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Co-evolution between parthenogenesis-inducing *Wolbachia* and its hosts

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Chapter 1

General Introduction

Sexual versus Asexual Reproduction

Sexual reproduction is the predominant mode of reproduction among eukaryotes. Therefore, it is generally thought that sexual reproduction is the optimal strategy for reproduction. There are many theories that explain the success of sexual reproduction. These theories fall into two broad categories (Maynard Smith 1978, Bell 1982). Mutational theories argue that sexual recombination facilitates the shedding of deleterious mutations from the genome, while under asexual reproduction these mutations accumulate and cause a genetic load (Muller 1964, Kimura & Maruyama 1966, Maynard Smith 1978, Kondrashov 1988, Crow 1994). Ecological theories suggest that sexual reproduction allows faster adaptation during antagonistic co-evolution with other organisms, such as parasites, predators and competitors (Van Valen 1973, Glesener & Tilman 1978, Bell 1982, 1985, Hamilton *et al.* 1990, Lively *et al.* 1990, Scheu & Drossel 2007). All of these theories support the general thought that sexual reproduction allows faster adaptation to a complex environment than asexual reproduction. In addition, over a long period of time sexual reproduction will be more advantageous than asexual reproduction.

However, sexual reproduction also has a lot of costs. Given the large fitness disadvantage of sexual reproduction, known as the twofold cost of sex (Maynard Smith 1978), it is remarkable that it is the predominant mode of reproduction in eukaryotes. The twofold cost of sex describes the fitness advantages of asexual reproduction (and fitness disadvantages of sexual reproduction), both from a population dynamical view and from a genetical view. Because an asexual female only produces daughters, all of her offspring will contribute to the next generation (in theory, each individual will produce at least one individual in the next generation). In contrast, only half of the offspring (with a 50/50 sex ratio) of a sexually reproducing female will contribute to the next generation (two individuals, a male and a female, are needed for one individual in the next generation). Therefore, an asexual population can grow faster than a sexual population and when they are in competition, the asexual population should outcompete the sexual one (Maynard Smith 1978). Also,

asexual females transmit all of their genome to each offspring, while a sexual female transmits only half of her genome to each offspring. Therefore, an asexual female has a higher fitness than a sexual female and a higher mother-offspring relatedness should favour asexual reproduction (Maynard Smith 1978).

Given the advantages and disadvantages of sexual and asexual reproduction, it can be predicted that asexual reproduction should be more advantageous in simplified environments with few biotic interactions and/or over a short period of time, while sexual reproduction should be more advantageous in complex environments and/or over a long period of time. Although sexual reproduction is the predominant mode of reproduction, asexual reproduction may be relatively common in simplified environments (Glesener & Tilman 1978, Bürger 1999, Haag & Ebert 2004, Scheu & Drossel 2007, Becks & Agrawal 2010). In support of this idea, recent studies on invertebrates have shown a prevalence of asexual species and populations in agricultural and human-disturbed (simplified) environments, but not in natural (complex) environments (Haack *et al.* 2000, Hoffmann *et al.* 2008, Foucaud *et al.* 2009, Gilabert *et al.* 2009).

Asexual reproduction in invertebrates is often induced by infection with cytoplasmatically inherited microorganisms, for example *Wolbachia* (Huigens & Stouthamer 2003), *Cardinium* (Zchori-Fein & Perlman 2004) and *Rickettsia* (Perlman *et al.* 2006). In this thesis, I will focus on the microorganism *Wolbachia*.

Wolbachia

Wolbachia are intracellular, symbiotic bacteria belonging to the order Rickettsiales within the α-Proteobacteria. *Wolbachia* are known to infect a wide range of arthropods, including insects, spiders, mites, scorpions and isopods, and have also been found in nematodes (Rousset *et al.* 1992, Werren 1997, Stouthamer *et al.* 1999, Werren *et al.* 2008). The type species for the *Wolbachia* genus is *Wolbachia pipientis*, which was first described in the mosquito *Culex pipiens* (Hertig 1936). The genus *Wolbachia* can be divided into six, and possibly eight, major clades or supergroups (A-H) (Lo *et al.* 2002, Casiraghi *et al.* 2005, Baldo & Werren 2007, Werren *et al.* 2008). Supergroup C and D are only found in filarial nematodes. The other supergroups are primarily found in arthropods, in which supergroup A and B are the most common. It has been estimated that 66% of all insect species is (partly) infected with *Wolbachia* (Hilgenboecker *et al.* 2008). Various insect species harbour multiple *Wolbachia* strains, which may even belong to different supergroups (Werren *et al.* 1995, Vavre *et al.* 1999, Werren & Windsor 2000).

Wolbachia are maternally inherited, because a sperm cell contains too little cytoplasm to harbour the bacteria. Therefore, vertical transmission (from mother to daughter) is the main transmission mode of *Wolbachia* within established hosts. However, incongruence between the phylogenies of *Wolbachia* and their hosts suggests widespread horizontal transmission of *Wolbachia* between hosts (O'Neill *et*

al. 1992, Rousset *et al.* 1992, Werren *et al.* 1995, Schilthuizen & Stouthamer 1997, Vavre *et al.* 1999). Horizontal transmission has been achieved experimentally both within and between species (Grenier *et al.* 1998, Heath *et al.* 1999, Huigens *et al.* 2000, 2004) and a recent field study (Kraaijeveld *et al.* 2011a, Kremer & Huigens 2011) found evidence for horizontal transmission during the early stages of *Wolbachia* infection in a parasitoid wasp. This suggests that horizontal transmission not only plays a role in the spread of *Wolbachia* from one species to another, but also between individuals within newly infected species.

Because *Wolbachia* are maternally inherited, they benefit from female-biased sex ratios in its hosts. To enhance its own transmission, *Wolbachia* can induce various alterations of the reproduction mechanism of its host, such as cytoplasmic incompatibility, feminization, male-killing and parthenogenesis (Rousset *et al.* 1992, Werren 1997, Stouthamer *et al.* 1999, Werren *et al.* 2008).

Cytoplasmic incompatibility (CI) is the most widespread effect of *Wolbachia* infection and has been described in mites, isopods and many insect orders, such as Coleoptera, Diptera, Hemiptera, Hymenoptera, Lepidoptera and Orthoptera (Werren 1997, Stouthamer *et al.* 1999, Werren *et al.* 2008). CI is an alteration of the reproduction mechanism that causes incompatibility between sperm and egg cells. CI-*Wolbachia* modify the sperm of *Wolbachia*-infected males during spermatogenesis. Eggs of CI-*Wolbachia*-infected females harbour a rescue mechanism for this sperm modification. However, when the right rescue mechanism is not present in the egg, because the female is not infected or infected with a different *Wolbachia* strain than the male, sperm and egg will be incompatible. When the sperm modification is not rescued, an asynchrony between the male and female pronuclei occurs during the first embryonic mitotic division. In diploid species this will result in embryonic death. In haplodiploid species only male offspring will develop (Werren 1997, Stouthamer *et al.* 1999, Werren *et al.* 2008). Two forms of CI exist. Unidirectional incompatibility occurs when the sperm of a *Wolbachia*-infected male fertilizes an egg of an uninfected female. The egg of a *Wolbachia*-infected female and sperm of an uninfected male are compatible. Bidirectional incompatibility occurs when a male and a female are infected with different strains of *Wolbachia* that are mutually incompatible. In this case, the sperm and egg cell are only compatible when a male and a female are infected with the same *Wolbachia* strain (Werren 1997, Stouthamer *et al.* 1999, Werren *et al.* 2008).

Feminization-inducing *Wolbachia* have been found in isopods and the insect orders Hemiptera and Lepidoptera (Werren 1997, Bouchon *et al.* 1998, Stouthamer *et al.* 1999, Hiroki *et al.* 2002, Negri *et al.* 2006, Werren *et al.* 2008). *Wolbachia*-induced feminization causes genetic males to be converted into functional females, for example by suppression of the androgenic glands (Werren 1997, Stouthamer *et al.* 1999, Werren *et al.* 2008).

Wolbachia-induced male-killing has been described in pseudoscorpions and the

insect orders Coleoptera, Diptera and Lepidoptera (Hurst *et al.* 1999, Stouthamer *et al.* 1999, Dyer & Jaenike 2004, Zeh *et al.* 2005, Werren *et al.* 2008). *Wolbachia*-infected males are killed during embryogenesis, possibly to provide more nutrients to the female offspring (Stouthamer *et al.* 1999, Werren *et al.* 2008).

Parthenogenesis-inducing (PI) *Wolbachia* have so far only been found in haplodiploid organisms, such as hymenopterans, thrips and mites (Stouthamer *et al.* 1990a, Werren 1997, Stouthamer *et al.* 1999, Arakaki *et al.* 2001, Weeks & Breeuwer 2001, Huigens & Stouthamer 2003, Werren *et al.* 2008). In uninfected haplodiploid organisms, fertilized eggs develop into diploid daughters and unfertilized eggs develop into haploid sons (arrhenotoky). PI-*Wolbachia* cause diploidization of the haploid eggs by disrupting the segregation of the homologous chromosomes during the first mitotic division after meiosis (Stouthamer & Kazmer 1994, Huigens & Stouthamer 2003, Pannebakker *et al.* 2004a) resulting in the production of daughters from unfertilized eggs (thelytoky).

