using specific PCR primers on template derived from the wasps. Genomic DNA of wasps was extracted with 50 µl of 5 % Chelex-100 solution and 2 µl of proteinase K and incubated for 3 hours at 56°C and for 10 min at 95°C (Mastercycler® gradient, eppendorf).

To determine infection with *Wolbachia*, PCR with a *Wolbachia*-specific primer set (*wsp*) was performed. The temperature profile was 94°C for 3 min (1cycle); 94°C for 1 min, 50°C for 1 min and 72°C for 1 min (40 cycles) on a thermocycler (Mastercycler® gradient, eppendorf) (Braig *et al.,* 1998).
To determine if a male carried PSR, PCR with a PSR-specific primer set was performed (van Vugt *et al*., in prep). The temperature profile was 94°C for 2 min (1cycle); 94°C for 1 min, 61°C for 1min and 72°C for 1 min (35 cycles) on a thermocycler (Mastercycler® gradient, eppendorf). The PCR products were run on a 1 % agarose gel and were visualized on the UV light.

Mating scheme and observation of mating

One day before the wasps emerged, parasitized *E. kuehniella* eggs in the black stage were placed individually in glass tubes (7.5×1cm). Freshly emerged wasps less than 24 hours old were used for the crossing experiments.
It is critical to observe the matings of the wasps, in order to confirm if a mated female used sperm or not. Each pair in each experiment was observed under a stereomicroscope (magnification 10×) until they mated. In the SWA♀×SWA♂ and the SWT♀×SWA♂ crosses, as soon as a pair mated, the male was taken out of the tube and frozen to later determine if it was infected with *Wolbachia* and harbored PSR. Especially the SWA♀×SWA♂ cross was not only used as a control, but was also used to determine if the females always used sperm when they mated.
In a previous study the transmission of PSR was not efficient in *T. deion* (Chapter 4). Therefore, each infected and uninfected PSR male was given two chances to mate to determine whether there was variation in transmitting PSR among PSR males (Table 1).

Table 1. Mating scheme and expected outcome of the crosses

Crosses (*T. deion*)		Expected outcome	
		Fertilized egg	Unfertilized egg
♀Un×	♂Un	♀Un	♂Un
♀In ×	♂Un	♀In	♀In
♀Un×	♂Inpsr	♂Unpsr	♂Un
♀In ×		♂Inpsr	♀In
♀Un×	♂Unpsr	♂Unpsr	♂Un
♀In ×		♂Inpsr	♀In

A PSR male was introduced into a tube in which an uninfected female had emerged. As soon as the mating was observed, the male was taken out and after a 2 hrs interval he was introduced into a tube containing an infected virgin female. As soon as they mated, the male was taken out of the tube and frozen to later determine if it was infected with *Wolbachia* and harbored PSR.

Determination of polymorphism in the transmission of PSR and categorization of polymorphism

After 1 day the females that had been confirmed to mate were allowed to lay eggs in 120～160 *E. kuehniella* eggs for about 72 hrs. When either one of the females, mated with a particular PSR male, died during the egg laying period or did not produce offspring, the offspring sex ratios of both females of their shared male were excluded from the analysis. After 72 hours of egg laying, the females were frozen to preserve the specimens for later determination of their *Wolbachia* infection status.

There are four possible combinations of offspring sex ratio produced by an uninfected female and a corresponding infected female mated with the same PSR male, which is either infected or uninfected (Table 2). The sex ratio pairs of offspring originating from the same father emerged during the first three days were assigned to one of the 4 categories.

Table 2. Four possible combinations of offspring production by SWA and SWT mated with Inpsr and Unpsr

Mother	Categories			
	a	b	c	d
SWA	♂	♀/♂	♂	♀/♂
SWT	♀/♂	♀/♂	♀	♀

The PSR status of males from the Unpsr line was determined afterwards. If a male from the Unpsr line was a normal male the offspring of this male was excluded from the data analysis.

Category a: Infected females mated with the PSR male produce male and female offspring, while uninfected females mated with the PSR male produce male offspring exclusively. In this category PSR appears to function as expected. **Category b**: Both the infected and uninfected females produce both male and female offspring. This situation could arise when the PSR male produces both sperm containing the PSR chromosome and sperm without the PSR. **Category c**: Here the PSR father produces no sperm, or the sperm contains PSR but it only expresses itself in the offspring of uninfected females. Alternatively the cross between the SWA female and PSR male may have been successful while the cross with the SWT female did not result in offspring. Finally **category d**: It comes about when the PSR male has completely lost the ability to pass on the PSR, therefore when a male mates with an uninfected female the offspring consists of both males and females while when mated with an infected female only female offspring is produced.

Results

Detection of Wolbachia *and PSR*

SWA and Unpsr are not infected with *Wolbachia*, and SWT and Inpsr are infected with *Wolbachia* (data not shown). Inpsr harbors PSR. The Unpsr group of males consists of normal males as well as uninfected PSR males. Only uninfected PSR males were selected from the Unpsr male group using PCR with a PSR specific primer set, and the result of their matings is reported in table 3. A

random sample of 40 males from the Inpsr line was tested to determine if they all carried the PSR chromosome. They were all positive for PSR.

Accuracy of the observation of the matings and egg fertilization

All the 40 SWA♀×SWA♂ crosses produced female offspring confirming that the observation of their matings resulted in insemination and subsequent fertilization of some eggs (Table 3). Consequently the observation of mating is assumed to be a sufficient indication that females are inseminated. Virgin SWA♀ produced only male offspring and virgin SWT♀ produced all female offspring as expected (Table 3). SWT♀ produced fewer offspring than SWA♀, probably because of the fecundity loss caused by *Wolbachia* infection (Tagami *et al*., 2001).

Table 3. Mean values with standard deviation of offspring production of the crosses between females (infected and uninfected) and males (normal, infected PSR, uninfected PSR)

♀×♂	Categories	N	M.♀	M.♂	M.O	%♂	Total
SWA		40	0	2.2± 4.9	32.2± 4.9[a]	100	1288
SWA×SWA		40	23.7± 4.6	6.7± 2.5	30.4± 4.3[a]	22.1	1214
SWA×Inpsr	a	24	0	28.3± 7.3	28.3± 7.1[a]	100	680
SWA×Inpsr	b	163	14.3± 7.6	18.5± 7.2	32.8± 6.0[a]	56.4	5340
SWA×Inpsr	c	6	0	32.2± 8.6	32.2± 8.6[a]	100	193
SWA×Inpsr	d	30	23.2± 6.8	10.9± 5.7	34.1± 6.8[a]	31.9	1022
SWA×Unpsr	a	27	0	32.8± 7.5	32.8± 7.5[a]	100	886
SWA×Unpsr	b	31	6.3± 4.1	27.2± 8.0	33.6± 7.9[a]	81.2	1040
SWT		38	21.2± 4.9	0	21.2± 4.9[b]	0	804
SWT×SWA		40	24.6± 6.3	0	24.6± 6.3[b]	0	984
SWT×Inpsr	a	24	6.6± 3.8	13.5± 5.5	20.0± 6.6[b]	67.2	481
SWT×Inpsr	b	163	16.4± 6.6	6.6± 4.6	23.0± 6.0[b]	28.7	3752
SWT×Inpsr	c	6	20.2± 3.8	0	20.2± 3.8[b]	0	121
SWT×Inpsr	d	30	21.3± 7.0	0	21.3± 7.0[b]	0	638
SWT×Unpsr	a	27	6.8± 3.1	17.1± 5.0	23.9± 6.8[b]	71.5	645
SWT×Unpsr	b	31	10.4± 4.9	10.5± 5.6	20.9± 8.9[b]	50.3	648

N: number of replications; M.♀: mean number of female offspring±standard deviation; M.♂: mean number of male offspring±standard deviation; M.O: mean number of offspring±standard deviation; %♂: sex ratio

M.O followed by the same letter does not differ significantly from each other.

In SWA: ANOVA $P < 0.05$, Tukey HSD a

In SWT: ANOVA $P > 0.05$, Tukey HSD b

Categorization of PSR males by their offspring sex ratios

Males in the Inpsr group have been categorized to all different categories, whereas the Unpsr males fall only in category a and b (Table 3 and Figure 1). In the Inpsr group the males in category b and d father some female offspring in the SWA females they mate with, which is 86.6 % of all the Inpsr males while the Unpsr males of category b father some female offspring with SWA females they mate with which is 53.5% of all the Unpsr males. The males of category c and d fail to induce any male production in the infected females they mate with and males of category c do not produce any daughters with the SWA females they mate with. The relative frequency of those categories where PSR is still transmitted (i.e. a and b) differs for the Inpsr and Unpsr males. The category a for Unpsr males is relatively larger than that for Inpsr males (Figure 1).

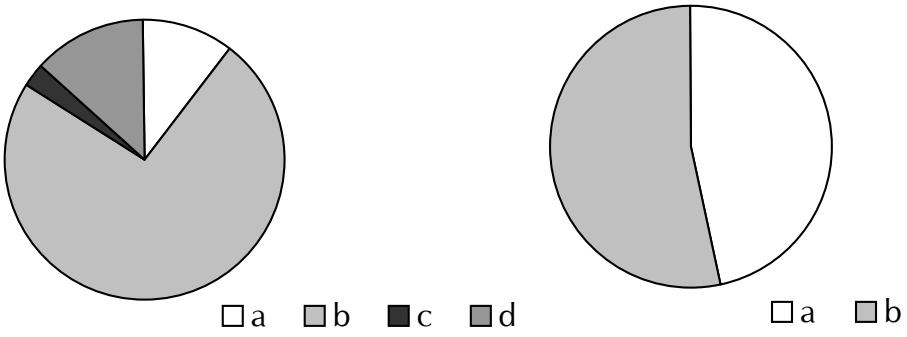

Figure 1. Proportions of 1) Inpsr and 2) Unpsr fathers belonging to different categories

1) SWA subgroup

In the SWA group the number of offspring of at least one group is different among the categories (ANOVA $P<0.05$). But this is not supported by the post hoc analysis (Tukey HSD test $P=0.082$) (Table 3). Category b and d produced female offspring, but the sex ratio is significantly more male biased in category b (Mann Whitney U test $P<0.001$). The sex ratio of category d is different from

that of the SWA♀×SWA♂ and is more male biased (Mann Whitney U test $P<0.01$).

The sex ratio of category b of the SWA♀×Inpsr♂ cross is more female biased than that of b of the SWA♀×Unpsr♂ cross (Mann Whitney U test $P<0.001$). The fertilization rate, i.e. 100–sex ratio = 77.9%, of the SWA♀×SWA♂ cross is used to estimate the proportion of the fertilized eggs of category b. First in the SWA♀×Inpsr♂ cross (category b) 77.9% of the 32.8 eggs i.e. 25.5 eggs should be fertilized and among them 14.3 eggs developed into female offspring and the rest 11.2 eggs developed into male offspring. Second for the SWA♀×Unpsr♂ cross the percentage of fertilized eggs is similarly estimated using the fertilization rate of the SWA♀×SWA♂ cross, consequently 26.2 eggs should be fertilized and among them 6.3 eggs developed into female offspring and the rest 19.9 eggs into male offspring (Table 4).

Table 4. Estimated values of female and male offspring from fertilized eggs

♀×♂	Category	M.O	E. fr	E. fe	M.♀	M.♂*	% F. af
SWA×Inpsr	b	32.8	68.2	23.3	14.3	9[a]	61.4
SWA×Unpsr	b	33.6	68.2	22.9	6.3	16.6[a]	27.5
SWT×Inpsr	b	23.0	67.2	15.5	8.9[b]	6.6	57.4
SWT×Unpsr	b	20.9	67.2	14.1	3.6[b]	10.5	25.5

E. fr: estimated fertilization rate followed by that of the category d of SWA×Inpsr in case of the category b of SWA×Inpsr and b of SWA×Unpsr and by that of the category a of SWT×Inpsr in case of the category b of SWT×Inpsr and b of SWT×Unpsr; E. fe: estimated number of fertilized eggs; [a] = E. fe–M.♀; [b] = E. fe–M.♂; % F. af: % of females among fertilized eggs; *: M.♂ is mean number of PSR in the category.

2) SWT subgroup

In the SWT group the number of offspring is not different among the categories (ANOVA $P>0.05$). This is supported by the post hoc analysis (Tukey HSD test $P=0.462$) (Table 3).

The sex ratios of category a of the SWT♀×Inpsr♂ cross and the SWT♀×Unpsr♂ cross are not different from each other (Mann Whitney U test $P>0.1$), but the number of offspring is different (T test $P<0.001$). There is no effect of the host

genetic background and the *Wolbachia* infection in category a. All the fertilized eggs in the category are thought to develop into PSR male offspring.

In the SWT♀×Unpsr♂ cross the sex ratio of a and b categories is different (Mann Whitney U test $P<0.001$). The sex ratio of category a of the SWT♀×Inpsr♂ cross is not different from that of the category a of the SWT×Unpsr♂ cross (Mann Whitney U test $P>0.1$). The sex ratio of the category b of the SWT♀×Inpsr♂ cross differs from that of the category b of the SWT♀×Unpsr♂ cross (Mann Whitney U test $P<0.001$). The fertilization rate, i.e. sex ratio, of category a is used to estimate the proportion of the fertilized eggs of category b. First in the SWT♀×Inpsr♂ cross 15.5 eggs should be fertilized and among them 6.6 developed into PSR male offspring and the rest 8.9 eggs into female offspring. Second in the SWT♀×Unpsr♂ cross 14.1 eggs should be fertilized and among them 10.5 eggs developed into PSR male offspring and the rest 3.6 eggs into female offspring (Table 4).