In this thesis, I will focus on PI-*Wolbachia* in hymenopterans. More specifically, I studied the dynamics, causes and consequences of *Wolbachia*-induced parthenogenesis in two parasitoid wasp species, *Tetrastichus coeruleus* and *Asobara japonica*.

Consequences of *Wolbachia*-induced parthenogenesis

In most cases of *Wolbachia*-induced parthenogenesis, the infection is fixed and the entire host population consists of females (Huigens & Stouthamer 2003). In the absence of males and sexual reproduction, genes involved in sexual reproduction are not actively maintained by selection. Accumulation of neutral mutations or selection against the maintenance of costly sexual traits may lead to their loss or deterioration (Carson *et al.* 1982, Pijls *et al.* 1996, Pannebakker *et al.* 2005, Kraaijeveld *et al.* 2009). Because male traits are not expressed in parthenogenetic populations, they are likely to degenerate due to neutral mutations. Female traits, which are expressed but not used, may also be selected against when they are costly to maintain (Pijls *et al.* 1996). In addition, females may loose the ability to reproduce sexually due to 'functional virginity mutations' that may spread concomitantly with the *Wolbachia* infection through a population (Stouthamer *et al.* 2010, King & Hurst 2010). Mutations that prevent females from fertilizing their eggs will have a selective advantage in the presence of PI-*Wolbachia*-infected females because they induce the bearer of these mutations to produce more sons which will have many mating opportunities. Virginity mutations arise during the early stages of PI-*Wolbachia* infection and affect traits in females involved in sexual reproduction, e.g. mating or egg fertilization. Accumulation of neutral mutations or selection against the maintenance of costly traits arise after a longer period of parthenogenetic reproduction and can potentially affect all traits involved in sexual reproduction, both in males and females, e.g. courtship behaviour or pheromone production.

Sexual traits may evolve to be costly when the reproductive interests of males and

females are different. Sexual conflict may result in sexually antagonistic coevolution, in which males evolve adaptations that manipulate female behaviour and females evolve resistance to male manipulation (Rice 1996, Chapman *et al.* 2003, Arnqvist & Rowe 2005). In the absence of males, selection on manipulative male traits and costly female resistance traits will be absent. Adaptations evolved under sexually antagonistic coevolution are released from selection and alleles that were favoured can be replaced by others due to accumulation of random mutations (Carson *et al.* 1982, Pijls *et al.* 1996) or antagonistic pleiotropy (Pijls *et al.* 1996, Pannebakker *et al.* 2005). Therefore, parthenogenetic females may be either more receptive to mating when they have been selected in the absence of males or not receptive at all when they have been selected in the presence of a small number of males.

When deterioration or loss of genes involved in sexual reproduction can evolve over a long period of time, asexual species or populations may no longer be capable of sexual reproduction. Many insect species with female-only populations exhibit deterioration or loss of female sexual traits, as expected by theory (e.g. Carson *et al.* 1982, Pijls *et al.* 1996, Gottlieb & Zchori-Fein 2001, Kraaijeveld *et al.* 2009).

Tetrastichus coeruleus

Tetrastichus coeruleus (Hymenoptera: Eulophidae) is a gregarious egg-larval parasitoid of the common asparagus beetle (*Crioceris asparagi*). *T. coeruleus* both feeds on and parasitizes the eggs of *C. asparagi* (Capinera & Lilly 1975a, van Alphen 1980). This (and own observations) indicates that *T. coeruleus* is synovigenic, meaning that it produces eggs during all or most of its adult life (Heimpel & Collier 1996).

C. asparagi lives on the asparagus plant (*Asparagus officinalis*), which grows on sandy soils, such as coastal dune areas, and as a crop in monoculture on agricultural fields. *A. officinalis* is native to western Asia, Europe and northern Africa and has been cultured for thousands of years (Audas & Heywood 1981, Weeda *et al.* 1991). *C. asparagi* is known as a pest species in asparagus agriculture and *T. coeruleus* can be used as a biological control agent against these beetles (Capinera & Lilly 1975b). *A. officinalis* has been introduced to the United States for culturing (Weeda *et al.* 1991) and in 1859 *C. asparagi* was first noticed in northeastern United States (Capinera & Lilly 1975a). Later, probably already in 1863, but certainly in 1909, *T. coeruleus* also was recorded and was noticed to control the asparagus beetle population by feeding on and ovipositing in the eggs of *C. asparagi* (Fernald 1909, Russell & Johnston 1912, Johnston 1915, Capinera & Lilly 1975a, 1975b). Russell & Johnston (1912) and Johnston (1915) only recorded females of *T. coeruleus* in the population, both in the field and when they were reared in the lab for multiple generations, showing that *T. coeruleus* occurs in parthenogenetic populations on agricultural fields in northeastern United States. Moreover, previous work in our lab suggested that several *T. coeruleus* populations in The Netherlands are infected with *Wolbachia* that cause parthenogenesis (B. Wielaard, pers. comm.).

Asobara japonica

Asobara japonica (Hymenoptera: Braconidae) is a solitary larval-pupal parasitoid of drosophilid flies (Ideo *et al.* 2008). *A. japonica* naturally occurs in Japan. Populations of *A. japonica* on the temperate main islands of Japan exhibit highly female-biased sex ratios (92.7% - 99.2% females), whereas population sex ratios on the smaller subtropical southern islands are not biased (Mitsui *et al.* 2007). The populations on the main islands are infected with parthenogenesis-inducing *Wolbachia*, while the populations on the smaller southern islands are not (Kremer *et al.* 2009).

Wolbachia in *T. coeruleus* and *A. japonica*

Interestingly, both *T. coeruleus* and *A. japonica* have populations that are infected with *Wolbachia*, while they also have populations that are not infected (*T. coeruleus*: chapter 2, Reumer *et al.* 2010; *A. japonica*: Mitsui *et al.* 2007, Kremer *et al.* 2009). In both species, *Wolbachia*-infected populations reproduce through parthenogenesis, whereas uninfected populations reproduce sexually. Also, in both species a small number of male offspring is regularly produced in the otherwise parthenogenetically reproducing populations (*T. coeruleus*: chapter 2, Reumer *et al.* 2010; *A. japonica*: Mitsui *et al.* 2007, chapter 6, Reumer *et al.* 2012).

Only a few other parasitoid wasp species are known in which both sexual and parthenogenetic populations occur. In the parasitoid wasps *Apoanagyrus diversicornis* (Pijls *et al.* 1996), *Telenomus nawai* (Arakaki *et al.* 2000) and *Leptopilina clavipes* (Pannebakker *et al.* 2004b) PI-*Wolbachia*-infected and uninfected populations occur allopatrically. Mixed populations of PI-*Wolbachia*-infected and uninfected individuals are limited to a number of species of the genus *Trichogramma* (Stouthamer *et al.* 1990a, 1990b, 2001). In most cases of *Wolbachia*-induced parthenogenesis, the infection is fixed and the entire host population consists of females that reproduce parthenogenetically (Huigens & Stouthamer 2003).

In this thesis, I will investigate the dynamics of *Wolbachia*-infected and uninfected populations of *T. coeruleus* and *A. japonica*. In addition, I will study the occasional male production in otherwise parthenogenetic populations of *A. japonica*.

Thesis Overview

In this thesis, I investigated *Wolbachia*-induced parthenogenesis in two parasitoid wasp species, *Tetrastichus coeruleus* (chapter 2, 3, 4 & 5) and *Asobara japonica* (chapter 6 & 7). I studied the population genetics of infected and uninfected populations of both species in order to determine population dynamics and shed more light on the origin of the *Wolbachia* infection (chapter 3 & 6). I studied the differences between infected and uninfected populations, in terms of *Wolbachia* infection frequency, mode of reproduction, ecology and life history traits (chapter

2 & 4). In addition, I studied the consequences of a PI-*Wolbachia* infection by investigating the sexual functionality in *T. coeruleus* females (chapter 5) and *A. japonica* males (chapter 7).

In **chapter 2**, I tested whether there is a correlation between ecology and mode of reproduction in populations of *T. coeruleus*. Classical ecological theories for the maintenance of sexual reproduction state that sexual reproduction facilitates adaptation to complex environments with many biotic interactions, whereas simplified environments are expected to favour asexual reproduction. Because *T. coeruleus* occurs both in natural (complex) and agricultural (simplified) environments, I investigated whether different reproductive modes can be found in different habitats and whether the possible correlation followed the classical theories. In addition, I investigated whether *Wolbachia* infection caused parthenogenesis in female-biased populations.

I sampled 13 populations of *T. coeruleus* in The Netherlands, France and Massachusetts, USA. In contrast to the general pattern, in Dutch and French natural areas I found *Wolbachia*-infected, highly female-biased populations that reproduce parthenogenetically (thelytoky), while populations on Dutch agricultural fields were not infected with *Wolbachia*, showed higher frequencies of males and reproduced sexually (arrhenotoky). However, I also found a female-only, *Wolbachia*-infected population on agricultural fields in Massachusetts. All *Wolbachia*-infected populations were infected with the same *Wolbachia* strain.

At this moment, I do not have a convincing explanation for the deviation of *T. coeruleus* populations in The Netherlands and France from the general association between ecology and mode of reproduction. The fixation of *Wolbachia* in the population from Massachusetts (where the species was introduced) may be due to founder effect and lack of uninfected, sexual source populations.

In **chapter 3**, I studied the population genetics of *Wolbachia*-infected and uninfected populations of *T. coeruleus*. Because *Wolbachia* are maternally inherited, vertical transmission is the main transmission mode within established hosts. However, horizontal transmission plays a major role in the spread of *Wolbachia* to novel hosts and within some newly infected host populations. I investigated the transmission mode with which *Wolbachia* has spread through the populations of *T. coeruleus*. In addition, I looked for evidence of recent gene flow between populations.

I studied the population genetics of 13 populations of *T. coeruleus*, using nuclear microsatellite markers and mitochondrial DNA. Two major genetic clusters were evident: a thelytokous cluster containing all the *Wolbachia*-infected populations and an arrhenotokous cluster containing all the uninfected populations.

Within the thelytokous cluster, there was no variation in mitochondrial DNA, suggesting that initially only one female became infected with *Wolbachia* and ver-

tical transmission was the main transmission mode for *Wolbachia* spread within *T. coeruleus*. However, nuclear markers displayed considerable genetic variation, suggesting that infected females mated with males, which would introduce nuclear DNA variation into the mitochondrial lineage. Thelytokous populations showed significant genetic substructure. Within the arrhenotokous cluster, both nuclear and mitochondrial DNA variation was present, but no population structure was recognized.

Several females from otherwise thelytokous populations were uninfected and/or heterozygous for one or more microsatellite loci. No infected females were found in any of the arrhenotokous populations. This suggests occasional migration from arrhenotokous to thelytokous populations, but not vice versa. I conclude that *Wolbachia* has spread via vertical transmission through the populations of *T. coeruleus*. The nuclear genetic variation in the *Wolbachia*-infected populations is due to occasional sex and not to horizontal transmission of *Wolbachia*.

In **chapter 4**, I studied the differences between two populations of *T. coeruleus* that differ in ecology, *Wolbachia* infection and mode of reproduction. Differences between populations may result in differences in life history traits and strategies in order to optimize reproduction and survival.

I quantified several life history traits in two populations of *T. coeruleus*, one from a natural (Meijendel) and one from an agricultural (Brabant) environment: clutch size, life span, female weight and nutrient concentrations (proteins, lipids, sugars and glycogen).

Females from Brabant laid larger clutches, had longer life spans and were heavier than females from Meijendel. There was no difference in the relative amounts of proteins, lipids or sugars, but females from Meijendel had relatively more glycogen than females from Brabant.

The two populations therefore exhibit markedly different life history strategies. Females from Brabant invest relatively more in survival, body size and clutch size, whereas females from Meijendel seem to be more active or fly longer distances. Further studies are needed to determine if and how these life history differences are related to differences in ecology, *Wolbachia* infection or mode of reproduction.

In **chapter 5**, I studied the consequences of a PI-*Wolbachia* infection in *T. coeruleus* females. In most cases of *Wolbachia*-induced parthenogenesis, the infection is fixed and the entire host population consists of females. In the absence of males and sexual reproduction, genes involved in sexual reproduction are not actively maintained by selection. Accumulation of neutral mutations or selection against the maintenance of such traits may lead to their loss or deterioration. In addition, females may loose the ability to reproduce sexually due to 'functional virginity mutations'.

First, I examined whether mating is costly to females. Then, I compared the sex-

ual functionality of arrhenotokous and thelytokous *T. coeruleus* females. I measured their attractiveness and receptiveness to males and I compared the morphology of their spermathecae.

Mated females had a shorter life span than virgin females, showing that either mating or sperm storage was costly. Several sexual traits of thelytokous females have degraded compared to arrhenotokous females. Arrhenotokous and thelytokous females were equally attractive to males, but thelytokous females were unreceptive to males and there was a clear difference in spermathecal morphology between arrhenotokous and thelytokous females.

Selection against the maintenance of costly sexual traits appears to have resulted in the degradation of receptivity and spermathecal morphology of thelytokous females. However, I cannot exclude that these traits have degraded due to functional virginity mutations or accumulation of neutral mutations.

In **chapter 6**, I investigated the origin of male offspring production by PI-*Wolbachia*-infected females in *A. japonica*. Because all females in the field are infected and infected *A. japonica* females are not capable of sexual reproduction, male production seems to be maladaptive in thelytokous populations. In addition, I studied the population genetics of *Wolbachia*-infected and uninfected populations of *A. japonica*.

I tested three hypotheses for the origin of male production: high rearing temperatures could result in higher offspring sex ratios (more males), low *Wolbachia* titer of the mother could lead to higher offspring sex ratios and/or the *Wolbachia* infection is of relatively recent origin and not enough time has passed to allow complete co-adaptation between *Wolbachia* and host.

One third of the *Wolbachia*-infected females produced males and more than half of these males were also infected with *Wolbachia*. Neither offspring sex ratio nor male infection frequency were affected by rearing temperature or *Wolbachia* concentration of the mother. The mitochondrial DNA sequence of one of the uninfected populations was identical to that of two of the infected populations. Therefore, the initial *Wolbachia* infection of *A. japonica* must have occurred recently. Mitochondrial sequence variation among the infected populations suggests that the spread of *Wolbachia* through the host populations involved horizontal transmission.

I conclude that the occasional male production by *Wolbachia*-infected *A. japonica* females is most likely a maladaptive side-effect of incomplete co-evolution between symbiont and host in this relatively young infection.

In **chapter 7**, I studied the consequences of a PI-*Wolbachia* infection in *A. japonica* males. Recent studies have shown that male-killing-*Wolbachia* strains can induce cytoplasmic incompatibility (CI) when introgressed into a resistant host. Phylogenetic studies suggest that transitions between CI and other *Wolbachia* phenotypes

have occurred frequently, raising the possibility that latent CI may be widespread among *Wolbachia*. I investigated whether a PI-*Wolbachia* strain also can induce CI.

PI-*Wolbachia*-infected *A. japonica* females regularly produce male offspring, which may either be infected or not. First, I tested whether infected males and females harboured the same *Wolbachia* strain. Then, mating experiments were performed between uninfected arrhenotokous females and males that were either infected or uninfected. Uninfected males were obtained naturally from infected thelytokous populations and through removal of *Wolbachia* using antibiotics. In addition, males from a naturally uninfected arrhenotokous *A. japonica* population were used.

Infected males and females harboured the exact same *Wolbachia* strain, indicating that the *Wolbachia* strain that induces parthenogenesis in females also was present in infected males. Uninfected arrhenotokous females that had mated with *Wolbachia*-infected males produced a slightly more male-biased sex ratio than females that had mated with uninfected males. This effect was strongest in females that had mated with males with a relatively high *Wolbachia* concentration. Because *Wolbachia*-infected males did not show higher ratios of nuclear versus mitochondrial DNA content than uninfected males, I concluded that *Wolbachia* does not cause diploidization of cells in infected males.

I conclude that the *Wolbachia* strain that induced parthenogenesis in females induced CI in infected males. However, the effect was very small. This suggests that if CI is induced in *A. japonica* males by the PI-*Wolbachia* strain, the ability of CI-induction may have degenerated through the accumulation of mutations. Although the results are consistent with CI, other alternatives such as production of abnormal sperm by *Wolbachia*-infected males cannot completely be ruled out.

In **chapter 8**, I summarized the most important results presented in this thesis and I compared the findings about *Wolbachia*-induced parthenogenesis for the two studied parasitoid wasp species: *T. coeruleus* and *A. japonica*. The main conclusions of this thesis are that different scenarios may occur for the spread of a parthenogenesis-inducing *Wolbachia* infection in different host species, different barriers may prevent migration and gene flow between *Wolbachia*-infected and uninfected populations in different host species, different ages of the PI-*Wolbachia* infection may have different consequences for the host species, and a PI-*Wolbachia* infection can have severe consequences for the sexual functionality of infected males and females.