Discussion

The study clearly shows that the *Wolbachia* infection and some host factor influence the transmission of PSR in *T. deion*. In the Inpsr group, only 10.8 % of the males appear to completely pass on the PSR, while this is 46.6 % in the Unpsr group. In all the tests used in this study the uninfected PSR males were tested using PCR to determine the presence of the PSR chromosome. In the Inpsr group the males were extracted from a culture that had been maintained with infected females and PSR males. In that culture the crosses that we categorized here as a, b, c and d have taken place for generations. Therefore, the males that are present in the culture are all the offspring of category a or b males. We did not test however if the individual Inpsr males used in the crosses are all carriers of PSR. Therefore it could be possible that some of the Inpsr males we used in our crosses did not carry PSR at all. However, we think that is unlikely because when we randomly tested 40 PSR males from our Inpsr culture all carried PSR. Consequently, all the males in our experiments are assumed to be carriers of PSR. From this it becomes clear that the infected PSR males produce in some cases only PSR sperm (Cat a), mixture of normal and PSR sperm (Cat b), only

normal sperm (Cat d) or these males are unable to produce functional sperm (Cat c) (Table 5). In the Unpsr group the transmission of the PSR factor was better, because only males of category a and b were present. The difference between the Inpsr and Unpsr in their ability to transmit the PSR is not only visible through the lack of cat c and d males in the Unpsr group but also if we compare the relative frequencies of the transmitting males (cat a and b). In the Inpsr group the relative frequency of the group with the best transmission (category a) is 10.8 %, whereas in the Unpsr group this category has a frequency of 46.6%. So there is a negative effect of the *Wolbachia* infection in the males on their ability to express or transmit PSR. We do not have any data if there are any differences in the transmission of PSR by the PSR offspring from category a and category b. We expect that a relatively larger fraction of category a would father more PSR sons than those from category b.

Table 5. Transmission efficiency of PSR in each category

	Categories			
	a	b	c	d
PSR transmission	Efficient	Partially efficient	Non functional sperm or low sperm production	Inefficient
Fertilized egg	PSR♂	♀and PSR♂	?	♀

Among the crosses the SWA♀×Inpsr♂ cross is analogous to the CI cross, where death of offspring or development of haploid male offspring results from the cross between an uninfected female and an infected male (Hoffmann and Turelli, 1997; Vavre *et al*., 2000). To detect the effects of CI a comparison needs to be made between the SWA♀×Inpsr♂ cross with the SWT♀×Inpsr♂ cross, however these transmission rates did not differ indicating that the *Wolbachia* infection in the males does not cause CI. There is a clear difference however between the transmission rate of PSR through the Unpsr males and the Inpsr males.

The meiotic instability is important for B chromosome evolution, because any Bs would be very unstable in meiosis. In the case of PSR the ability to induce paternal genome loss is important to avoid meiosis (Werren and Stouthamer,

2003). In *T. deion* once PSR exists in sperm, PSR seems to effectively degenerate the paternal genome after fertilization, resulting in PSR male offspring. So the unstable generation of PSR, i.e. mitotic instability, is the major limiting factor of PSR proliferation in the species.

In *Nasonia*, female production may result from the production of normal sperm by PSR males. The PSR males that did not transmit PSR should be somatic mosaic (Beukeboom and Werren, 1993). Cell mosaicism is also a possible explanation for the polymorphism in this experiment. If the host genetic background in *T. deion* induced unstable mitosis in the early development, normal sperm could occur resulting in female offspring. If it occurred in spermatogenesis, it could also induce female offspring production. It, however, is still unclear how the *Wolbachia* infection influences the increase in somatic or germ line mosaic.

It has been argued that the interspecific transmission of PSR from *T. kaykai* to *T. deion* should happen occasionally in the field. Based on argument concerning the mating structure of the *T. deion* population in the field together with the low infection frequency of *T. deion*, the authors concluded that PSR was unable to be maintained in *T. deion* (Huigens, 2003). If we add to these arguments the reduced transmission efficiency through *T. deion*, it becomes even more unlikely that PSR can play any role in keeping the *Wolbachia* infection at a low level in this species.

One of the reasons why PSR is getting much attention is its application as a suppressor of a haplodiploid pest population and as a potential vehicle for genetic engineering of haplodiploids (Werren and Stouthamer, 2003). Its application is theoretically possible with its mode of action in a haplodiploid sex determination system under the condition that it maintains its function in the novel host genetic background (Werren and Stouthamer, 2003). But as seen from the results here it may not always be the case.

Acknowledgements

Many thanks are given to Peter de Jong, Joop C. van Lenteren, Isabel Silva, and Joke van Vugt for careful review. The authors thank Mohammad Ardeh, Ties Huigens and Bart Pannebakker for useful comments. Leo Koopman and Koppert B.V. are acknowledged for providing *E. kuehniella* eggs. The authors thank Peter de Jong and Leo Koopman for sharing experimental space. This study is supported in part by Nexgen Biotechnologies Inc. (Korea).

References

Arakaki N., Miyoshi T., and Noda H., 2001, *Wolbachia*-mediated parthenogenesis in the predatory thrips *Franklinothrips vespiformis* (Thysanoptera: Insecta). *Proc. R. Soc. Lond. B*. **268**:1011-1016.

Bandi C., Anderson T.J.C., Genchi C., and Blaxter M.L., 1998, Phylogeny of *Wolbachia* in filarial nematodes. *Proc. R. Soc. Lond. B*. **265**:2407-2413.

Beukeboom L.W., and Werren J.H., 1993, Transmission and expression of the parasitic paternal sex ratio (PSR) chromosome. *Heredity*. **70**:437-443.

Braig H.R., Zhou W., Dobson S.L., and O'Neill S.L., 1998, Cloning and characterization of a gene encoding the major surface protein of the bacterial endosymbiont *Wolbachia*. *J. Bacteriol*. **180**:2373-2378.

Breeuwer J.A.J., and Werren J.H., 1993, Cytoplasmic incompatibility and bacterial density in *Nasonia vitripennis*. *Genetics*. **135**:565-574.

Dobson S.L., and Tanouye M.A., 1998, Interspecific movement of the paternal sex ratio chromosome. *Heredity*. **81**:261-269.

Gottlieb Y., Zchori-Fein E., Werren J.H., and KarrT.L., 2002, Diploidy restoration in *Wolbachia*-infected *Muscidifurax uniraptor* (Hymenoptera: Pteromalidae). *J. Invertebrate Pathol*. **81**:166-174.

Hoffmann A.A., and Turelli M., 1997, Cytoplasmic incompatibility in insects. In: O'Neill S.L., Hoffmann A.A., and Werren J.H. (eds), '*Influential Passengers: Inherited microorganisms and arthropod reproduction*'. Oxford University Press: Oxford. pp42-80.

Huigens M.E., 2003, *On the evolution of* Wolbachia*-induced parthenogenesis in* Trichogramma *wasps*. Ph.D. thesis. Wageningen University.

Hulskes A., 2002, *The origin of the paternal sex ratio chromosome in* Trichogramma kaykai. M.Sc. thesis. Wageningen University.

Stouthamer R., 1997, *Wolbachia*-induced parthenogenesis. In: O'Neill S.L., Hoffmann A.A., and Werren J.H. (eds), '*Influential Passengers: Inherited microorganisms and arthropod reproduction*'. Oxford University Press: Oxford. pp102-124.

Stouthamer R., Breeuwer J.A.J., and Hurst G.D.D., 1999, *Wolbachia pipientis*: microbial manipulator of arthropod reproduction. *Annu. Rev. Microbiol.* **53**:71-102.

Stouthamer R., and Kazmer D.J., 1994, Cytogenetics of microbe-associated parthenogenesis and its consequences for gene flow in *Trichogramma* wasps. *Heredity*. **73**:317-327.

Stouthamer R., van Tilborg M., de Jong J.H., Nunney L., and Luck R.F., 2001, Selfish element maintains sex in natural populations of a parasitoid wasp. *Proc. R. Soc. Lond. B*. **268**:617-622.

Tagami Y., Miura K., and Stouthamer R., 2001, How does infection with parthenogenesis-inducing *Wolbachia* reduce the fitness of *Trichogramma*? *J. Invertebr. Pathol*. **78**:267-271.

Van Vugt J.J.F.A., Salverda M., de Jong H., and Stouthamer R., 2003, The paternal sex ratio chromosome in the parasitic wasp *Trichogramma kaykai* condenses the paternal chromosomes into a dense chromatin mass. *Genome*. **46**(4):580-587.

Werren J.H., Nur U., and Eickbush D., 1987, An extrachromosomal factor causing loss of paternal chromosomes. *Nature*. **327**:75-76.

Werren J.H., and Stouthamer R., 2003, PSR (paternal sex ratio) chromosomes: the ultimate selfish genetic elements. *Genetica*. **117**:85-101.

Chapter 6

Sex ratio distorter-associated selective mating in *Trichogramma kaykai*

Sex ratio distorter-associated selective mating in *Trichogramma kaykai*

Abstract

The haplodiploid parasitoid wasp *Trichogramma kaykai* hosts two sex ratio distorters that manipulate the sex ratio in a different direction. The cytoplasmic bacterium *Wolbachia* induces gamete duplication in the first mitotic division resulting in the development of an infected female from an unfertilized egg in this species. Besides *Wolbachia*, a paternal sex ratio chromosome exists in males which destroys the paternal chromosome set in a fertilized egg. The egg develops into a male carrying PSR. The bacterium is inherited via egg cytoplasm. PSR, on the other hand, is only inherited via sperm. In situations where the two sex ratio distorters are present, three heritable components have different optimal mate choices. Both infected and uninfected females should avoid mating with PSR males because mating with a PSR male results in a severe loss of fitness for both the nuclear genome and the *Wolbachia*. For males either PSR or normal the choice seems less important, however the argument could be made that PSR males should prefer infected females, while uninfected males should prefer uninfected females. The results of our choice experiments run against the expectation. In crossing experiments with normal males and infected and uninfected females the mating was random, while in crosses involving PSR males and infected and uninfected females a significantly higher proportion of the matings took place between PSR males and uninfected females. Either PSR males have a preference for uninfected females or infected females prefer not to mate with PSR males, adaptive explanations for the latter case would require that the *Wolbachia* infection somehow would influence the mating behaviour of their hosts.

Keywords

Trichogramma kaykai, *Wolbachia*, PSR, non random mating

Introduction

Sex ratio distorters are factors that manipulate their hosts' sex ratio in favor of their own transmission (Werren *et al*, 1988). In populations polymorphic for the presence of a sex ratio distorter, mating with an individual that carries the sex ratio distorter, or is free of it, has vastly different fitness consequences. For instance in some populations of *Acraea encedon* and *A. encedana* in East Africa a male killing *Wolbachia* can attain high frequencies resulting in an extremely female biased sex ratio in the populations (Jiggins *et al*., 2000; Jiggins *et al*., 2001). The few males that are present will have a much higher fitness if they mate with an uninfected female versus mating with an infected female. Indeed Jiggins *et al*. (2001) have shown that males have a clear preference for uninfected females. Similarly in the phytophagous mite *Tetranychus urticae*, polymorphic for an infection with a *Wolbachia* strain inducing cytoplasmic incompatibility, the uninfected females showed preference for uninfected males over infected ones. Infected females did not show the preference (Vala, 2001). This behavior is adaptive because uninfected females have a higher fitness when they mate with uninfected males, while for infected females the infection status of the male is not important.

Trichogramma kaykai is a minute lepidopteran egg parasitoid found in the Mojave Desert in California, USA (Pinto *et al*., 1997). The species is infected with *Wolbachia* that induce parthenogenesis resulting in all-female broods in infected individuals (Stouthamer *et al*., 1990; Huigens *et al*., 2000). The infected and uninfected individuals of the species co-occur in the field (Huigens, 2003). Recently a second sex ratio distorter is found in this species (Stouthamer *et al*., 2001); the paternal sex ratio (PSR) chromosome which is an extremely selfish genetic element causing the destruction of the paternal set of chromosome in a fertilized egg (van Vugt *et al*., 2003; Werren and Stouthamer, 2003). Eggs fertilized by PSR sperm do not develop into females but grow out to be males again carriers of the PSR chromosome. Infected eggs fertilized by PSR sperm become infected PSR males. The presence of PSR is largely responsible for keeping the *Wolbachia* infection at low levels in the parasitoid *T. kaykai* (Stouthamer *et al*., 2001).

Female *T. kaykai* can mate several times under laboratory conditions and it is often observed that a female mates more than twice within 20 minutes after a male has been introduced. Multiple mating may occur in the field as well (Huigens, 2003). *T. kaykai* females in the field parasitize relatively large host eggs. Per host egg a female will produce on average a clutch of 4 ~ 5 eggs, which normally consists of a single unfertilized egg and 3 ~ 4 fertilized eggs. When an uninfected female mates with a normal male she will produce 3 ~ 4 daughters and a single male from a host egg. Subsequently most of these females will mate with their brother before dispersing. However, if an uninfected female mates with a PSR male then the brood will consist of 3 ~ 4 PSR males from the fertilized eggs and one normal male from the unfertilized egg, in order to acquire matings these males will have to compete in the population for females. Only about 30 percent of all the females is estimated to mate with a male that is not their brother (Huigens, 2003). A PSR male has therefore limited mating opportunities. However, if an infected female mates with a PSR male, her clutch will consist of a single female from the unfertilized egg and 3 ~ 4 PSR males from the fertilized egg. Under these circumstances the PSR males also have the opportunity to mate with their sister upon emergence. Infected PSR males therefore have an advantage over their uninfected PSR conspecifics, albeit small, to mate with a female. If offered the choice and everything else being equal, the PSR factor gains a selective advantage, when the male mates with the infected female. Similarly the argument can be made that a normal male should avoid mating with an infected female if given the choice and everything else being the same. Alternatively, all females should try to avoid mating with a PSR male if they have the choice.

Here we tested if *Wolbachia* and PSR could modify the male host's mating preference/acceptance by females. The bacteria in males gain no selective advantage due to their exclusive maternal inheritance. We expected the infection not to affect the male hosts' mating preference. Here we show that uninfected normal males mate equally frequent with infected versus uninfected females, but males harboring PSR, regardless of their *Wolbachia* infection status, mate more frequently with uninfected females. This pattern can also be caused by a higher

rejection rate of PSR males by *Wolbachia* infected females than by uninfected females. The results are not expected. We propose possible explanations about the results.

Materials and methods

Trichogramma *species and lines*

Wolbachia infected *T. kaykai* was collected at Last Chance Canyon, Kern County, California, is abbreviated to LCT. The sexual isofemale line is cured through antibiotic treatment and abbreviated to LCA. The infected PSR line is maintained on the LCT genetic background by mating a LCT female with an infected PSR male and abbreviated to Inpsr. The uninfected PSR males are obtained by mating a LCA female with an Inpsr for the test and abbreviated to Unpsr. In the laboratory all the lines including Unpsr were reared on *Ephestia kuehniella* (Lepidoptera: Pyralidae) eggs provided by Koppert B.V. at 23±1°C, 16:8 LD, and 60±10 % RH.