Chapter 2

Ecology, *Wolbachia* infection frequency and mode of reproduction in the parasitoid wasp *Tetrastichus coeruleus* (Hymenoptera: Eulophidae)

Barbara M. Reumer, Jacques J.M. van Alphen & Ken Kraaijeveld

Whereas sexual reproduction may facilitate adaptation to complex environments with many biotic interactions, simplified environments are expected to favour asexual reproduction. In agreement with this, recent studies on invertebrates have shown a prevalence of asexual species in agricultural (simplified) but not in natural (complex) environments. We investigated whether the same correlation between reproductive mode and habitat can be found in different populations within one species. The parasitoid wasp *Tetrastichus coeruleus* forms an ideal model to test this question, since it occurs both in natural and agricultural environments. Further, we investigated whether *Wolbachia* infection caused parthenogenesis in female-biased populations.

In contrast to the general pattern, in Dutch and French natural areas we found *Wolbachia*-infected, highly female-biased populations that reproduce parthenogenetically. By contrast, populations on Dutch agricultural fields were not infected with *Wolbachia*, showed higher frequencies of males and reproduced sexually. However, we also found a female-only, *Wolbachia*-infected population on agricultural fields in northeastern USA. All *Wolbachia*-infected populations were infected with the same *Wolbachia* strain.

At this moment, we do not have a convincing explanation for this deviation from the general pattern of ecology and reproductive mode. It may be that asparagus agricultural fields differ from other crop fields in ways that favour sexual reproduction. Alternatively, *Wolbachia* may manipulate life history traits in its host, resulting in different fitness pay-offs in different habitats. The fixation of *Wolbachia*

in the USA populations (where the species was introduced) may be due to founder effect and lack of uninfected, sexual source populations.

Introduction

Sexual reproduction is the predominant mode of reproduction among eukaryotes. This is remarkable given the large fitness disadvantage of sexual reproduction, known as the twofold cost of sex (Maynard Smith 1978). Because an asexual female only produces daughters, all of her offspring will contribute to the next generation, while only half of the offspring (with a 50/50 sex ratio) of a sexually reproducing female will contribute to the next generation. Therefore, an asexual population can grow faster than a sexual population and when they are in competition, the asexual population should outcompete the sexual one (Maynard Smith 1978). Also, asexual females transmit all of their genome to each offspring, while a sexual female transmits only half of her genome to each offspring. Thus, higher mother-offspring relatedness should favour asexual reproduction (Maynard Smith 1978).

Theories that explain the success of sexual reproduction fall into two broad categories (Maynard Smith 1978, Bell 1982). Mutational theories argue that sexual recombination facilitates the shedding of deleterious mutations from the genome, while under asexual reproduction these mutations accumulate and cause a genetic load (Muller 1964, Kimura & Maruyama 1966, Maynard Smith 1978, Crow 1994). Ecological theories suggest that sexual reproduction allows faster adaptation during antagonistic coevolution with other organisms, in particular parasites, but also competitors or predators (Van Valen 1973, Glesener & Tilman 1978, Bell 1985, Hamilton *et al.* 1990, Lively *et al.* 1990). This predicts that the advantage of sex should be smaller in simplified environments with fewer biotic interactions. Asexual reproduction may thus be relatively common in such environments (Glesener & Tilman 1978, Bürger 1999, Haag & Ebert 2004). In support of this idea, recent studies on invertebrates have shown a prevalence of asexual species and populations in agricultural and human-disturbed (simplified) environments, but not in natural (complex) environments (Haack *et al.* 2000, Hoffmann *et al.* 2008, Foucaud *et al.* 2009, Gilabert *et al.* 2009). In order to understand the mechanisms behind this pattern, it is necessary to study populations within one species that occur in different environments. The parasitoid wasp *Tetrastichus coeruleus* (Hymenoptera: Eulophidae) forms an ideal model to test ecological theories for the maintenance of sexual reproduction, because it occurs both in natural (complex) and in agricultural (simplified) environments.

T. coeruleus is an egg-larval parasitoid of the common asparagus beetle (*Crioceris asparagi*). *C. asparagi* lives on the asparagus plant (*Asparagus officinalis*), which grows on sandy soils, such as coastal dune areas, and as a crop in monoculture on agricultural fields. *A. officinalis* is native to western Asia, Europe and northern Africa and has been cultured for thousands of years (Audas & Heywood 1981, Weeda *et al.* 1991). *C. asparagi* is known as a pest species in asparagus agriculture and *T.*

coeruleus can be used as a biological control agent against these beetles (Capinera & Lilly 1975b). *A. officinalis* has been introduced to the United States for culturing (Weeda *et al.* 1991) and in 1859 *C. asparagi* was first noticed in northeastern United States (Capinera & Lilly 1975a). Later, probably already in 1863, but certainly in 1909, *T. coeruleus* also was recorded and was noticed to control the asparagus beetle population by feeding on and ovipositing in the eggs of *C. asparagi* (Russell & Johnston 1912, Johnston 1915, Capinera & Lilly 1975a, 1975b). Russell & Johnston (1912) and Johnston (1915) only recorded females of *T. coeruleus* in the population, both in the field and when they were reared in the lab for multiple generations, showing that *T. coeruleus* occurs in parthenogenetic populations on agricultural fields in northeastern United States.

Asexual reproduction in invertebrates is often induced by infection with cytoplasmatically inherited microorganisms, such as *Wolbachia*. *Wolbachia* are intracellular, symbiotic bacteria belonging to the order Rickettsiales within the α-Proteobacteria. *Wolbachia* is known to infect a wide range of arthropods, including insects, spiders, mites, scorpions and isopods, and has also been found in nematodes. A recent analysis estimated that 66% of all insect species is infected with *Wolbachia* (Hilgenboecker *et al.* 2008). *Wolbachia* is maternally inherited, because a sperm cell contains too little cytoplasm to harbour the bacteria. Therefore, they benefit from female-biased sex ratios in their hosts. To enhance its own transmission, *Wolbachia* can induce various alterations of the reproduction mechanism of its host, such as cytoplasmic incompatibility, feminization, male-killing and parthenogenesis. *Wolbachia*-induced parthenogenesis is most commonly found in haplodiploid organisms, such as Hymenoptera (Werren 1997, Stouthamer *et al.* 1999, Werren *et al.* 2008).

Like all Hymenoptera, *T. coeruleus* is haplodiploid. Fertilized eggs develop into diploid daughters, while unfertilized eggs develop into haploid sons. This form of sexual reproduction is called arrhenotoky. Haplodiploid organisms can relatively easily shift to asexual reproduction. All that is required is diploidization of the haploid eggs, for example by alteration of meiotic and/or mitotic processes. It has been shown that *Wolbachia* can induce parthenogenesis in haplodiploid organisms by disrupting the segregation of the homologous chromosomes during the first mitotic division after meiosis in unfertilized eggs (Huigens & Stouthamer 2003, Pannebakker *et al.* 2004a). This form of parthenogenetic reproduction in haplodiploids is called thelytoky. Technically speaking, in sexually reproducing haplodiploids sons are also produced parthenogenetically, because they develop from unfertilized haploid eggs. In thelytokous reproducing haplodiploids, daughters are produced parthenogenetically from unfertilized diploid eggs. For convenience, in this paper we will use the terms sexual reproduction and parthenogenesis in stead of arrhenotoky and thelytoky.

Here, we investigate whether the ecological pattern found by Hoffmann *et al.* (2008) is also applicable within species. According to this pattern, parthenogenetic

populations of *T. coeruleus* should be found in agricultural environments, while sexual populations would be expected in natural environments. In support of this, Russell & Johnston (1912) and Johnston (1915) found parthenogenetic populations of *T. coeruleus* on the agricultural fields in northeastern United States. Therefore, we predicted that European agricultural populations of *T. coeruleus* would be similarly parthenogenetic. We further expected that natural populations of *T. coeruleus* might reproduce sexually. In addition, we investigated whether *Wolbachia* infection is the cause of parthenogenetic reproduction in *T. coeruleus*.

We sampled different populations of *T. coeruleus*. For each population, we determined field sex ratio and *Wolbachia* infection status. Parthenogenetic populations turned out to be infected with *Wolbachia*. Because we found differences in sex ratio and *Wolbachia* infection frequency between populations, we sequenced *Wolbachia* to see whether different populations were infected with different strains of *Wolbachia*.

Materials and Methods

Field sampling

In the spring and summer of 2007 and 2008, eleven populations of *Tetrastichus coeruleus* were sampled throughout The Netherlands and Belgium (Fig. 2.1). Several locations were visited in both years and multiple times per year. Five populations were sampled in coastal dune areas, five populations were sampled from agricultural fields and one population was sampled in a private allotment garden. Because the sampled agricultural field population from Antwerpen, Belgium, lies very close to the Dutch border and for convenience in this paper, we will talk about five Dutch agricultural fields. *T. coeruleus* was not found in dune areas north of the Noord-Hollandse Duinen and south of Goeree Overflakkee. In the summer of 2006 a population of *T. coeruleus* was sampled in a natural area in the Camargue in southern France (between Albaron and Saintes Maries de la Mer, on *Asparagus maritimus*) and in the spring of 2008 *T. coeruleus* was sampled on agricultural fields in Massachusetts, USA. In all locations, except the Camargue, adult wasps were caught in the field, giving the operational sex ratio of the population. In the Camargue and some locations in The Netherlands (Noord-Hollandse Duinen, Kennemerduinen, Meijendel, Gelderland and Brabant), larvae of *C. asparagi* were collected and reared in the lab. Some of these had been parasitized by *T. coeruleus*. Wasps emerging from the pupae of *C. asparagi* give the primary sex ratio of the population.

The sex of each wasp (caught in the field or emerged from pupae of *C. asparagi*) was determined by looking under a binocular for the presence of a groove on the ventral side of the abdomen which envelopes the ovipositor. In males this groove is absent. All wasps were then stored individually in 70% ethanol in a 1.5 ml Eppendorf tube and kept at -20°C.
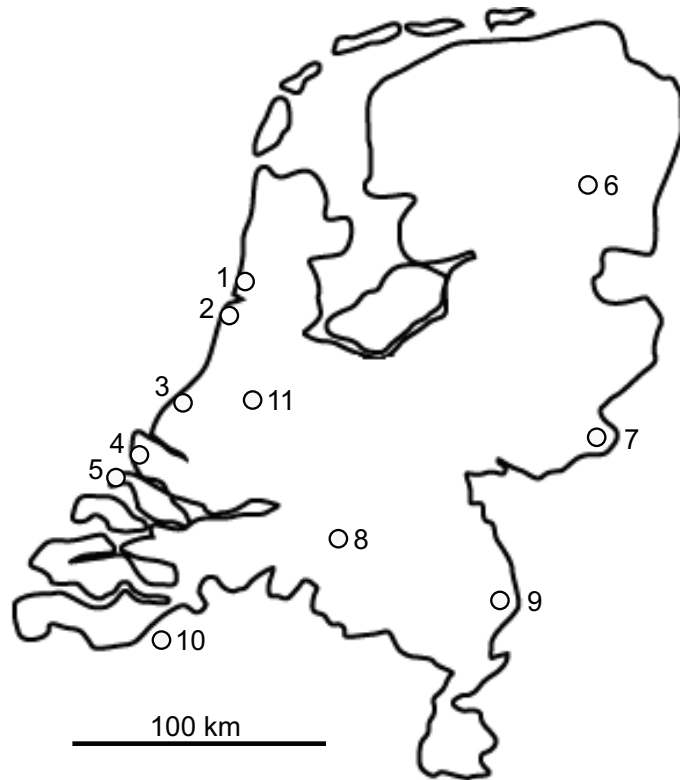
Figure 2.1: Map of collection sites of *Tetrastichus coeruleus* in The Netherlands. 1-5: Dune areas (1: Noord-Hollandse Duinen, 2: Kennemerduinen, 3: Meijendel, 4: Voornse Duinen, 5: Goeree Overflakkee), 6-10: Agricultural fields (6: Drenthe, 7: Gelderland, 8: Brabant, 9: Limburg, 10: Antwerpen), 11: Private allotment garden (Alphen aan den Rijn).

DNA extraction

DNA extractions were performed using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol, using mini spin columns. Before starting the DNA extraction, each wasp was transferred to a new 1.5 ml Eppendorf tube. After evaporation of remaining ethanol, tissue lysis buffer (ATL) was added to the tube and the wasp was crushed using a plastic pestle. The tissue was incubated overnight in proteinase K at 56°C. The DNA was dissolved in 100 µl elution buffer (AE).

Table 2.1: Primers used to amplify genes for *Wolbachia* detection and *Wolbachia* characterization (sequencing) in *Tetrastichus coeruleus*. For each gene the forward (F) and reverse (R) primer sequences with the specific annealing temperature T_a (°C) are given. Note that for the genes *wsp* and *ftsZ* different primers were used for sequencing than for *Wolbachia* detection.

Gene	Primer name	Primer sequence (5' → 3')	T_a	Reference
Wolbachia detection				
wsp	*wsp* 81F	TGG TCC AAT AAG TGA TGA AGA AAC	55	Braig *et al.* 1998
	wsp 691R	AAA AAT TAA ACG CTA CTC CA		Zhou *et al.* 1998
ftsZ	*ftsZ* F	GGA CCG GAT CCG TAT GCC GAT TGC AGA GCT TG	55	Holden *et al.* 1993
	ftsZ R	GGA CCG AAT TCG CCA TGA GTA TTC ACT TGG CT		Sinkins *et al.* 1995
Wolbachia characterization				
gatB	*gatB* Asp F1	TTT AGA GCA AGA TGC AGG RAA GAG CG	64	Baldo *et al.* 2006
	gatB R1	TGG YAA YTC RGG YAA AGA TGA		MLST website
gatB	*gatB* Bsp F1	TAA GAA TCG CAA GAA TTC AC	62	Baldo *et al.* 2006
	gatB R1	TGG YAA YTC RGG YAA AGA TGA		MLST website
coxA	*coxA* Asp F1	ATA CCC ACC TTT ATC ACA GG	56	Baldo *et al.* 2006
	coxA R1	CTA AAG ACT TTK ACR CCA GT		MLST website
coxA	*coxA* Bsp F1	ATA CCC ACC TYT RTC GCA AA	54	Baldo *et al.* 2006
	coxA R1	CTA AAG ACT TTK ACR CCA GT		MLST website
hcpA	*hcpA* F1	GAA ATA RCA GTT GCT GCA AA	55	Baldo *et al.* 2006
	hcpA Asp R1	TTC TAR YTC TTC AAC CAA TGC		MLST website
hcpA	*hcpA* F1	GAA ATA RCA GTT GCT GCA AA	55	Baldo *et al.* 2006
	hcpA Bsp R1	TTC TTT GTC GCT MAC TTY AAT CAK G		MLST website
ftsZ	*ftsZ* Asp F1	AAA GAT AGT CAT ATG CTT TTC	55	Baldo *et al.* 2006
	ftsZ Asp R1	CAT CGC TTT GCC CAT CTC G		MLST website
ftsZ	*ftsZ* Bsp F1	AAA GAT AGC CAT ATG CTC TTT	59	Baldo *et al.* 2006
	ftsZ Bsp R1	CAT TGC TTT ACC CAT CTC A		MLST website
fbpA	*fbpA* Asp F1	TTA ACC CTG ATG CTT ATG AC	55	Baldo *et al.* 2006
	fbpA R1	CCR CCA GAR AAA AYY ACT ATT C		MLST website
fbpA	*fbpA* Bsp F1	GTT AAC CCT GAT GCT TAC GAT	58	Baldo *et al.* 2006
	fbpA R1	CCR CCA GAR AAA AYY ACT ATT C		MLST website
wsp	*wsp* F1	GTC CAA TAR STG ATG ARG AAA C	59	Baldo *et al.* 2006
	wsp R1	CYG CAC CAA YAG YRC TRT AAA		MLST website

Wolbachia detection

Wasps were tested for *Wolbachia* infection by amplifying the *Wolbachia*-specific *wsp* gene, with forward primer *wsp*-81F and reverse primer *wsp*-691R (Braig *et al.* 1998, Zhou *et al.* 1998; for primer sequences and annealing temperatures see Table 2.1). When amplification of *wsp* was weak and infection status ambiguous, the *Wolbachia*-specific *ftsZ* gene was also amplified (Holden *et al.* 1993, Sinkins *et al.* 1995; for primer sequences and annealing temperatures see Table 2.1). Polymerase Chain Reactions (PCR) for both the *wsp* gene and the *ftsZ* gene were performed in a total volume of 20.0 µl, containing 1x PCR-buffer (Qiagen), 62.5 µM dNTPs, 1 unit Taq polymerase, 250 nM forward primer, 250 nM reverse primer and 1.0 µl DNA template. A PTC-200 DNA Engine Thermal Cycler PCR machine (MJ Research, Waltham, MA, USA) was used for all PCRs. PCR conditions for the *wsp* gene were as follows: 3 min at 94°C, then 35 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C, and finally 5 min at 72°C. PCR conditions for the *ftsZ* gene were as follows: 3 min at 94°C, then 35 cycles of 45 sec at 94°C, 1 min at 55°C and 1 min at 72°C and finally 5 min at 72°C. All PCR products were run on a 2% agarose gel and visualized using ethidium bromide staining.