Mating experiments

One day before the wasps emerged, parasitized *E. kuehniella* eggs were individually placed in glass tubes (7.5×1cm). When the female wasps emerged, an uninfected female and an infected female were transferred in a new glass tube. A male wasp was introduced in the tube. When the male mated with a female, the other unmated female was taken out of the tube and killed. The remaining two wasps were frozen for further molecular analysis. This experiment was done using first uninfected normal males, secondly for infected PSR carrying males and lastly for uninfected PSR carrying males.

Detection of Wolbachia *and PSR*

Genomic DNA of the wasps was individually extracted with 50 µl of 5 % Chelex-100 solution and 2 µl of proteinase K and incubated at 56°C for 3 hours and at 95°C for 10 min (Mastercycler® gradient, eppendorf). To determine infection with *Wolbachia* of the females and Inpsr, PCR with a *Wolbachia*-specific primer set (*wsp*) was performed. The temperature profile was 94°C for 3

min (1cycle); 94°C for 1 min, 50°C for 1 min and 72°C for 1min (40 cycles) on a thermocycler (Mastercycler® gradient, eppendorf) (Braig *et al.,* 1998).

To determine if the males from Inpsr and Unpsr carried PSR, PCR with a PSR-specific primer set was performed (van Vugt *et al*., in prep). The temperature profile was 94°C for 2 min (1cycle); 94°C for 1 min, 61°C for 1min and 72°C for 1 min (35 cycles) on a thermocycler (Mastercycler® gradient, eppendorf). The PCR products were run on a 1% agarose gel and were visualized on the UV light.

Sometimes insufficient amounts of DNA were extracted from the individual wasps to determine the host's infection status and/or the presence of PSR. To assure that negative results were not caused by low DNA titers the DNA concentration in the sample was determined by using a spectrophotometer (SmartSpec™ 3000, Bio-rad). When sufficient DNA (more than 30ng/µl) was not present the individual was excluded from further analysis.

Results

Mating experiments

The normal males mated with equal frequencies with infected and uninfected females (Figure 1). 26 out of 54 (48.1%) males mated with the infected females (χ^2 test $P>0.1$). Infected PSR males significantly mated more frequently with uninfected females (Figure 1), 34 out of 90 (37.8%) males mated with infected females (χ^2 test $P<0.01$). Similarly, the uninfected PSR males significantly mated more frequently with uninfected females (Figure 1), 36 out of 103 (35%) chose infected females (χ^2 test $P<0.01$).

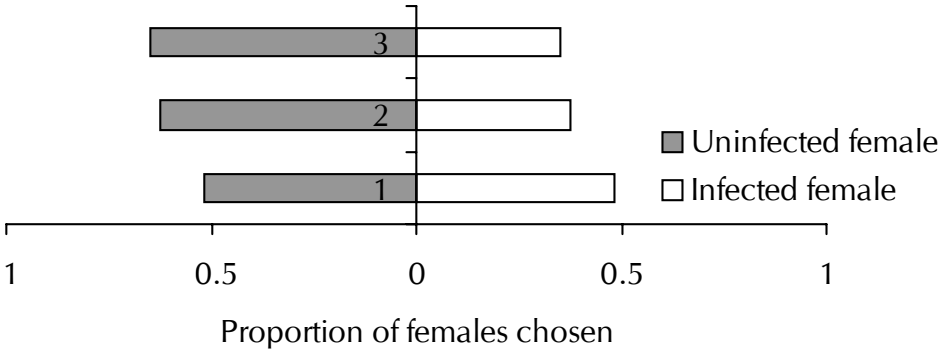

Figure 1. Relative proportion of females that mated in an experiment where a single male (a normal male, a *Wolbachia* infected PSR male or an uninfected PSR male) was placed in a glass tube with a *Wolbachia* infected and an uninfected female.

Number of males tested: 1: normal 54; 2: Inpsr 90; 3: Unpsr 103

Discussion

The result shows that in the laboratory condition no assortative mating takes place when normal males are placed with infected and uninfected females. However when PSR males are placed with infected and uninfected females a significantly higher number of matings takes place with the uninfected females. This mating preference is found independent of the *Wolbachia* infection status of the PSR males. Because of its mode of action to destroy the paternal chromosome, PSR can not select host any particular genotypes. Even though PSR is assumed to have non-functional genes on it (Werren and Stouthamer, 2003), if the males are the choosy sex the mating preference of the PSR males would have its origin in PSR. Choosiness of a sex may be favored when mating is costly to the sex and when there is variation in quality among members of the opposite sex (Amundsen and Forsgren, 2003; Kirkpatrick and Barton, 1997). Theoretically low quality individuals of both sexes are expected to be less choosy (Parker, 1983). The result, however, does not mean that the normal males are phenotypically or genetically in low quality. We do not know if females or males are the choosy sex in *Trichogramma*. If males are the choosy sex, then it appears that PSR males are choosy. Alternatively if females are the choosy sex, then the

argument could be made that *Wolbachia* infected females are choosier than normal females.

Assuming that the males are the choosy sex then the choice of the PSR males for uninfected females appears to run against our argument that infected females would result in a better transmission for the PSR. This argument is based on the fact that by mating with an infected female the PSR father will offer his PSR sons a single female to mate within its brood. This is assumed to be an improvement over the alternative when the PSR sons emerge together with a single uninfected PSR brother.

Under the alternative explanation of the female being the choosy sex, the *Wolbachia* infected female would be choosier than the normal female. This could only come about if the presence of *Wolbachia* would somehow enable the female to "distinguish" PSR males from normal males. Under field conditions *Wolbachia* infected females run a higher risk of mating with a PRS male than normal females do, therefore any *Wolbachia* variant that would allow the female to discriminate between normal and PSR males would have a selective advantage. Of course the same argument can be made for normal females, however the selective advantage of such a trait is much higher for *Wolbachia* infected females because of their higher risk of mating with PSR males in the field (Stouthamer *et al*., 2001).

In several studies clear adaptive explanations can be found for assortative mating when reproductive parasites such as *Wolbachia* are present (Jiggins *et al*., 2001; Vala, 2001). There have been few studies that fail to find assortative mating at all (O'Neill, 1991) or fail to find an adaptive explanation for part of the assortative mating that they do find (Moreau *et al*., 2001). In *Drosophila* species no evidence was found for assortative mating between infected and uninfected individuals (O'Neill, 1991). In populations of the woodlouse, *Armadillidium vulgare,* that is polymorphic for *Wolba*chia causing genetic males to develop into functional females, uninfected males prefer uninfected females over infected ones having the ZZ genotype. But there was no difference in preference between infected females of the ZW genotype and uninfected females. They concluded

that instead of *Wolbachia* infection, the genotype of the females may influence the mating preference of the males (Moreau *et al*., 2001).

If our hypothesis that *Wolbachia* infected females are capable of distinguishing between normal and PSR males is true, we would expect that infected females in the field would mate with PSR males at a lower frequency than is predicted by the frequency of PSR males in the population. Future fieldwork will determine if this is the case. This reluctance of infected females to mate with PSR males may also explain the lower than expected PSR frequency among males in the field (Huigens, 2003; Stouthamer *et al*., 2001). If this trait can gain expression in the infected females, it may lead to the complete loss of PSR from the population, which then would result in the fixation of the *Wolbachia* infection in the field populations (Stouthamer *et al*., 2001).

Acknowledgement

The authors are grateful to Mohammad Ardeh and Ties Huigens for their useful comments. Many thanks are given to Mohammad Ardeh, Nina Fatouros, and Ties Huigens for their critical reviews of early manuscripts. Leo Koopman and Koppert B.V. are acknowledged for providing *E. kuehniella* eggs. The authors thank Peter de Jong and Leo Koopman for sharing experimental space. This study is supported in part by Nexgen Biotechnologies Inc. (Korea).

References

Amundsen T., and Forsgren E., 2003, Male preference for colourful females affected by male size in a marine fish. *Behav. Ecol. Sociobiol.* **54**:55-64.

Braig H.R., Zhou W., Dobson S.L., and O'Neill S.L., 1998, Cloning and characterization of a gene encoding the major surface protein of the bacterial endosymbiont *Wolbachia*. *J. Bacteriol.* **180**:2373-2378.

Huigens M.E., Luck R.F., Klaassen R.H.G., Maas M.F.P.M., Timmermans M.J.T.N., and Stouthamer R., 2000, Infectious parthenogenesis. *Nature*. **405**:178-179.

Huigens M.E., 2003, *On the evolution of* Wolbachia*-induced parthenogenesis in* Trichogramma *wasps*. Ph.D. thesis. Wageningen University.

Jiggins F.M., Hurst G.D.D., Dolman C.E., and Majerus M.E.N., 2000, High prevalence male-killing *Wolbachia* in the butterfly *Acraea encedana*. *J. Evol. Biol*. **13**(3):495-501.

Jiggins F.M., Hurst G.D.D., and Majerus M.E.N., 2001, Sex-ratio-distorting *Wolbachia* causes sex-role reversal in its butterfly host. *Proc. R. Soc. Lond. B*. **267**:69-73.

Kirkpatrick M., and Barton N.H., 1997, The strength on indirect selection on female mating preferences. *Proc. Natl. Acad. Sci. USA*. **94**:1282-1286.

Moreau J., Bertin A., Caubet Y., and Rigaud T., 2001, Sexual selection in an isopod with *Wolbachia*-induced sex reversal: males prefer real females. *J. Evol. Biol*. **14**:388-394.

O'Neill S.L., 1991, Cytoplasmic incompatibility in *Drosophila* populations: Influence of assortative mating on symbiont distributions. *J. Invertebr. Pathol*. **58**:436-443.

Parker G.A., 1983, Mate quality and mating decisions. In: Bateson P. (eds), *Mate choice*. Cambridge University Press: Cambridge. pp141-166.

Pinto J.D., Stouthamer R., and Platner G.R., 1997, A new cryptic species of *Trichogramma* (Hymenoptera: Trichogrammatidae) from the Mojave Desert of California as determined by morphological, reproductive and molecular data. *Proc. Entomol. Soc. Wash*. **99**(2):238-247.

Stouthamer R., and Luck R.F., 1993, Influence of microbe-associated parthenogenesis on the fecundity of *Trichogramma deion* and *T. pretiosum*. *Entomol. Exp. Appl*. **67**:183-192.

Stouthamer R., van Tilborg M., de Jong J.H., Nunney L., and Luck R.F., 2001, Selfish element maintains sex in natural populations of a parasitoid wasp. *Proc. R. Soc. Lond. B*. **268**:617-622.

Tagami Y., Miura K., and Stouthamer R., 2001, How does infection with parthenogenesis-inducing *Wolbachia* reduce the fitness of *Trichogramma*? *J. Invertebr. Pathol*. **78**:267-271.

Vala F., 2001, *Genetic conflicts between cytoplasmic bacteria and their mite host*. Ph.D thesis. University of Amsterdam.

Van Vugt J.J.F.A., Salverda M., de Jong H., and Stouthamer R., 2003, The paternal sex ratio chromosome in the parasitic wasp *Trichogramma kaykai* condenses the paternal chromosomes into a dense chromatin mass. *Genome*. **46**(4):580-587.

Werren J.H., Nur U., and Wu C.I., 1988, Selfish genetic elements. *Trends Ecol. Evol*. **3**(11):297-302.

Werren J.H., and Stouthamer R., 2003, PSR (paternal sex ratio) chromosomes: the ultimate selfish genetic elements. *Genetica*. **117**:85-101.

Chapter 7

Summarizing discussion

Summarizing discussion

Eukaryotic cells evolved with competing microorganisms, and in some cases endosymbiotic relationship between them has coevolved (Kahn *et al.*, 2002). An eminent example of such a symbiosis is mitochondria. Its genome sequence is related to that of the α-proteobacteria (Kurland and Andersson, 2000). Another α-proteobacteria group contains *Wolbachia* a common endosymbiont of invertebrates that manipulates its hosts' sex ratio (for review see O'Neill *et al.*, 1997; Jeyaprakash and Hoy, 2000; Stouthamer *et al.*, 1999; Werren, 1997; Werren *et al.*, 1995).

Besides the four sex ratio distortions (male killing, feminization, parthenogenesis and crossing incompatibility) other unique effects of the *Wolbachia* strains on their diverse hosts indicate close interactions between the bacteria and their hosts. Normally hosts can be cured from the skewed sex ratio caused by *Wolbachia*, when the bacteria are removed (Stouthamer *et al.*, 1990). But it is increasingly evident that in some cases the relationship is so tight that the host cannot reproduce successfully without their symbiont. For instance, in *Asobara tabida*, a *Wolbachia* strain is essential for oogenesis of the wasp (Dedeine *et al.*, 2001), and in the filarial nematodes, *Litomosoides sigmodontis* and *Onchocerca volvulus*, loss of *Wolbachia* causes sterility (Hoerauf *et al.*, 1999; Hoerauf *et al.*, 2000). These specific interactions are most likely only the tip of the iceberg in the relationships between the symbionts and their hosts; many remain to be found.

In addition to microorganisms, other heritable elements such as driving chromosomes and B-chromosomes are important factors influencing the sex ratio and - with that - the evolution of sex ratio and sex determination (Hurst and Werren, 2001; Jaenike, 2001; Stouthamer *et al.*, 2001; Werren and Stouthamer, 2003). Among them the PSR chromosome occurs only in species with a haplodiploid sex determination system and can severely reduce the population growth rate by converting fertilized eggs that would normally become females into males that are again carriers of the PSR (Reed and Werren, 1995; van Vugt *et al.*, 2003; Werren and Stouthamer, 2003).

In this thesis I show close interactions between host genes and *Wolbachia*, *Wolbachia* and PSR, and PSR and host genes. Such seemingly complex interactions are the results of symbiotic or parasitic nature of the sex ratio distorters and of reaction of their host genes.