Wolbachia characterization

Wolbachia from three populations were characterized by sequencing six genes specific for *Wolbachia*. Two infected females were used from each population: the dune area of Meijendel in The Netherlands, the natural area in the Camargue in France and the agricultural fields in Massachusetts, USA. For each female, all six genes were sequenced. *Wolbachia* was characterized by sequencing a set of five Multi Locus Sequence Typing (MLST) genes: *gatB*, *coxA*, *hcpA*, *ftsZ* and *fbpA* (Baldo *et al.* 2006; see also the MLST website: http://pubmlst.org/wolbachia/, Jolley *et al.* 2004). For these five genes, specific primers for both the A- and B-type *Wolbachia* alleles were tested. In addition to these MLST genes, the *Wolbachia*-specific *wsp* gene was sequenced (Baldo *et al.* 2006). For primer sequences and annealing temperatures see Table 2.1. Note that for the genes *wsp* and *ftsZ* different primers were used for sequencing than for *Wolbachia* detection. PCRs for both the MLST genes and the *wsp* gene were performed in a total volume of 40.0 µl, containing 1x PCR-buffer (Qiagen), 200 µM dNTPs, 0.5 unit Taq polymerase, 1.0 µM forward primer, 1.0 µM reverse primer and 2.0 µl DNA template. A PTC-200 DNA Engine Thermal Cycler PCR machine (MJ Research) was used for all PCRs. PCR conditions for both the MLST genes and the *wsp* gene were as follows: 2 min at 94°C, then 37 cycles of 30 sec at 94°C, 45 sec at the specific annealing temperature and 90 sec at 72°C, and finally 10 min at 72°C. All PCR products were run on a 2% agarose gel and visualized using ethidium bromide staining.

Sequencing was performed by Macrogen Inc. (Seoul, Korea). Sequences were checked with Sequencher software (version 4.2; Gene Codes, Ann Arbor, MI, USA)

and aligned with BioEdit software (version 7.0.9; Hall 2007). The resulting sequences were compared with each other and with other available sequences in the *Wolbachia* MLST profiles database on the MLST website (http://pubmlst.org/wolbachia/, Jolley *et al.* 2004), using several query functions on the website. The Allelic Profile Query was used to compare the combination of our five MLST alleles with other available allele combinations. The Similarity and Compare functions were used to compare the sequences of single alleles with other available alleles of the same gene in *Wolbachia*. For the *wsp* gene similar comparisons could be made using the *wsp* database on the MLST website.

Statistical analysis

Statistical analyses were performed in R (version 2.8.0; R Development Core Team 2008). Generalized linear models (glm) with a binomial error distribution were used to test for differences in sex ratio and infection frequency between populations. In the sex ratio model, the number of males was used as the response variable and the total number of individuals as the binomial denominator. Location was nested within habitat as an explanatory variable. Five habitats were included: Dutch dune areas (with five locations), Dutch agricultural fields (with five locations), the allotment garden, the natural area in France and the agricultural fields in the USA. To test for differences in collection method (wasps or larvae), only two habitats were considered (Dutch dunes and Dutch fields) and collection method was nested within habitat. In the infection frequency model, the number of infected females was used as the response variable and the total number of females as the binomial denominator (males were never infected). Location was nested within habitat as an explanatory variable. The same five habitats as in the sex ratio model were included. Significance of explanatory variables was tested by dropping terms from the model or lumping levels within terms and comparing the resulting change in deviance to a χ^2-distribution. This was done for each explanatory variable, until the simplest model remained, from which all non-significant terms and levels had been removed.

### Results

Field sampling

The results of the field sampling are summarized in Table 2.2. At three sites we also collected *Tetrastichus crioceris*, a close relative of *T. coeruleus* that parasitizes the spotted asparagus beetle (*Crioceris duodecimpunctata*) that also lives on asparagus plants: three individuals from the Noord-Hollandse Duinen, ten from Meijendel and one from Antwerpen.

Table 2.2: Sex ratio and *Wolbachia* infection frequency for the field samples of *Tetrastichus coeruleus*. In almost all locations adult wasps were field-caught (column 2-9), while in some locations larvae of *C. asparagi* were collected and reared in the lab (column 10-14). Column 1 shows all locations. Column 2-9 give numbers (n) and percentages (%) of field-caught adult males and females, and infected and uninfected females. Column 10 indicates the number of pupae from the collected larvae that developed wasps. Column 11-14 give numbers (n) and percentages (%) of males and females that emerged from these pupae.

Location	Males n	Males %	Females n	Females %	Inf. females n	Inf. females %	Uninf. females n	Uninf. females %	Pupae n	Males n	Males %	Females n	Females %
Noord-Hollandse Duinen	0	0	12	100	11	91.67	1	8.33	2	0	0	14	100
Kennemerduinen	0	0	18	100	18	100	0	0	7	6	10.17	53	89.83
Meijendel	0	0	60	100	59	98.33	1	1.67	16	7	6.67	98	93.33
Voornse Duinen	1	3.85	25	96.15	24	96	1	4	-	-	-	-	-
Goeree Overflakkee	0	0	15	100	14	93.33	1	6.67	-	-	-	-	-
Total Dunes (NL)	1	0.76	130	99.24	126	96.92	4	3.08	25	13	7.3	165	92.7
Drenthe	6	7.69	72	92.31	0	0	72	100	-	-	-	-	-
Gelderland	8	7.34	101	92.66	0	0	101	100	63	219	45.82	259	54.18
Brabant	9	8.11	102	91.89	0	0	58	100	102	347	43.38	453	56.63
Limburg	6	17.65	28	82.35	0	0	28	100	-	-	-	-	-
Antwerpen	0	0	22	100	0	0	22	100	-	-	-	-	-
Total Fields (NL)	29	8.19	325	91.81	0	0	281	100	165	566	44.29	712	55.71
Alphen aan den Rijn (NL)	2	12.5	14	87.5	0	0	14	100	-	-	-	-	-
Camargue (FR)	-	-	-	-	69	100	0	0	16	0	0	69	100
Massachusetts (USA)	0	0	213	100	213	100	0	0	-	-	-	-	-

Sex ratio

In four of the five Dutch dune areas only females were caught. Only in the Voornse Duinen one male was collected (Table 2.2). On four of the five Dutch agricultural fields both males and females were found, although this operational sex ratio was highly female-biased on all four fields (Table 2.2). From the agricultural field in the province of Antwerpen only females were collected. Within the habitats Dutch dune areas and Dutch agricultural fields, there was no significant difference in sex ratio between locations (deviance = 10.29, $df = 8$, $p = 0.25$). Therefore, locations within these habitats were pooled for further analyses. In the Dutch allotment garden, both males and females were collected, with a highly female-biased sex ratio (Table 2.2). Both in the natural area in the Camargue (France) and on the agricultural fields in Massachusetts (USA) only females were found (Table 2.2). There was a significant difference in sex ratio between the five habitats (Dutch dunes, Dutch agricultural fields, the allotment garden, the Camargue and Massachusetts: deviance = 42.84, $df = 4$, $p < 0.0001$; Fig. 2.2). The sex ratios within locations appeared constant between seasons and years.
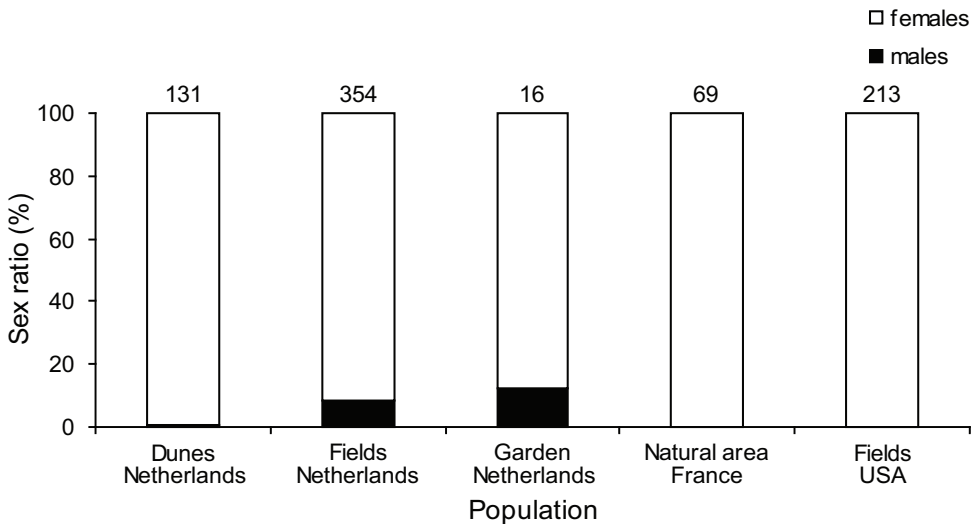
Figure 2.2: Sex ratio in five populations of *Tetrastichus coeruleus*: three field-caught populations from The Netherlands (one from five dune areas along the Dutch coast, one from five agricultural fields in the Dutch and Belgian inlands, and one from a private allotment garden), one lab-reared population from a natural area in the Camargue in southern France and one field-caught population from agricultural fields in Massachusetts (USA). Black areas represent percentage collected males and white areas represent percentage collected females. Sample sizes for each population are indicated above the bars.

The sex ratio in the Dutch dune population and the Dutch field population differed significantly from each other (deviance = 12.67, $df = 1$, $p < 0.001$). The sex ratio in the allotment garden did not differ significantly from that in the Dutch field population (deviance = 0.33, $df = 1$, $p = 0.57$), but did differ significantly from that in the Dutch dune population (deviance = 5.49, $df = 1$, $p = 0.02$). The sex ratios in France and the USA did not differ significantly from that in the Dutch dune population (France: deviance = 0.85, $df = 1$, $p = 0.36$; USA: deviance = 1.94, $df = 1$, $p = 0.16$), but differed significantly from that in the Dutch field population (France: deviance = 10.74, $df = 1$, $p = 0.001$; USA: deviance = 28.26, $df = 1$, $p < 0.0001$). The sex ratios between France and the USA did not differ significantly from each other (deviance < 0.0001, $df = 1$, $p = 0.99$).