1. Summary of the results

Chapter 2. *Why can't we establish sexual lines from infected populations?*

In *Telenomus nawai*, the *Wolbachia* infected form is geographically isolated from the uninfected form (Arakaki *et al*., 2000). I tested whether a sexual line could be established from the infected line by antibiotic treatment. Just as was found earlier by Arakaki *et al*. (2000) this was not possible. This confirms the pattern that has been observed before. In *Trichogramma* species sexual lines can be derived from infected populations but not in other taxa. This failure to establish a sexual line is caused by the fact that females from the infected line are unsuccessful in fertilizing their eggs, either because they did not mate or because of some post insemination problem with the fertilization of the eggs. Males from the infected line obtained by antibiotic treatment are functional and capable of fathering offspring when mated with the females of the sexual line. Huigens and Stouthamer (2003) have theorized that during the spread of the parthenogenesis inducing (PI) *Wolbachia* in a population a mutant that will allow females to produce male offspring has initially a large selective advantage because of sex ratio selection. Later, most of the population consists of females that are infected and are homozygous for this mutation. The mutation will go to fixation because practically all the males that are still produced are offspring of the mutant infected females when they produce eggs with a low *Wolbachia* titer. The ultimate result of this mutation is that once the population has gone to fixation all the infected females are mutant and therefore they are unable to produce offspring from fertilized eggs. Here we test this hypothesis by introgressing the "mutant" genotype into the sexual line to determine the genetic basis of the trait. The result of this introgression shows that the trait is recessive and has a simple genetic basis either involving one major locus with some modifiers, or it involves two loci with two alleles each. It is expected that this is a general phenomenon

in those cases where it is not possible to establish sexual lines from originally infected populations. Why *Trichogramma* is so different from other species in this respect remains an unanswered question. However, if this scenario is as common as we think, once a population has gone this route it will be practically impossible to re-establish sexual reproduction in these populations (Figure1).

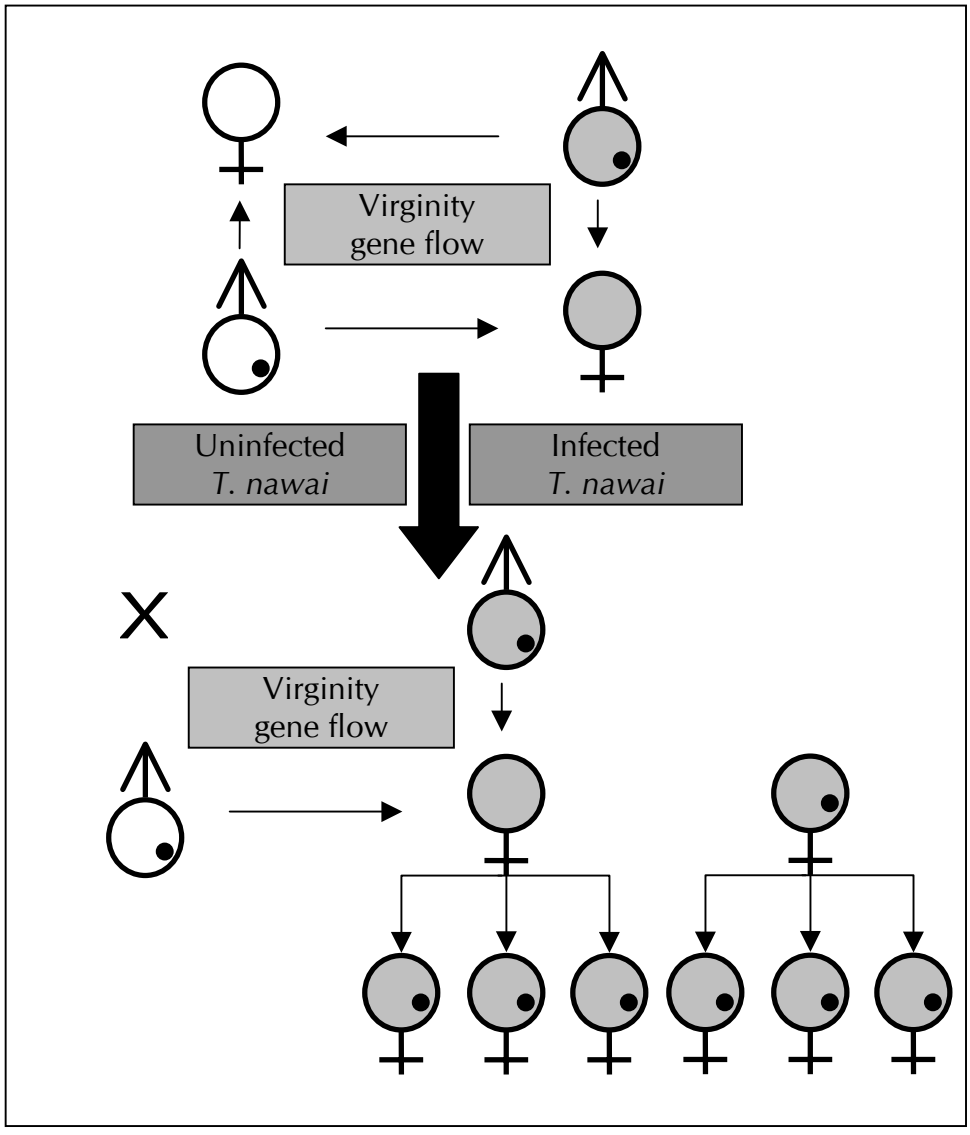

Figure 1. The fate of uninfected and infected individuals in a mixed population with the virginity mutation. ◯ : Uninfected; ● : Infected; ● :virginity mutation

Chapter 3. *PSR beyond reproductive isolation?*

In 1922, Haldane published one of the most general and influencing rules in evolutionary biology. It is commonly called the Haldane's rule where, in interspecific hybridization, the heterogametic sex is normally sterile or inviable (Haldane, 1922). Hybrid sterility and inviability are the most important features for populations as initial steps in the speciation process. The difference between the two may come from the level of chromosomal compatibility, when incompatible the hybrid dies, otherwise it survives but is sterile. Apart from chromosomal compatibility, recent findings suggest the importance of the components of cytoskeleton such as spindle and dynein etc. in normal development of cells (Beaudouin *et al*., 2002; Bornens and Piel, 2002; Robinson *et al*., 1999; Salina *et al*., 2002). If these cytoplasmic organelles do not function correctly in a fertilized egg, the egg will die, even if the two chromosome sets are compatible.

I investigated whether the two distinctive postzygotic isolation mechanisms differentially affected the interspecific transmission of PSR in *Trichogramma*. The results show that sterility and inviability between two species are overcome by PSR (Figure 2). An interesting asymmetry was found in the efficiency of PSR transmission between the original host *T. kaykai* and the novel host *T. deion*. While the transmission from *kaykai* to *deion* is efficient, the transmission from *deion* to *kaykai* is much lower. This is partly due to the inefficient transmission of PSR within *T. deion* (chapter 4 and 5) (Figure 2). However, the incompatibility between a *T. kaykai* egg and *T. deion* sperm may have an influence on the inefficient transmission of PSR as well. This may not be caused by nuclear incompatibility. Cytoplasmic factors, that are essential for cell development such as centrosomes i.e. microtubule organizing center (Kellog *et al*., 1994), or dynein, that connects microtubules to chromosomes in meiosis and mitosis (Schliwa and Woehlke, 2003), may not be functional in the cross or their action is not triggered by the heterospecific sperm penetration.

Another unusual outcome of these experiments was that in the cross between *T. deion* females and *T. kaykai* males "hybrid" females were produced that

appeared to be phenotypically and genetically entirely of paternal origin. These results require further scrutiny.

Chapter 4. *A sex ratio distorter suffering from conflicts*

A sex ratio of 1:1 is the evolutionary stable strategy (ESS) for nuclear chromosomes of sexual organisms (Fisher, 1930; West and Herre, 2002), when the cost for producing a son or a daughter is equal. But the existence of factors that do not follow the ESS form a complex context in the evolution of the sex ratio. For example cytoplasmic factors are transmitted through females, and nuclear factors through both females and males in sexual reproduction. This non-Mendelian transmission of cytoplasmic factors may cause genetic conflicts (Cosmides and Tooby, 1981). Normally cytoplasmic factors will cause a female bias. Genetic conflicts can be an evolutionary force, which can lead for example to a change of genetic systems, eukaryotic evolution, speciation, extinction, and social behavior (Abbot *et al*., 2001; Hurst, 1992; Hurst Schilthuizen, 1998; Hurst and Werren, 2001; Keller and Ross, 1998).

PI-*Wolbachia* is strictly maternally inherited, while PSR is only inherited via males. These opposing transmission routes of the two sex ratio distorters may cause a genetic conflict. As seen in the results, in the natural host, *Trichogramma kaykai*, the host's genetic background and PI-*Wolbachia* have no negative effect on the transmission of PSR. In this case it appears there are no suppressors present for any of the factors, the PSR "wins" over both the nuclear genome and the *Wolbachia*, while the *Wolbachia* "wins" over the nuclear genome. On the other hand in the novel host, *T. deion*, the transmission of PSR is impeded by the host's genetic background. Furthermore, *Wolbachia* infection enhances the inefficiency of the PSR transmission. The result of interaction is the production of some female offspring by PSR males (Figure 2). The different efficiency of transmission of PSR in the two species indicates that either PSR simply does not function very well in the novel host, or this inefficient transmission may be the remainder of a nuclear and/or *Wolbachia* based suppressor of the PSR. Under this last hypothesis the reason that the PSR is not found in *T. deion* field populations is that suppressors have evolved against this element in the past

(Figure 2) that are still functional in the population. In addition, the effect of the *Wolbachia* on the PSR transmission can be seen as a novel effect of PI-*Wolbachia* infection other than sex ratio distortion.

Chapter 5. *Wolbachia and host genes interfere with PSR?*

I have been able to show how *Wolbachia* infection and the host genetic background in the novel host, *Trichogramma deion* affects the transmission efficiency of PSR. *Wolbachia* and the host genome can overcome the effect of PSR leading to the recovery of female production from eggs inseminated by PSR fathers. PSR is not found in *T. deion* in the field, yet there should have been possibilities for PSR to enter *T. deion* through interspecific matings with *T. kaykai* PSR males. This tells us that the ability of the host genome and *Wolbachia* to suppress PSR may be the remnant of a previous presence of PSR in this species (Figure 2). PSR males of *T. deion* showed variation in their ability to pass on the PSR. Four different categories of males were detected in *Wolbachia* infected PSR males, and only two in uninfected PSR males. As noted earlier the transmission of PSR was a lot lower through *Wolbachia* infected males than in males free of the infection. In infected PSR males the transmission of PSR ranged from perfect to a seemingly lack of sperm production all together. While in uninfected males the transmission was either efficient or inefficient, some of the males also fathered some female offspring. Unfortunately we did not determine if the PSR male offspring of a father with perfect transmission, differed in his ability to pass on PSR from a PSR male that was the offspring of a father with imperfect transmission. Cytogenetic studies are needed to determine if the sperm of the imperfectly transmitting males is polymorphic for the presence of the PSR chromosome or if somehow the effect of the PSR in the first mitotic division is modified leading to the loss of the PSR instead of to the loss of the normal paternal chromosomes. All experiments done thus far have been limited to a single PSR line. Consequently we do not know if there is variation in expression levels between different PSR lines.

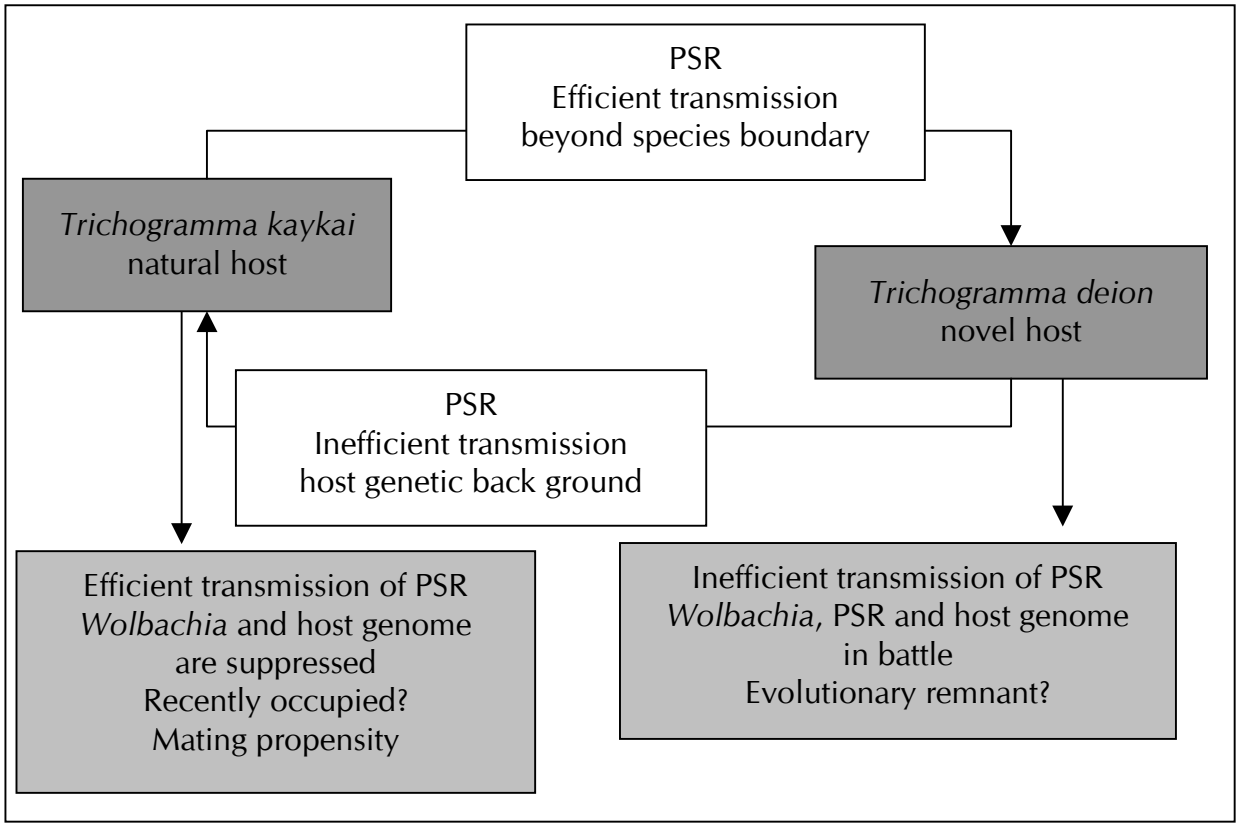

Figure 2. Genetic conflicts in the *Wolbachia*-PSR-host genome complex in *Trichogramma kaykai* and *T. deion*.

Chapter 6. *Mating preference*.

Conspecific mate choice is important in maintaining the integrity of a sexual population (Kokko *et al.*, 2003). Obtaining a fitter mate will cause the choosing mate a higher probability of transmitting fitter genes to descendants. Mating preference depends on phenotypes (Sinervo, pers. com.). I have tried to determine whether the different phenotypes caused by the *Wolbachia* infection and the presence of PSR resulted in a non random mating. In *Trichogramma* it is not known which of the partners is the choosy sex. When we exposed normal males to an infected and an uninfected female, there was no deviation from random in the female they mated with. However, when PSR males were used, a substantially higher number of matings took place with uninfected females. This may be caused by a male's preference for uninfected females or by the preference or discrimination in the females. If the male were the choosy sex, we would have expected the PSR male to mate with the infected female. If the

females are the choosy sex, it may mean that the *Wolbachia* infected females prefer not to mate with PSR males. Because PSR is a major factor in suppressing the *Wolbachia* infection frequencies in *T. kaykai* populations, and because the frequency of PSR males mating with infected females is relatively high in the field, a mutant form of *Wolbachia* that would somehow be able to discriminate against PSR males would have a selective advantage. If this were truly a general pattern in these populations, this would be an example of one sex ratio distorter evolving in response to the presence of an opposing second sex ratio distorter.

2. Future research with sex ratio distorters

2-1. PI-*Wolbachia*

Even though the research on *Wolbachia* has been growing exponentially (Webspirs search hits 514 articles since 1996), there are still many unexplored fields. *Trichogramma* is the only taxon where infected and uninfected individuals are found together. Suppressor genes and PSR are proposed as well as other factors influencing the *Wolbachia* dynamics (Huigens, 2003). The high failure rate of gamete duplication seems too high in the results reported by Tagami *et al*. (2001). It should therefore be tested in a larger number of lines, in addition the mechanism of the failure should be studied with the more advanced cytogenetic techniques that are now available (Gerlich *et al*., 2001; Francis-Lang *et al*., 1999).

There are several mechanisms to restore diploidy resulting from the *Wolbachia* infection. Nuclear fusion in *Muscidifurax* and gamete duplication in *Trichogramma* and *Leptopilina clavipes* have been found (Gottlieb *et al*., 2002; Pannebakker *et al*., in press; Stouthamer and Kazmer, 1994). Gamete duplication seems to occur in *Diplolepis rosae* as well (Stille and Dävring, 1980). Additional mechanisms for the restoration of diploidy may exist.

In gamete duplication an unanswered question remains how fertilization effectively obstructs the action of *Wolbachia* in *Trichogramma*. A factor (or factors) of paternal origin may nullify the gamete duplication by *Wolbachia*. Or it may be an effect of timing. If in a fertilized egg the sperm arrives and initiates the diploidization of the egg in x minutes through the fusion with the pronucleus,

and in an unfertilized infected egg the initiation of gamete duplication starts at a time later than x, then the fertilization with sperm may preclude the *Wolbachia* effect. Gamete duplication only occurs in the first mitotic division. It should also be verified if gamete duplication does not occur in some later mitotic divisions, which may explain the high incidence of gynandromorphs in PI-*Wolbachia* infected wasps (Stouthamer, 1997). Other novel effects of PI-*Wolbachia* such as regulation of PSR shown here remain to be discovered in other taxa (chapter 4 and 5).

Wolbachia proteomics is also very fascinating. Once a protein (cluster) responsible for gamete duplication is identified and its structure is revealed, the gene (cluster) encoding the protein (cluster) can be traced. When the currently running *Wolbachia* genome projects are published, the tasks will be easier.

Finally in biological control using parasitoids, only a female wasp is effective in controlling pests. Parthenogenetic wasps are cheaper in mass production and are more easily established (Huigens, 2003; Silva, 1999; Stouthamer, 2003; van Meer, 1999). It will be advantageous for biological control, if a tool can be developed to transmit PI-*Wolbachia* horizontally to novel hosts.

2-2. PSR

PSR has so far been found in *Nasonia* and *Trichogramma* (Stouthamer *et al*., 2001; Werren *et al*., 1987). The PSR like factors may cause such a reduction in the population growth rate that the population goes to extinction. The conditions for spread of PSR like factors in populations are such that many more cases of PSR are expected in haplodiploids (Werren and Stouthamer, 2003).

The mechanism induced by PSR in *Trichogramma* is very similar to that in *Nasonia*, even though their origin is totally different (Werren and Stouthamer, 2003). So far knowledge of its mechanism is fragmentary. Cytogenetic and molecular studies may show us how PSR influences the paternal chromosome, why cytoplasmic cell organizers can not recognize the malfunction *etc*.

It has been suggested that PSR can be introduced as a biological control agent of haplodiploid pests or a vehicle for genetic engineering of haplodiploids (Werren and Stouthamer, 2003). Obviously the mechanism of PSR destroying the paternal

chromosome set is very attractive especially when a target organism is an economically important haplodiploid organism. But there are several prerequisites. First, and foremost, it should easily be transmitted into the target organism (chapter 3). We do not know if PSR continues to function stably in other taxa. Our results in *T. deion* suggest it does not, however we do not know how general this declined functionality is. Secondly if PSR is incompatible with the genome of its novel host, PSR could be lost (chapter 4 and 5). Finally, we have to know the factors that can potentially interfere with the action of PSR (chapter 5).

Acknowledgements

Mohammed Ardeh, Ties Huigens, Richard Stouthamer and Joop van Lenteren are thanked for their important contributions to this chapter

References

Abbot P., Withgott J.H., and Moran N.A., 2001, Genetic conflict and conditional altruism in social aphid colonies. *Proc. Natl. Acad. Sci. USA*. **98**(21):12068-12071.

Arakaki N., Noda H., and Yamagishi K., 2000, *Wolbachia*-induced parthenogenesis in the egg parasitoid *Telenomus nawai*. *Entomol. Exp. Appl*. **96**:177-184.

Beaudouin J., Gerlich D., Daigle N., Eils R., and Ellenberg J., 2002, Nuclear envelope breakdown proceeds by microtubule-induced tearing of the lamina. *Cell*. **108**:83-96.

Bornens M., and Piel M., 2002, Centrosome inheritance: Birthright or the privilege of maturity? *Curr. Biol*. **12**:R71-R73.

Cosmides L.M., and Tooby J., 1981, Cytoplasmic inheritance and intragenomic conflict. *J. Theor. Biol*. **89**:83-129.

Dedeine F., Vavre F., Fleury F., Loppin B., Hochberg M.E., and Boulétreau M., 2001, Removing symbiotic *Wolbachia* bacteria specifically inhibits oogenesis in a parasitic wasp. *Proc. Natl. Acad. Sci. USA*. **98**:6247-6252.

Fisher R.A., 1930, *The genetical theory of natural selection*. Oxford University Press: Oxford.

Franscis-Lang H., Minden L., Sullivan W., and Oegema K., 1999, Live confocal analysis with fluorescently labeled proteins. *Methods Mol. Biol*. **122**:223-239.

Gerlich D., Beaudouin J., Gebhard M., Ellenberg J., and Eils R., 2001, Four-dimensional imaging and quantitative reconstruction to analyse complex spatiotemporal processes in live cells. *Nat. Cell Biol*. **3**:852-855.

Gottlieb Y., Zchori-Fein E., Werren J.H., and Karr T.L., 2002, Diploidy restoration in *Wolbachia*-infected *Muscidifurax uniraptor* (Hymenoptera: Pteromalidae). *J. Invertebr. Pathol*. **81**:166-174.

Haldane J.B.S., 1922, Sex ratio and unisexual sterility in hybrid animals. *J. Genet*. **12**:101-109.

Hoerauf A., Nissen-Pähle K., Schmetz C., Henkle-Dürsen K., Blaxter M.L., Büttner K.W., Gallin M.Y., Al-Qaoud K.M., Lucius R., and Fleischer B., 1999, Tetracycline therapy targets intracellular bacteria in the filarial nematode *Litomosoides sigmodontis* and results in filarial infertility. *J. Clin. Invest*. **103**(1):11-17.

Hoerauf A., Volkmann L., Hamelmann C., Adjei O., Autenrieth I.B., Fleischer B., and Büttner D.W., 2000, Endosymbiotic bacteria in worms as targets for a novel chemotherapy in filariasis. *Lancet*. **355**:1242-1243.

Huigens M.E., 2003, *On the evolution of* Wolbachia*-induced parthenogenesis in* Trichogramma *wasps*. Ph.D. thesis. Wageningen University.

Huigens M.E., and Stouthamer R., 2003, Parthenogenesis associated with *Wolbachia*. In Bourtzis K., and Miller T.A. (eds), *'Insect symbiosis'*. CRC Press: Boca Raton. pp247-266.

Hurst L.D., 1992, Intragenomic conflict as an evolutionary force. *Proc. R. Soc. Lond. B*. **248**:135-140.

Hurst G.D.D., and Schilthuizen M., 1998, Selfish genetic elements and speciation. *Heredity*. **80**:2-8.

Hurst G.D.D., and Werren J.H., 2001, The role of selfish genetic elements in eukaryotic evolution. *Nat. Rev. Genet*. **2**:597-606.

Jaenike J., 2001, Sex chromosome meiotic drive. *Annu. Rev. Ecol. Syst.* **32**:25-49.

Jeyaprakash A., and Hoy M.A., 2000, Long PCR improves *Wolbachia* DNA amplification: *wsp* sequences found in 76% of sixty-three arthropod species. *Insect Mol. Biol.* **9**:393-405.

Kahn R.A., Fu H., and Roy C.R., 2002, Cellular hijacking: A common strategy for microbial infection. *Trends Biochem. Sci.* **27**(6):308-314.

Keller L., Ross K.G., 1998, Selfish genes: a green beard in the red fire ant. *Nature*. **394**:573-575.

Kellog D.R., Moritz M., and Alberts B.M., 1994, The centrosome and cellular organization. *Annu. Rev. Biochem*. **63**:639-674.

Kokko H., Brooks R., Jennions M.D., and Morley J., 2003, The evolution of mate choice and mating biases. *Proc. R. Soc. Lond. B*. **270**:653-664.

Kurland C.G., and Andersson S.G.E., 2000, Origins and evolution of the mitochondrial proteome. *Microbiol. Mol. Biol. Rev*. **64**:786-820.

O'Neill S.L., Hoffmann A.A., and Werren J.H., 1997, *Influential passengers: Inherited microorganisms and arthropod reproduction*. Oxford University Press: Oxford.

Pannebakker B.A., Pijnacker L.P., Zwaan B.J., and Beukeboom L.W., Cytology of *Wolbachia*-induced parthenogenesis in *Leptopilina clavipes* (Hymenoptera: Figitidae). *Genome*. In press.

Reed K.M., and Werren J.H., 1995, Induction of paternal genome loss by the paternal sex ratio chromosome and cytoplasmic incompatibility bacteria (*Wolbachia*): A comparative study of early embryonic events. *Mol. Reprod. Dev*. **40**:408-418.

Robinson J.T., Wojcik E.J., Sanders M.A., McGrail M., and Hays T.S., 1999, Cytoplasmic dynein is required for the nuclear attachment and migration of centrosomes during mitosis in *Drosophila*. *J. Cell. Biol*. **146**(3):597-608.

Salina D., Bodoor K., Eckley D.M., Schroer T.A., Rattner J.B., and Burke B., 2002, Cytoplasmic dynein as a facilitator of nuclear envelope breakdown. *Cell*. **108**:97-107.

Schliwa M., and Woehlke G., 2003, Molecular motors. *Nature*. **422**:759-765.

Silva I.M.M.S., 1999, *Identification and evaluation of* Trichogramma *parasitoids for biological pest control*. Ph.D. thesis. Wageningen University.

Stille B., and Dävring L., 1980, Meiosis and reproductive strategy in the parthenogenetic gall wasp *Diplolepis rosae*. *Hereditas*. **92**:353-362.

Stouthamer R., 2003, The use of unisexual wasps in biological control. In: van Lenteren J.C. (ed), *'Quality control and production of biological control agents: Theory and testing procedures'*. CABI Publishing: Oxon. pp93-114.

Stouthamer R., Breeuwer J.A.J., and Hurst G.D.D., 1999, *Wolbachia pipientis*: Microbial manipulator of arthropod reproduction. *Annu. Rev. Microbiol*. **53**:71-102.

Stouthamer R., and Kazmer D.J., 1994, Cytogenetics of microbe-associated parthenogenesis and its consequences for gene flow in *Trichogramma* wasps. *Heredity*. **73**:317-327.

Stouthamer R., Luck R.F., and Hamilton W.D., 1990, Antibiotics cause parthenogenetic *Trichogramma* to revert to sex. *Proc. Natl. Acad. Sci. USA*. **87**:2424-2427.

Stouthamer R., van Tilborg M., de Jong H., Nunney L., and Luck R.F., 2001, Selfish element maintains sex in natural populations of a parasitoid wasp. *Proc. R. Soc. Lond. B*. **268**:617-622.

Tagami Y., Miura K., and Stouthamer R., 2001, How does infection with parthenogenesis-inducing *Wolbachia* reduce the fitness of *Trichogramma*? *J. Invertebr. Pathol*. **78**(4):267-271.

Van Meer M.M.M., 1999, *Phylogeny and host-symbiont interactions of thelytoky inducing* Wolbachia *in Hymenoptera*. Ph.D thesis. Wageningen University.

Van Vugt J.J.F.A., Salverda M., de Jong H., and Stouthamer R., 2003, The paternal sex ratio chromosome in the parasitic wasp *Trichogramma kaykai* condenses the paternal chromosomes into a dense chromatin mass. *Genome*. **46**(4):580-587.

Werren J.H., 1997, Biology of *Wolbachia*. *Annu. Rev. Entomol*. **42**:587-609.

Werren J.H., Nur U., and Eickbush D., 1987, An extrachromosomal factor causing loss of paternal chromosomes. *Nature*. **327**:75-76.

Werren J.H., and Stouthamer R., 2003, PSR (paternal sex ratio) chromosomes: The ultimate selfish genetic elements. *Genetica*. **117**:85-101.

Werren J.H., Windsor D., and Guo L.R., 1995, Distribution of *Wolbachia* among neotropical arthropods. *Proc. R. Soc. Lond. B*. **262**:197-204.

West S.A., and Herre E.A., 2002, Using sex ratios: Why bother? In: Hardy I.C.W. (ed), '*Sex ratios: Concepts and research methods*'. Cambridge University Press: Cambridge. pp399-413.

Summary in English Dutch and Korean

Summary

Sex ratio of various organisms can be manipulated by selfish genetic elements. Among such sex ratio distorters *Wolbachia* bacteria have drawn much attention due to their ability to induce several sex ratio manipulations such as cytoplasmic incompatibility, feminization, male killing and parthenogenesis. Such manipulations of host sex ratio are only for the proliferation of the bacteria leading to female biased sex ratio. Aside from these major phenomena, the infection appears to be obligatory in some species. This strongly suggests that there has been a close relationship between the bacteria and their hosts.