When larvae of *C. asparagi* were collected and reared in the lab, a higher percentage of *T. coeruleus* males emerged from the pupae than when wasps were field caught. This was true for two dune areas (Kennemerduinen and Meijendel; Table 2.2) and for two agricultural fields (Gelderland and Brabant; Table 2.2). Within both habitats the sex ratios of lab-reared populations (the primary sex ratios) were significantly higher (more males) than the sex ratios of field-caught populations (the operational sex ratios) (deviance = 194.76, $df = 2$, $p < 0.0001$). Within the two habitat categories there was no significant difference in primary sex ratio between locations (deviance = 3.55, $df = 3$, $p = 0.31$), but there was a significant difference in primary sex ratio between the dune areas and the agricultural fields (deviance = 108.99, $df = 1$, $p < 0.0001$).

Wolbachia detection

Almost all females in the five Dutch dune areas were infected with *Wolbachia*. In each of four dune areas a single uninfected female was found (Table 2.2). In the Kennemerduinen only infected females were found. Unexpectedly, none of the females collected on the Dutch agricultural fields were infected with *Wolbachia* (Table 2.2). Within the habitats Dutch dune areas and Dutch agricultural fields, there was no significant difference in infection frequency between locations (deviance = 2.92, $df = 8$, $p = 0.94$). Therefore, locations within these habitats were pooled for further analyses. None of the females in the Dutch allotment garden were infected with *Wolbachia*, while both in the natural area in the Camargue (France) and on the agricultural fields in Massachusetts (USA) all females were infected (Table 2.2). There was a significant difference in infection frequency between the five habitats (Dutch dunes, Dutch agricultural fields, the allotment garden, the Camargue and Massachusetts; deviance = 927.51, $df = 4$, $p < 0.0001$; Fig. 2.3).

The infection frequencies in the Dutch dune population and the Dutch field population differed significantly from each other (deviance = 470.90, $df = 1$, $p < 0.0001$). The infection frequency in the allotment garden did not differ significantly from that in the Dutch field population (deviance < 0.0001, $df = 1$,
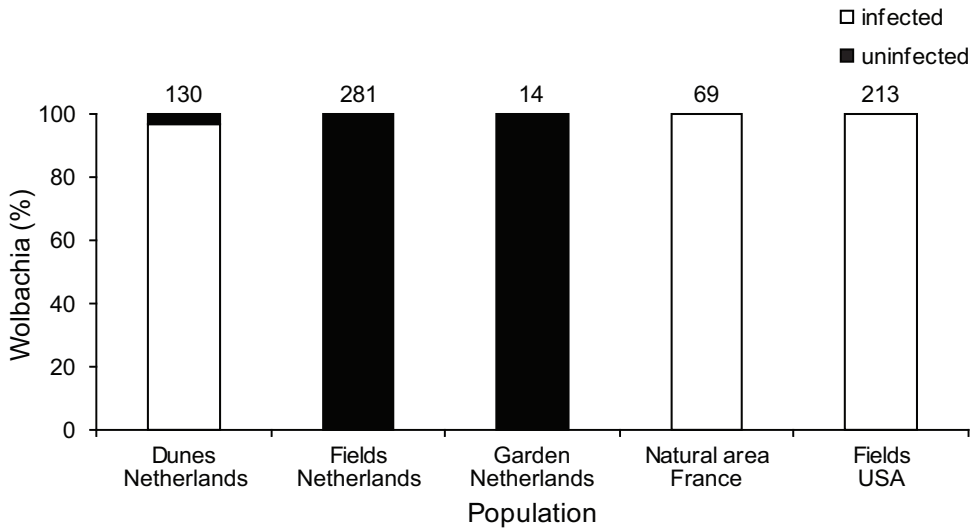
Figure 2.3: *Wolbachia* infection frequency of females in five populations of *Tetrastichus coeruleus*: three field-caught populations from The Netherlands (one from five dune areas along the Dutch coast, one from five agricultural fields in the Dutch and Belgian inlands, and one from a private allotment garden), one lab-reared population from a natural area in the Camargue in southern France and one field-caught population from agricultural fields in Massachusetts (USA). White areas represent percentage *Wolbachia*-infected females and black areas represent percentage uninfected females. Sample sizes for each population are indicated above the bars.

$p = 1$), but was significantly different from that in the Dutch dune population (deviance $= 72.78$, $df = 1$, $p < 0.0001$). The infection frequencies in France and the USA did not differ significantly from each other (deviance < 0.0001, $df = 1$, $p = 1$). The infection frequency in the USA differed significantly from that in the Dutch dune population (deviance $= 7.84$, $df = 1$, $p = 0.005$). However, the infection frequency in France did not differ significantly from that in the Dutch dune population (deviance $= 3.45$, $df = 1$, $p = 0.06$). This is probably due to the smaller sample size from the Camargue compared to that from the dune population. Both infection frequencies in France and the USA differed significantly from that in the Dutch field population (France: deviance $= 4974.0$, $df = 1$, $p < 0.001$; USA: deviance $= 15354.6$, $df = 1$, $p < 0.001$).

Wolbachia characterization

Wolbachia from three infected populations (Dutch dunes, natural area in France and agricultural fields in USA) were characterized by sequencing six *Wolbachia*-specific genes. For the five MLST genes (*gatB*, *coxA*, *hcpA*, *ftsZ* and *fbpA*) all B-type *Wolbachia*-specific alleles were amplified, while for *fbpA* also the A-type specific allele was amplified. However, both sequences for the *fbpA* gene were exactly the same. It can thus be concluded that *T. coeruleus* is infected with *Wolbachia* belonging to *Wolbachia*-supergroup B and is infected with only one *Wolbachia* strain. *Wolbachia* from the three different populations of *T. coeruleus* were genetically identical (100% similarity). This *Wolbachia* strain, which we name *wTcoer* has sequence type (ST) 37 with alleles *gatB*-9, *coxA*-9, *hcpA*-6, *ftsZ*-8 and *fbpA*-10, and *wsp*-63, with highly variable regions HVR1-19, HVR2-17, HVR3-24 and HVR4-33. The sequences belonging to *wTcoer* have been submitted to the GenBank database (accession numbers GU724211-GU724216) and to the MLST website (id 293; http://pubmlst.org/wolbachia/).

Discussion

We investigated whether the pattern found by Hoffmann *et al.* (2008) also is applicable within the species *Tetrastichus coeruleus*. According to this pattern, parthenogenetic populations should be found in agricultural environments, while sexual populations would be expected in natural environments.

The results showed that there are two main populations of *T. coeruleus* in The Netherlands. One population was found on wild asparagus plants in the dune areas. This population consisted almost exclusively of females (less than 1% males in the field and 7% males emerging from pupae) and was infected with *Wolbachia*. The populations from the five different dune areas did not differ in sex ratio or *Wolbachia* infection status. Previous work in our lab showed that females from the dune population reproduce through parthenogenesis, but that their offspring is not 100% female (B. Wielaard, pers. comm.). The other population is found on cultivated asparagus plants in agricultural fields. This population contained both males and females, although with a strongly female-biased operational sex ratio in field-caught adults (8% males). However, the primary sex ratio among the emerging wasps from *C. asparagi* larvae that were collected in the field and reared in the lab was much higher (44% males). An explanation for this difference between operational and primary sex ratio might be that males have a smaller probability of being found on the asparagus plants and are therefore less easily caught, or that females live longer than males. None of the wasps sampled from this population were infected with *Wolbachia*. Again, we found no differences between the five populations from the agricultural fields in terms of sex ratio or *Wolbachia* infection status. The population in the allotment garden resembled those in the Dutch agricultural fields: both males and females were found and none of them were infected with *Wolbachia*.

This population has probably been transported from the agricultural fields to this garden, most likely as eggs or pupae in the root stems of asparagus plants. Both the French natural population and the American agricultural field population consisted entirely of females, which were all infected with *Wolbachia*. Given the perfect match between *Wolbachia* infection status and sex ratio, we suggest that *Wolbachia* causes *T. coeruleus* to reproduce through parthenogenesis (Russell & Johnston 1912, Johnston 1915, B. Wielaard, pers. comm.).