Another extremely selfish element is the paternal sex ratio (PSR) chromosome that is only found in Hymenoptera. It drives a population to a male bias, because of its unique nature of paternal inheritance.

These two sex ratio distorters have different evolutionary interests, because of their different pattern of inheritance. Especially when they share their hosts, conflicts should arise. The aim of this thesis is to elucidate interactions between the sex ratio distorters and their hosts.

Until now it has been impossible to establish a sexual line from wasps infected with parthenogenesis inducing (PI) *Wolbachia* except for the genus *Trichogramma*. In chapter 2, it is demonstrated that the inability to restore sexuality from asexuality is genetically based in the *Wolbachia* infected wasp *Telenomus nawai*. The infected individuals are mutants that can not fertilize their eggs. The mutants, therefore, depends on the infection to maintain themselves. The mutant gene can drive the uninfected sexual individuals to extinction when arising in a population in which infected and uninfected wasps coexist.

The transmission of PSR is achieved by destroying the paternal genome in the fertilized egg. This fertilized egg develops into a male carrying PSR. With its mode of action PSR is thought to move beyond the species boundary relatively easily in haplodiploid organisms. Reproductive isolation is the most important factor in the biological species concept. Between two closely related minute wasp species *Trichogramma kaykai* and *T. deion* there are complex postzygotic reproductive isolation i.e. hybrid sterility and hybrid inviability. In chapter 3 it

was determined whether the two reproductive isolation mechanisms affected the interspecific transmission of PSR between these two species. PSR could overcome reproductive isolation mechanisms. But when the donor was *T. deion*, the transmission efficiency decreased. This implies that there is an effect of host genetic background on the PSR transmission.

It is thought that interspecific mating between geographically overlapping *T. kaykai* and *T. deion* occurs in the field. But PSR has not yet been found in *T. deion*. I compared the transmission efficiency of PSR in *T. deion*, the novel host, with that of *T. kaykai*, the natural host in chapter 4. The transmission efficiency of PSR in the novel host is very low. In addition when the PSR male of the novel host is infected with *Wolbachia*, it is even lower. It can, therefore, be concluded that there are conflicts among the host genome, *Wolbachia* and PSR in the novel host.

As demonstrated in chapter 4 the transmission efficiency of PSR in *T. deion* is largely hampered by the infection of *Wolbachia*. In chapter 5, I tried to determine whether variation in transmitting PSR occurred in the species. The results show that the transmission efficiency can be divided into four distinctive categories by their offspring sex ratio i.e. perfect transmission, imperfect transmission, non-functional sperm production or low sperm production, and complete lack of transmission. In uninfected PSR males the first two are found. Combined with the results in chapter 4, we suggest that the ability of the host genome and *Wolbachia* to suppress PSR in *T. deion* may be the remnant of conflicts in the evolutionary past.

Choosiness in mating can result in different fitness consequences in the presence of a sex ratio distorter. If males carrying PSR chose PI-*Wolbachia* infected above uninfected females to mate with, PSR male offspring have an advantage, because they would arise together with infected sisters. The results, however, show that PSR males prefer uninfected females. We interpret that choosiness of infected females may come up with these results. If infected females are able to distinguish normal males from males carrying PSR, and consequently choose the normal males to mate with, both the bacteria and the genome of the females will have the benefit. The results, therefore, suggest that if this is the real pattern in

these populations, this will be an example of one sex ratio distorter evolving in response to the presence of an opposing second sex ratio distorter.

Besides summarizing the important findings, in this thesis I focussed on future work to be carried out with PI-*Wolbachia* and PSR in chapter 7. PI-*Wolbachia* are considered for the improvement of biological control agents. The causal protein inducing gamete duplication is not yet explored and can be of special interest. PSR can directly be used to control haplodiploid pests, once it proves its ability to induce the paternal genome loss in target hosts.

The main conclusion from the thesis is that close interactions among the host genes and *Wolbachia* and PSR are the results of symbiotic or parasitic nature of the sex ratio distorters and of reaction of their host genes. The female virginity mutation in *Telenomus nawai* helped *Wolbachia* go fast to fixation. Conversely *Wolbachia* infection is obligatory to the mutants because they can not maintain themselves without infection. In *Trichogramma kaykai* PSR keeps the frequency of *Wolbachia* at low levels, on the other hand the cooperation between the host genes and *Wolbachia* effectively suppress PSR in *T. deion*. We suggest that the trait shown in *T. deion* may be the remnant of the conflicts between the host genes and *Wolbachia*, and PSR in the evolutionary past.

With the results from this thesis potential interactions i.e. cooperation or conflicts, among the host genes and *Wolbachia* and PSR should be of special importance when the sex ratio distorters are considered to improve the production of biological control agents and controlling pests.

Samenvatting

Sex ratios van verscheidene organismen kunnen worden gemanipuleerd door egoïstische genetische elementen. Te midden van deze sex ratio verstoorders hebben *Wolbachia* bacteriën veel aandacht getrokken doordat ze verschillende manipulaties van een sex ratio kunnen veroorzaken zoals cytoplasmatische incompatibiliteit, feminizatie, het doden van mannen en parthenogenese. Deze manipulaties van de sex ratio van hun gastheren zorgen voor een optimale transmissie van de bacteriën en leiden daarbij tot een door vrouwtjes gedomineerde, oftewel female biased, sex ratio. Naast deze belangrijke fenomenen, kan de infectie ook obligatorisch of verplichtend zijn in sommige soorten. Dit suggereert een nauw verband tussen de bacteriën en hun gastheren.
Een ander extreem egoïstisch element is het paternale (vaderlijke) sex ratio (PSR) chromosoom dat tot nu toe alleen in de Hymenoptera is gevonden. Het leidt een populatie naar een meerderheid van mannetjes doordat het chromosoom alleen maar via de vader kan worden overgedragen.
Deze twee sex ratio verstoorders hebben verschillende evolutionaire interesses vanwege hun tegenovergestelde overerving. Zeker wanneer ze een gastheer delen kan dit tot conflicten leiden. Het doel van dit proefschrift is het ophelderen van de interacties tussen sex ratio verstoorders en hun gastheren.

Met uitzondering van het genus *Trichogramma* is het tot nu toe onmogelijk gebleken om een sexuele lijn van wespen te genereren uit een parthenogenetsiche, met *Wolbachia* geïnfecteerde, lijn. In hoofdstuk 2, is aangetoond dat de onmogelijkheid om sexualiteit uit asexualiteit te genereren genetisch is bepaald in de met *Wolbachia* geïnfecteerde wesp *Telenomus nawai*. De geïnfecteerde individuen zijn mutanten die hun eieren niet kunnen bevruchten. Deze mutanten zijn daarom afhankelijk van de infectie om zich te handhaven. Het mutante gen kan de ongeïnfecteerde sexuele individuen laten uitsterven als het ontstaat in een populatie waarin geïnfecteerde en ongeïnfecteerde individuen naast elkaar voorkomen.
De overdracht van PSR wordt tot stand gebracht door de vernietiging van paternale genoom in een bevrucht ei. Dit bevruchte ei ontwikkelt zich tot een

PSR-dragend mannetje. Door deze manier van handelen wordt verondersteld dat PSR relatief makkelijk tussen soorten kan worden overgedragen in haplodiploïde organismen. Reproductieve isolatie is de belangrijkste factor in het biologische soortsconcept. Tussen de twee nauwverwante wespensoorten *Trichogramma kaykai* en *T. deion* bestaan er complexe vormen van post-zygotische reproductieve isolaties, namelijk de steriliteit en sterfte van hybriden. In hoofdstuk 3 is bepaald of deze reproductieve isolatie mechanismen de interspecifieke overdracht van PSR tussen *T. kaykai* en *T. deion* beïnvloeden. PSR kon beide reproductieve isolatie mechanismen overwinnen. Echter, als de donor *T. deion* was, nam de transmissie efficiëntie van PSR af. Blijkbaar kan de genetische achtergrond van de gastheer de overdracht van PSR beïnvloeden.

Hoogstwaarschijnlijk komen in de natuur interspecifieke paringen tussen de geografisch gezien overlappende *T. kaykai* en *T. deion* voor. PSR is echter nog niet in *T. deion* gevonden. In hoofdstuk 4 heb ik de efficiëntie van PSR overdracht in *T. deion*, de nieuwe gastheer, vergeleken met die in *T. kaykai*, de natuurlijke gastheer. De transmissie efficiëntie van PSR in de nieuwe gastheer is erg laag. Als een PSR mannetje van de nieuwe soort geïnfecteerd is met *Wolbachia*, is deze zelfs nog lager is. Er kan daarom worden geconcludeerd dat er in de nieuwe gastheer conflicten bestaan tussen het genoom van de gastheer, *Wolbachia* en PSR.

Zoals is gedemonstreerd in hoofdstuk 4 wordt de transmissie efficiëntie van PSR in *T. deion* grotendeels tegengegaan door een infectie met *Wolbachia*. In hoofdstuk 5, heb ik geprobeerd te bepalen of er variatie in de overdracht van PSR bestond in deze soort. De resultaten tonen aan dat de transmissie efficiëntie in vier verschillende categoriën kan worden onderverdeeld, namelijk perfecte overdracht, imperfecte overdracht, niet-functionele spermaproductie of lage spermaproductie, en het complete ontbreken van overdracht. In ongeïnfecteerd PSR mannnetjes vinden we de eerste twee categoriën. Dit, combinerend met de resultaten van hoofdstuk 4, leidt ons tot de hypothese dat de mogelijkheid van het genoom van de gastheer en *Wolbachia* in *T. deion* om PSR te onderdrukken een overblijfsel is van conflicten in het evolutionaire verleden.

Kieskeurigheid bij het kiezen van een partner kan resulteren in verschillende consequenties voor de fitness in de aanwezigheid van een sex ratio verstoorder. Als PSR-dragende mannetjes met PI-*Wolbachia* geïnfecteerde vrouwtjes verkiezen boven ongeïnfecteerde vrouwtjes om mee te paren, hebben de PSR-dragende zonen een voordeel omdat ze directe toegang hebben tot het paren met geïnfecteerde zussen. De resultaten laten echter zien dat PSR mannetjes ongeïnfecteerde vrouwtjes prefereren. Dit zou kunnen komen door de kieskeurigheid van geïnfecteerde vrouwtjes. Als geïnfecteerde vrouwtjes normale mannetjes van PSR mannetjes kunnen onderscheiden, en dan vervolgens de normale mannetjes kiezen om mee te paren, zullen zowel de bacteriën als het genoom van de vrouwtjes hiervan profiteren. De resultaten suggereren daarom dat, wanneer dit een echt patroon in deze populaties is, dit een voorbeeld is van een sex ratio verstoorder die is geëvolueerd als antwoord op de aanwezigheid van een andere "vijandelijke" sex ratio distorter.

Naast het samenvatten van de belangrijkste vondsten in dit proefschrift richt ik mij in hoofdstuk 7 op toekomstig onderzoek aan PI-*Wolbachia* en PSR. PI-*Wolbachia* zouden kunnen worden gebruikt ter verbetering van de biologische plaagbestrijding. Het eiwit dat gameet duplicatie, de vorm van parthenogenese bij *Trichogramma* wespen, veroorzaakt, is nog niet onderzocht en verdient veel aandacht in de toekomst. PSR kan direct worden gebruikt tegen haplodiploïde plagen, zodra het z'n vermogen toont om ook het vaderlijke genoom te vernietigen bij een bepaalde "doelwit"plaag.

De belangrijkste conclusie in dit proefschrift is dat nauwe interacties tussen genen van de gastheer, *Wolbachia* en PSR, het resultaat zijn van het symbiotische of parasitaire karakter van de sex ratio verstoorders en van de reactie van de genen van de gastheer. De "maagdelijkheid" mutatie in *Telenomus nawai* heeft *Wolbachia* geholpen om naar fixatie te gaan, een situatie waarin alle individuen in een populatie geïnfecteerd zijn. Anderzijds is de *Wolbachia* infectie obligatorisch voor de mutanten omdat zij zichzelf niet kunnen handhaven zonder de infectie. In *Trichogramma kaykai* houdt PSR de infectie frequency met *Wolbachia* op een laag niveau, maar in *T. deion* wordt PSR onderdrukt door een samenwerking tussen de genen van de gastheer en

Wolbachia. Wij suggereren dat die eigenschap in *T. deion* een overblijfsel is van de conflicten tussen de genen van de gastheer, *Wolbachia* en PSR in het evolutionaire verleden.

De potentiële interacties tussen de genen van de gastheer, *Wolbachia* en PSR –in dit geval samenwerkingsverbanden of conflicten- zijn vooral belangrijk om in ogenschouw te nemen wanneer de sex ratio verstoorders worden gezien als een manier om de productie van biologische plaagbestrijders en de plaagbestrijding te verbeteren.

성비, 성비교란인자와 결과요약

벌목의 곤충 - 개미, 벌, 기생벌 등 -은 성비 연구의 중심이 되어왔다. 그 이유는 어미가 자손의 성비를 임의로 조절할 수 있는 능력에 기인한다. 즉, 수정된 알에서는 배수체(diploid) 암컷의 자손이, 수정이 되지않은 알에서는 반수체(haploid) 수컷의 자손이 태어나기 때문이다(haplodiploid sex determination).

초기 성비에 관한 연구는 1930 년대 Fisher 의 1:1 성비론(Fisherian sex ratio)이 주류를 이루었다. 그의 이론에 의하면 유성생식을 하는 생물(sexual organisms)에서 핵 유전자(nuclear genes)의 적합도(fitness)는 암컷과 수컷의 자손에게 자원(resource)이 동등하게 투자(investment)될 경우 진화적으로 안정적이다(evolutionary stable strategy). 이러한 연구경향은 Hamilton(1967)의 벌목의 성비에 관한 연구에 의해 크게 바뀐다. Hamilton 은 근친교배(sibmating)하는 벌목의 성비는 암수 약 3:1 일 때 가장 적합하다는 것을 수학적으로 증명하였다(Hamiltonian sex ratio). 또한 그는 성비는 성비교란인자에 의해 변형될 수 있다는 것을 인지하였다. 현재 밝혀진 성비교란인자는 크게 핵 성비교란인자(nuclear chromosomal factors) - driving X chromosome(X chromosome meotic drive), paternal sex ratio chromosome (PSR)- 와 세포질 성비교란인자(cytoplasmic factors) – *Wolbachia* 박테리아, Cytophaga-Flexibacter-Bacteroides(CFB), *Microsporidia*, *Rickettia*, γ-Proteobacteria 종류 등-로 나눌 수 있다. 이외에도 숙주의 성비를 교란하는 인자들은 계속 발견될 것으로 예상된다.

이중 *Wolbachia* 박테리아는 특별한 관심을 받고 있는데, 그 이유는 다른 인자와는 달리 여러가지 성비교란 작용을 동시에 가지고 있기 때문이다.

본 논문은 벌목 곤충의 성비와 성비교란인자(*Wolbachia* 와 PSR)의 상호관계에 관한 연구이며 본 장에서는 성비교란인자중 PSR 과 *Wolbachia* 박테리아에 관한 일반 정보와 본 논문의 결과를 요약하였다. 다른 성비교란인자에 관한 학술 논문은 본 논문 각 장의 참고문헌에서 찾을 수 있다.

1. The paternal sex ratio (PSR) chromosome

PSR 은 B-염색체의 일종으로 극단적으로 이기적인 유전자이며, 현재 벌목 2 종의 곤충- *Nasonia* 와 *Trichogramma*-의 수컷에서만 발견되었다. 그 숙주의 수정된 알은 배수체(diploid) 암컷으로 발생하지만, PSR 을 가진 정자에 의해 수정된 알에서는 PSR 이 첫번째 유사분열이 시작되기 전에 부계 염색체(paternal chromosome)를 응축시켜 제거함으로써 그 알은 반수체(haploid)의 PSR 염색체를 가진 수컷으로 발생한다. 이러한 극단적인 성의 전환은 숙주 생물군(Hymenoptera)의 독특한 성결정 방식 때문에 가능하다(haplodiploid sex determination) (Table 1a, b).

Table 1a. Paternal genome loss (PGL) induced by PSR in *Nasonia*

Crosses	Virgin	$Unpsr_{♂}$		$Inpsr_{♂}$	
	U	F	U	F	U
$Un_{♀}$	$Un_{♂}$	$Unpsr_{♂}$	$Un_{♂}$	$Un_{♂}$	$Un_{♂}$
$In_{♀}$	$In_{♂}$	$Inpsr_{♂}$	$In_{♂}$	$Inpsr_{♂}$	$In_{♂}$

U: unfertilized; F: fertilized; Un: uninfected; In: infected; Unpsr: uninfected PSR; Inpsr: infected PSR

Table 1b. General scheme of paternal genome loss (PGL) induced by PSR in *Trichogramma*

Crosses	Virgin	$Unpsr_{♂}$		$Inpsr_{♂}$	
	U	F	U	F	U
$Un_{♀}$	$Un_{♂}$	$Unpsr_{♂}$	$Un_{♂}$	$Unpsr_{♂}$	$Un_{♂}$
$In_{♀}$	$In_{♀}$	$Inpsr_{♂}$	$In_{♀}$	$Inpsr_{♂}$	$In_{♀}$

U: unfertilized; F: fertilized; Un: uninfected; In: infected; Unpsr: uninfected PSR; Inpsr: infected PSR

현재 그 기원은 주 염색체(A-chromosome)에서 발생한 결실에 의해 생긴다는 가설과 우발적인 이종교배(interspecific hybridization)에서 살아남은 유전자의 비기능적 조각일 것이라는 가설이 있으나 증명되지는 않았다.

Nasonia 와 *Trichogramma* 에서 발견되는 PSR 의 진화적 기원은 다르나, 부계 염색체를 제거하는 방식은 놀라울 정도로 비슷하다. 이러한 방식으로 PSR 은 숙주 개체군의 성장을 저해하고, 결과적으로 멸종에 이르게 할 수 있다. 그러므로 PSR 은 특정 해충의 방제에 이용하거나 특정 유전자를 전달하는 유전자 조작(genetic engineering)에 이용할 수 있을 것으로 생각된다.

2. *Wolbachia* bacteria

Wolbachia 박테리아는 무척추동물-곤충강, 거미강, 갑각강, 선충강-의 세포질에 서식하는 α-프로테오박테리아 계통의 내부 공생체이다. 현재 무척추동물에 가장 광범위하게 분포된 공생체로 생각된다. 이 박테리아는 1924 년 Hertig 와 Wolbach 에 의해 모기, *Culex pipiens*,에서 처음으로 발견되었다. 그러나 그 숙주모기의 지역적 개체군간(strain)의 번식적 불합치(incompatibility)가 세포질에 서식하는 이 박테리아에 의해 일어난다는 사실이 밝혀지기까지(Yen and Barr, 1971), 이 박테리아는 단순히 한 종류의 공생체로 인식되어왔다. 숙주의 번식과 성비가 외부 유전물질인 *Wolbachia* 박테리아에 의해 교란된다는 사실은 20 세기 곤충학의 가장 중요한 발견의 하나가 되었다(Chapman, 2000).

이 박테리아는 분류학적으로 크게 5 개의 그룹으로 나뉘며 A, B, E 그룹은 절지동물문에서, C, D 그룹은 기생 선충류에서 발견되며, 향후 다양한 생물군을 조사할수록 더욱 더 많은 감염사례를 발견할 것이며 그에 따라 더 많은 그룹으로 분류할 수 있을 것으로 예상된다.

Wolbachia 와 숙주의 공생관계는 약 200 만년정도 된 것으로 추측하고 있으며 거의 대부분 모계에서 자손으로 수직으로 전이(vertical transmission)되지만, 이 박테리아의 계통수와 숙주의 계통분류는 일치하지 않는다. 이러한 사실은 진화적 시간 동안 숙주의 분류학적 분지(divergence) 후에도 개체와 개체간의 빈번한 수평적 전이(horizontal transmission)가 이루어졌다는 것을 뒷받침해 준다. 이것은 최근에 실험적으로 증명되었다(Huigens, 2003).

Wolbachia 의 감염은 크게 4 가지 성비교란을 일으킨다.

2-1. Cytoplasmic incompatibility (CI)

이것은 가장 광범위하게 나타나는 박테리아의 작용이다. 앞서 서술한 바와 같이 이 박테리아에 관한 본격적인 연구를 촉발시킨 Yen 과 Barr(1971)의 결과를 필두로 *Drosophila*, *Nasonia* 등 여러 곤충류에서 나타난다. 이의 작용메카니즘은 다음과 같다. 감염되지 않은 암컷이 감염된 수컷과 교미를 하면 수정(fertilization) 직후 부계의 염색체가 소실되어 수정된 알이 [배수배수체(diplodiploid) 생물과 일부 반수배수체(haplodiploid) 생물에서] 죽거나, 반수배수체(haplodiploid)에서 수컷으로 발생한다. 그 나머지 교배의 경우 모든 수정된 알들은 정상적으로 발생한다(Table 2a, b). 또한 이 현상은 배우자가 각각 다른 종류의 *Wolbachia* 에 감염되어 있는 경우에는 양방향으로 발생한다(bi-directional CI) (Table 2a, b).

Table 2a. Cytoplasmic incompatibility induced by *Wolbachia* infection in diplodiploid organisms and some haplodiploid organisms

Crosses	$Un_{♂}$	$In_{♂}^{a}$	$In_{♂}^{b}$	$In_{♂}^{ab}$
$Un_{♀}$	$Un_{♀}$, $Un_{♂}$	×	×	×
$In_{♀}^{a}$	$In_{♀}^{a}$, $In_{♂}^{a}$	$In_{♀}^{a}$, $In_{♂}^{a}$	×	×
$In_{♀}^{b}$	$In_{♀}^{b}$, $In_{♂}^{b}$	×	$In_{♀}^{b}$, $In_{♂}^{b}$	×
$In_{♀}^{ab}$	$In_{♀}^{ab}$, $In_{♂}^{ab}$	$In_{♀}^{ab}$, $In_{♂}^{ab}$	$In_{♀}^{ab}$, $In_{♂}^{ab}$	$In_{♀}^{ab}$, $In_{♂}^{ab}$

Un: uninfected; In: infected; $^{a, b}$: *Wolbachia* strain; ×: death of offspring

Table 2b. Cytoplasmic incompatibility in haplodiploid organisms

Crosses	Virgin	$Un_{♂}$		$In_{♂}^{a}$		$In_{♂}^{b}$		$In_{♂}^{ab}$	
	U	F	U	F	U	F	U	F	U
$Un_{♀}$	$Un_{♂}$	$Un_{♀}$	$Un_{♂}$	×	$Un_{♂}$	×	$Un_{♂}$	×	$Un_{♂}$
$In_{♀}^{a}$	$In_{♂}^{a}$	$In_{♀}^{a}$	$In_{♂}^{a}$	$In_{♀}^{a}$	$In_{♂}^{a}$	×	$In_{♂}^{a}$	×	$In_{♂}^{a}$
$In_{♀}^{b}$	$In_{♂}^{b}$	$In_{♀}^{b}$	$In_{♂}^{b}$	×	$In_{♂}^{b}$	$In_{♀}^{b}$	$In_{♂}^{b}$	×	$In_{♂}^{b}$
$In_{♀}^{ab}$	$In_{♂}^{ab}$	$In_{♀}^{ab}$	$In_{♂}^{ab}$	$In_{♀}^{ab}$	$In_{♂}^{ab}$	$In_{♀}^{ab}$	$In_{♂}^{ab}$	$In_{♀}^{ab}$	$In_{♂}^{ab}$

U: unfertilized; F: fertilized; Un: uninfected; In: infected; $^{a, b}$: *Wolbachia* strain; ×: death of offspring

Werren(1997)은 이 현상을 modification/rescue 모델로 설명하였다. 즉 감염 수컷의 정자는 변형되었고(mod+), 감염 암컷의 알은 변형된 정자를 치료할 수 있으나(res+), 감염되지 않은 암컷의 알은 치료할 수 없으므로(res-), 감염된 정자(mod+)에 의해 수정된 비감염 알은 생존하지 못한다는 것이다. 현재 mod+/res+, mod-/res+와 mod-/res-타입의 *Wolbachia* 가 발견되었다

(Bourtzis *et al*., 1998; Werren, 1997). 최근에 Tram 과 Sullivan(2001)은 이 작용을 세포생물학적으로 규명하였다. 비감염 암컷과 감염 수컷의 교배에서 수정 후 정확한 시기에 감염개체의 정자의 핵막이 터지지 않아(DNEB: delayed nuclear envelope breakdown), 방추사가 부계염색체에 접합하지 못함으로써 부계염색체가 소실된다. CI 는 *Wolbachia* 감염으로 나타나는 작용 중 원시적인 형태로, 나머지 성비교란작용, 즉 feminization, male killing, parthenogenesis 은 진화된 형태로 추측된다.

2-2. Feminization

자성화는 암컷의 성염색체가 이형(female heterogametic sex determination; the ZW sex determination system)인 생물에서 발생한다. 이 분야의 연구는 육상 등각류(terrestrial isopods), 나비류에서 활발하게 진행되고 있다. 이들 숙주에서 유전적 수컷(ZZ)이 감염에 의해 기능적 암컷으로 전환된다(Table 3). 감염된 기능적 암컷은 수컷과 교배해 감염된 기능적 암컷(ZZ)을 낳는다.

Table 3. Feminization induced by *Wolbachia* infection in Crustacea

Crosses	$Un_{♂ZZ}$
$Un_{♀ZW}$	$Un_{♀ZW}$, $Un_{♂ZZ}$
$In_{♀ZW}$	$In_{♀ZW}$, $In_{♀ZZ}$
$In_{♀ZZ}$	$In_{♀ZZ}$

Un: uninfected; In: infected; $_{ZW, ZZ}$: sex chromosome

이러한 성의 전환은 이 종류에서 암컷은 default 상태 즉, 수컷으로 발생하지 않으면 암컷으로 발생하게 되는 독특한 생리생화학적 구조 때문이며, 이는 웅성호르몬(androgenic hormone)의 표적기관을 차단하거나 호르몬 자체의 생성을 억제하므로써 이루어진다. 이러한 개체군 구조에서 진화적으로 W 유전자는 점점 감소해 사라지게 되며, 비감염 수컷 또한 개체군 유지를 위해 필수적이지만 감염이 확산될수록 수컷의 수가 줄어들게 된다. 이러한 교란에 대해 최근에 새로운 성결정 시스템의 진화가 *Armadillidium bulgare* 에서 관찰된다(Juchault 와 Mocquard, 1993). 또한 수컷은 ZW 암컷을 더 선호하는 경향을 보이기도 한다.

2-3. Male killing

몇몇 종의 딱정벌레와 나비류에서 수컷으로 발생하게 될 수정된 알이 감염된 경우 발생단계에서 죽게 된다(Hurst *et al.*, 1999) (Table 4). 이의 자세한 기작은 아직 밝혀지지 않았다. 암컷과 암컷내의 *Wolbachia* 는 수컷을 감염시킨 *Wolbachia* 의 이타적 자살로 다음과 같은 이득을 얻는다. 1) 암컷의 생존율이 높아진다. 2) 근친교배에 의한 개체군의 적합도 저하를 피할 수 있다. 3) 암컷은 더 많은 자원을 확보할 수 있다. 4) 암컷은 동종의 암컷과의 경쟁을 피할 수 있다.

대개 수컷이 암컷에게 구애행동을 하지만, 최근 이 종류의 *Wolbachia* 에 감염된 *Acraea encedon* 의 개체군에서 수컷의 부족에 따른 역전된 구애행동이 관찰되고 있다(Jiggins *et al.*, 2000).

Table 4. Male killing induced by *Wolbachia* infection

Crosses	$Un_{♂}$
$Un_{♀}$	$Un_{♀}$, $Un_{♂}$
$In_{♀}$	$In_{♀}$, ×

Un: uninfected; In: infected; ×: death of male offspring

2-4. Parthenogenesis

1990 년대 초 Stouthamer *et al.*(1990, 1993)는 두 종의 *Trichogramma* 속 기생벌에서 단위생식이 세포질내의 미생물에 의해 일어난다는 사실을 밝혀냈으며 그 후 이 미생물은 *Wolbachia* 로 판명되었다. 단위생식을 일으키는 *Wolbachia* 는 반수배수체 성결정 시스템(haplodiploid sex determination; 암컷은 배수체, 수컷은 반수체)을 가진 생물군-벌목, 총채벌레목, 진드기류-에서만 발견되었다.

현재 *Wolbachia* 에 의한 단위생식의 기작은 두 가지가 밝혀져 있다. 첫번째 두 종의 *Trichogramma*(Stouthamer and Kazmer, 1994)와 *Leptopilina clavipes*(Pannebakker *et al.*, in press)에서는 배우자 복제(gamete duplication)에 의해 미수정 알의 염색체는 배수체가 되며, *Muscidfurax uniraptor*(Gottlieb *et al.*, 2002)에서는 2 차 유사분열에서 단수체 핵의 융합(nuclear fusion)에 의해 배수체가 되어 암컷으로 발생한다.

단위생식을 일으키는 *Wolbachia* 에 감염된 *Trichogramma* 의 암컷은 감염이 되지 않은 수컷과 교미를 통해서도 감염된 암컷을 낳을 수 있어, *Wolbachia* 박테리아를 항생제로 제거하여도 유성생식(sexual reproduction)을 통해서 자손을 가질 수 있다(Table 5a). 이에 반해 현재까지 다른 생물군 즉, *Apoanagyrus diversicornis, Bryobia praetiosa, Eretmocerus mundus, Franklinothrips vespiformis, Muscidifurax uniraptor* 와 *Telenomus nawai* 에서는 그 박테리아를 제거해도 유성생식을 하지 못한다(Table 5b). 이와 관련된 하나의 가설은 하나의 개체군에서 암컷의 비율이 높은 경우 수컷을 많이 낳는 돌연변이는 선택적 이득을 얻게 된다. 수컷의 비율은 수정률을 낮추거나 교미를 하지 않는 경우에 높아질 수 있다. 이러한 돌연변이는 감염개체와 비감염개체가 혼합된 개체군에서 비감염개체의 멸종을 유도해 *Wolbachia* 박테리아의 신속한 고정(fixation)을 돕는다.

Table 5a. Parthenogenesis induced by *Wolbachia* infection in *Trichogramma*

Crosses	Virgin	$Un_{♂}^{b}$	
	U	F	U
$Un_{♀}^{a}$	$Un_{♂}^{a}$	$Un_{♀}^{ab}$	$Un_{♂}^{a}$
$In_{♀}^{a}$	$In_{♀}^{a}$	$In_{♀}^{ab}$	$In_{♀}^{a}$

U: unfertilized; F: fertilized; Un: uninfected; In: infected; $^{a, b}$: host genotype

Table 5a. Parthenogenesis in other organisms than *Trichogramma*

Crosses	Virgin	$Un_{♂}^{b}$	
	U	F	U
$Un_{♀}^{a}$	$Un_{♂}^{a}$	$Un_{♀}^{ab}$	$Un_{♂}^{a}$
$In_{♀}^{a}$	$In_{♀}^{a}$		$In_{♀}^{a}$

U: unfertilized; F: fertilized; Un: uninfected; In: infected; $^{a, b}$: host genotype

2-5. 성비교란 이외의 감염효과

Drosophila melanogaster 에서 *Sxl*(sex lethal)의 돌연변이에 의한 알 생성결여(defects in oogenesis)가 *Wolbachia* 의 감염에 의해 복구되며, *Asobara tabida* 의 암컷은 *Wolbachia* 가 제거되면 알을 낳지 못한다. 선충류에서도 *Wolbachia* 는 알의 생성에 밀접히 관여하며, André *et*

al.(2001)은 선충류감염에 의한 병변인 river blindness 의 직접적인 원인이 이 박테리아에 대한 숙주의 급성 면역반응에 의한 것임을 최근에 밝혔다. 이외의 몇몇 종에서 번식력을 높이거나 정자의 경쟁력을 향상시키는 등의 작용이 관찰되었다. 이러한 자료들은 특정의 숙주생물과 이 박테리아간의 긴밀한 상호작용이 있음을 시사한다.

결과 요약

이상에서 살펴본 바와 같이 성비교란인자들은 자신들의 진화적 이득(evolutionary interests)을 위해 숙주의 성비를 교란한다. 이러한 특수한 숙주와 공생자의 관계에서 여러가지 진화적 상호작용을 일으킨다. 특히 PSR 과 단위생식형(PI) *Wolbachia* 는 진화적으로 상충(evolutionary antagonistic) 한다. 그러므로 이 둘이 하나의 숙주에 존재하는 경우 마찰(genetic conflicts)이 예상된다.

본 논문 제 2 장에서는 *Telenomus nawai* 의 감염체가 박테리아의 치료후에도 유성생식을 하지 못하는 이유가 유전적으로 돌연변이임을 실험적으로 증명하였다.

제 3 장에서 PSR 의 독특한 작용으로 생물학적 종개념(biological species concept)의 핵심인 두 종간 격리기작(postzygotic reproductive isolation)-Hybrid sterility 와 inviability-을 극복하고 근연종으로 쉽게 전이됨을 증명하였다.

제 4 장에서 PSR 의 고유 숙주인 *Trichogramma kaykai* 와 실험적 숙주인 *T. deion* 에서 PSR 의 수직 전이(vertical transmission) 효율을 알아보았다. *T. kaykai* 에서 PSR 은 *Wolbachia* 의 감염여부에 관계없이 전이되나, *T. deion* 에서는 그 전이가 불완전했고, 특히 *Wolbachia* 에 감염된 경우 불완전한 전이는 심화되었다.

제 5 장에서는 제 4 장에 이어서 *Wolbachia* 감염이 *T. deion* 에서 어떻게 PSR 의 전이를 방해하는지 알아보았다. 그 결과 PSR 의 전이는 감염개체에 따라 완전히 사라지거나, 전이가 크게 저하되거나, 정자생산자체가 중단되거나, 저해 받지 않는 등 다양하게 나타났다. 제 4 장과 5 장의 결과를 종합하면 *Wolbachia* 와 PSR 간의 진화적 마찰의 결과 *T. kaykai* 에서는 PSR 이 *Wolbachia* 의 번성을 억제하며, *T. deion* 에서는 *Wolbachia* 와 숙주자체가 PSR 의 번성을 억제한다. 또한 이 결과는 *T. deion* 에서 *Wolbachia* 와 숙주의 유전자의 PSR 을 억제하는 표현형은 진화적 과거(evolutionary past)에 있었던 그 요소들간의 마찰(genetic conflicts)의 잔유물(remnant)일 것이라는 것을 암시한다.

제 6 장에서 두 가지 성비교란인자가 숙주의 교미행동에 영향을 주는지 실험하였다. 실험결과 PSR 을 가진 수컷은 *Wolbachia* 에 감염되지 않은 암컷을 선호하는 경향을 띠는 것으로 나타났으며 이는 반대로 *Wolbachia* 에 감염된 암컷이 PSR 을 가진 수컷을 배격하므로써 나타난 결과인 것으로 추측된다.

본 논문의 결론은 숙주와 *Wolbachia* 와 PSR 간의 밀접한 상호작용은 이들의 공생체적 기생체적 성질과 이에 대한 숙주의 반응의 결과이다. 이러한 결과들은 생물학적 방제에 쓰이는 기생벌의 생산성 향상 또는 해충을 직접적으로 방제할 경우에 발생가능한 저해요소로서 중요하게 고려해야 할 진화적 요소이다.

한글 요약은 박소현에 의해 교정, 향상되었슴.

Acknowledgements

During my stay here in Wageningen almost for 4 years I have gotten innumerable and invaluable help from all of you. I may accidentally miss some of you. You could understand that it is not my intention.

First of all I would to start to express my gratitude to my promoter Prof. Dr. Joop C. van Lenteren. Without your allowance I would not be able to study here. I am grateful for your supervision. You made a great contribution to my thesis.

I am really grateful to my co-promoter Prof. Dr. Richard Stouthamer. In the beginning when I first saw you, my impression was just that you were really tall. I realized that Dutch were tall as soon as I arrived at Schiphol Airport, but I never expected that I would have such a tall supervisor. Richard, your joke made me feel comfortable, and you sent my life to the exciting science field. You always kindly answered my (sometimes dull) questions. Even though you left for Riverside in my infant stage of research, your high standard of scholarship and enthusiasm toward research and remote but active discussion and suggestion via the lovely chatting program spurred me on to the terminus of my study in the Netherlands (Of course it should not be the termination of my research career!). At the last moment to submit my manuscripts to the reading committee, you had to spend your time for me even at a conference. I don't know how I can thank you for all your effort and patience!

I am vastly grateful to the president Sun Lee and my senior Namjun Chung. Without your mental and physical encouragement, I would not have been able to start my study. You have always been a great help. I deeply appreciate your incitement again and forever.

I thank Dr. Peter de Jong. You have been very helpful. You encouraged me by giving valuable comments on my manuscripts.

The *Wolbachia*-PSR group made me delightful in conducting experiments. Emmanuel, Fabrice, Joke, Raul, Ties and also Mohammad and Bertha and Patrick...! Thank all of you for your help. Emmanuel, I hope you good luck with your family there. Fabrice, I got lots of advice from you and it was a great lecture you gave us. Joke, your PSR markers are perfect. Raul, I am very happy that we are siblings born on 21th of January! Ties, you have been my teacher in the

absence of Richard. I learnt a lot from you. You helped me a lot in Greece, in Leiden and in Amsterdam ANYWHERE we were together. Except one thing! Bart and you were not there when I was drowning in the Mediterranean. Anyway I am still alive. Mohammad, I wish you good luck with your study and your wife. Bertha, you were the molecular teacher until Patrick came to us. You were also the first person with Maartje whom I met at the lab. Patrick, with your help I could perform molecular works in a safe and well organized condition. Olivier, you always kindly lead me to Ocidental culture and told me funny stories while doing molecular work. I was very happy when we talked about our hobby. I can not skip to mention Isabel! You and Ineke and Gerad helped me even before I came here. You arranged all things like official documents, visa, my room etc. After returning to the lab, Isabel, you kindly read early versions of my manuscripts and gave valuable comments on them. My previous officemates Antoon and Jetske, without your help, I would not be able to solve problems encountered in daily life. Even so, I had to leave, Maartje must know why. Sara, with your guidance Paul, Raul and I yielded a masterpiece in a mini-research. It resulted in the incredible mark as well. The PhD lunch meeting organizers, Jetske, Maartje and Mohammad, should be appreciated as well. Due to your effort I learnt what a researcher should consider in doing research and in writing a manuscript. Nina, I enjoyed parties, meetings, funny lyrics and what you organized, and thank you for your advice on choosing a sexy male!

Sander, we made a trip to Groningen and you showed how to fly a frisbee (?). Yutong, thank you for everything you have done for me. Wouter, my wife and I unfortunately except my son (he is almost carnivorous) really enjoyed your products. Yde and Tibor, thank you very much for organizing parties. Remco and Trefas, Sinter Klass this year was very nice. I will never forget.

Leo, Andre and Frans, I really thank you for your effort to supply materials on time. With the fresh eggs, my wasps have kept their health!

I can not leave my wasps and host eggs out! You deserve my appreciation. Whenever it rained and snowed, you shared joys and sorrows with me below ground. It was really cruel to press extra wasps down to kill and to stab *Ephestia*

and *Mamestra* larva with a fine pin. But I could not help doing. I am still sorry for that, you wasps and larva.

Prof. Hosa Lee, Prof. Yoochung Cho, Prof. Kitae Lee, Prof Hokeun Lee, Prof Chungchil Yoo, Prof. Jongphil Chu, Prof. Joongmyung Choi in Korea also encouraged me when I was in hard time. Special gratitude is given to emeritus Prof. Yoohang Shin who thought me what entomology was. With your guidance I could broaden my insight into entomology.

All the members at the Lab. of Entomology, and Hans, Byungha and Beomchul etc in Korea, your warm friendship helped me a lot go ahead.

It is time for me to return my family. All the family members in Korea are appreciated for all of your support. Especially I thank my father and father in law and mother and mother in law from the bottom of my heart.

That I want to express my best gratitude to my wife, Sohyun and my son, Seungjae is most proper. Sohyun, I even feel sorry for all what I imposed on you to achieve my goal. Without your sacrifice, I would definitely NOT reach this point. I am deeply and deeply in debt to you.

My son, Seungjae, I, your father, am really sorry that I did not do my best to you. You, Seungjae, did not receive enough love and guide of me. Even so you always welcomes me with a smiling face. I am really lucky that I have a son like you. Being with you is one of my happiest moments in my life. You two are my hope and my ultimate motive!!!

During your struggling against loneliness, homesickness and all the other difficulties and pain, I become a doctor. I dedicate all the glories and this thesis to you two.

Gilsang

At the lab of Entomology, Wageningen

5th of December, 2003

Curriculum vitae

Gilsang Jeong was born in Kongju City, Chungcheong South Province, South Korea on May 5, 1967. His family moved to the capital city, Seoul, when he was 4 years old. In the city he spent his young age. While his high school days, his interest toward insects was growing with the aid of his biology teacher.

As a result he started to study biology at Kyunghee University in Seoul from 1985 to 1993. In 1987 he paused his study to perform military duties. He returned to the university in 1991. After obtaining M.Sc. degree from Kyunghee Graduate School in 1995, he started to work as a research assistant at the Laboratory of Parasitology, at Kyunghee Medical School for 3 years. In 1999 he started to work at the Kyunghee natural history museum as a research assistant. In the meantime he decided to study abroad. In April 2000, he started a PhD joining the Graduate School of Production Ecology & Resource Conservation (PE & RC) at Wageningen University. He conducted research at the laboratory of Entomology for about 4 years. This thesis is the results of the research he carried out.

List of publications

Boeke S.J., Sinzogan A.A.C., de Almeida R.P., de Boer P.W.M., Jeong G., Kossou D.K., and van Loon J.J.A., 2003, Side effect of cowpea treatment with botanical insecticides on two parasitoids of *Callosobruchus maculatus*. *Entomologia Experimntalis et Applicata*.**108**:43-51.

Jeong G., and Stouthamer R., Genetics of female virginity in the *Wolbachia* infected wasp *Telenomus nawai*: to be submitted to *Heredity*

Jeong G., and Stouthamer R., No effect of reproductive isolation on the interspecific transmission of PSR in *Trichogramma*: to be submitted to *Journal of evolutionary biology*

Jeong G., and Stouthamer R., Inefficient transmission of the paternal sex ratio chromosome of *Trichogramma kaykai* in the novel host *T. deion*: to be submitted to *Evolution*

Jeong G., and Stouthamer R., Interaction between *Wolbachia* infection and transmission of the paternal sex ratio (PSR) chromosome in *Trichogramma deion*: to be submitted to *Heredity*

Sex ratio distorter-associated selective mating in *Trichogramma kaykai*: to be submitted

Printed by Ponsen & Looijen bv. Wageningen, The Netherlands
Cover design by Beomcheol An