In a recent study, Hoffmann *et al.* (2008) found a high incidence of parthenogenesis in agricultural pest species. They found that in North America and Italy 45-48% of insect agricultural pest species reproduce through parthenogenesis compared to an overall incidence of only 10-16% parthenogenesis in these genera. This difference is thought to be due to the agricultural environments being simplified, uniform and rich in resources, such that the same genotype may be continuously favoured over other genotypes. Also, agricultural fields generally are regularly cleared of all vegetation, offering an advantage to parthenogenetically reproducing species due to their greater colonization ability (Glesener & Tilman 1978, Maynard Smith 1978, Haag & Ebert 2004). The Dutch *T. coeruleus* populations show the opposite of Hoffmann *et al.*'s (2008) pattern within one species: the agricultural field population reproduced sexually, whereas the natural dune population reproduced parthenogenetically. In the French natural population, only infected females were found, similar to the Dutch natural dune population. The similarity between the Dutch and French populations suggests that the presence or absence of *Wolbachia* is due to ecological selection on either the mode of reproduction or *Wolbachia* infection. At this moment, we do not have a convincing explanation for this deviation from the general pattern of ecology and reproductive mode. It is possible that the culture of asparagus plants differs from that of other crops in ways that offer an advantage to sexual reproduction. Asparagus fields are typically used for 10 or 15 years without crop rotation, whereas other agricultural fields have a very high turn over rate of crop species. Alternatively, there may be important differences between phytophagous pest species and their natural enemies. Hoffmann *et al.* (2008) focused on phytophagous insect pests, while we studied a parasitoid. It is also possible that *Wolbachia* manipulates life history trade-offs in its host in ways that are beneficial in natural, but not in agricultural environments. *Wolbachia* has been shown to affect life history traits in several of its hosts (Alexandrov *et al.* 2007, Brownlie *et al.* 2009, Gross *et al.* 2009, Jia *et al.* 2009).

Our findings in the American agricultural fields, however, were inconsistent with the European pattern: we found exclusively females, which reproduce through parthenogenesis (Russell & Johnston 1912, Johnston 1915). The American population originates from a (presumably small) founder population that was introduced from Europe. The beetles and wasps were most likely transported as eggs or pupae in the root stems of asparagus plants. Possibly, all of the introduced individuals

of *T. coeruleus* were infected with *Wolbachia*, giving rise to a founding population that was fixed for *Wolbachia*. Although these parthenogenetic populations may have a disadvantage in the agricultural fields, no uninfected, sexual source populations were available to invade and outcompete the parthenogenetic populations. As a consequence, all of the populations of *T. coeruleus* that arose from this founding population are completely infected with *Wolbachia* and reproduce parthenogenetically. It seems likely that the asparagus plants that were introduced into America originated from agricultural fields in Europe. This suggests that *Wolbachia* used to be present in *T. coeruleus* populations on European agricultural fields, and now has disappeared. Alternatively, there may be *Wolbachia*-infected *T. coeruleus* populations on European agricultural fields that we do not know of.

Only a few other parasitoid wasp species are known in which both sexual and parthenogenetic populations occur. In the parasitoid wasps *Apoanagyrus diversicornis* (Pijls *et al.* 1996), *Telenomus nawai* (Arakaki *et al.* 2000), *Leptopilina clavipes* (Pannebakker *et al.* 2004b) and *Asobara japonica* (Kremer *et al.* 2009) infected and uninfected populations occur allopatrically. Mixed populations of infected and uninfected individuals are limited to a number of species of the genus *Trichogramma* (Stouthamer *et al.* 1990a, 1990b, 2001). In most cases of *Wolbachia*-induced parthenogenesis, the infection is fixed and the whole population reproduces parthenogenetically (Huigens & Stouthamer 2003). In the sampled French and American populations of *T. coeruleus* the *Wolbachia* infection is indeed fixed. However, the *Wolbachia* infection is not fixed in the Dutch dune population. We found several males and uninfected females (Table 2.2). The origin of these individuals is currently unclear.

A reason for the difference between the Dutch dune population and the French and American populations could have been that they are infected with different strains of *Wolbachia* that have different effects on their hosts. However, all three populations harbour exactly the same *Wolbachia* strain. There were no differences in the sequences of the five *Wolbachia*-specific MLST genes (*gatB*, *coxA*, *hcpA*, *ftsZ* and *fbpA*) or the *wsp* gene.

Another explanation for the few uninfected females and males in the dune population might be inefficient transmission of the *Wolbachia* bacteria from mother to daughter. Infected females may loose their *Wolbachia* when they become older or because of high temperatures (Cabello & Vargas 1985, Legner 1985). Eggs laid in a later stage of life or at high temperatures would then be deprived of *Wolbachia* bacteria. The temperature in the dunes can become very high in certain areas. However, in the French and American populations of *T. coeruleus* we only found females that are all infected with *Wolbachia*, suggesting that there is a very efficient transmission of *Wolbachia* in these populations, even though the summers in southern France and Massachusetts can be very hot.

In *Trichogramma*, *Wolbachia* is prevented from reaching fixation by the action

of a supernumerary B chromosome, called Paternal-Sex-Ratio (PSR) chromosome. This is a selfish genetic element that is only transmitted through males and its evolutionary interests are thus opposed to those of maternally-inherited *Wolbachia* (Werren 1991, Beukeboom & Werren 2000, Stouthamer *et al.* 2001, Werren & Stouthamer 2003). After fertilization of an egg with the sperm of a PSR-infected male, the whole paternal genome, except for PSR, forms a dense chromatin mass and is eventually lost (van Vugt *et al.* 2003). This results in a haploid egg, which develops into a male with the extra PSR chromosome (Werren 1991, Werren & Stouthamer 2003). Since the PSR chromosome only acts after fertilization of the egg and *Wolbachia* is already transmitted to the egg via the cytoplasm during egg formation, the males that would develop from these eggs should be infected with *Wolbachia* (Stouthamer *et al.* 2001). However, none of the *T. coeruleus* males were infected with *Wolbachia*. Also, the presence of a PSR chromosome in *T. coeruleus* would not explain the uninfected females in the dunes. We think it is unlikely that we will find a PSR chromosome in *T. coeruleus*.

We also discounted the possibility that *Wolbachia* in *T. coeruleus* from the Dutch dunes is infected with a bacteriophage (Gavotte *et al.* 2004) that kills the bacterium or diminishes the effect on its host *T. coeruleus*. No bacteriophage infections were found in *Wolbachia* in *T. coeruleus* (data not shown).

Perhaps the simplest explanation why *Wolbachia* is not fixed in the Dutch dunes is that the males and uninfected females we found migrated from the agricultural fields to the dune areas. If so, then migration appears to be unidirectional. No infected females were found on the agricultural fields.

Since only the B-type *Wolbachia*-specific alleles were amplified, we conclude that *T. coeruleus* is infected with a single *Wolbachia* strain belonging to *Wolbachia*-supergroup B. We name this strain *wTcoer*. Interestingly, the molecular profile of this *Wolbachia* was an exact match to that of the *Wolbachia* strain found in *Anthene emolus*, a lycaenid butterfly species from Malaysia (Russell *et al.* 2009). The presence of the same or very similar *Wolbachia* strains in very distantly related species is still poorly understood. In addition to vertical transmission, horizontal transmission seems to play an important role in the dispersal of *Wolbachia* (e.g. Vavre *et al.* 1999, Huigens *et al.* 2000, Noda *et al.* 2001, Kittayapong *et al.* 2003, Sintupachee *et al.* 2006, Raychoudhury *et al.* 2009).

In conclusion, we found that some populations of *T. coeruleus* are infected with a parthenogenesis-inducing *Wolbachia*. Infection status and mode of reproduction of European populations follow an ecological pattern, with infected, parthenogenetic populations being limited to natural areas and uninfected, sexual populations limited to agricultural fields. This is remarkable, since agricultural fields are generally thought to be simplified and favour asexual reproduction, while natural environments are considered to be complex and favour sexual reproduction.

Acknowledgements

Chapter 3

Population genetics of *Tetrastichus coeruleus* (Hymenoptera: Eulophidae) reveals occasional sex during the spread of a parthenogenesis-inducing *Wolbachia* infection

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Manuscript

Wolbachia are endosymbiotic bacteria known to manipulate the reproduction of their hosts. *Wolbachia* are maternally inherited. Therefore, vertical transmission is the main transmission mode within established hosts. However, horizontal transmission plays a major role in the spread of *Wolbachia* to novel hosts and within some newly infected host populations. In this paper, we investigate the transmission mode with which *Wolbachia* has spread through the populations of the parasitoid wasp *Tetrastichus coeruleus*.

We studied the population genetics of several populations of *T. coeruleus*, using nuclear microsatellite markers and mitochondrial DNA. Two major genetic clusters were evident: a thelytokous cluster containing all the *Wolbachia*-infected populations and an arrhenotokous cluster containing all the uninfected populations. Within the thelytokous cluster, there was no variation in mitochondrial DNA, suggesting that initially only one female became infected with *Wolbachia* and vertical transmission was the main transmission mode for *Wolbachia* spread within *T. coeruleus*. However, nuclear markers displayed considerable genetic variation, suggesting that infected females mated with males, which would introduce nuclear DNA variation into the mitochondrial lineage. Thelytokous populations showed significant genetic substructure. Within the arrhenotokous cluster, both nuclear and mitochondrial DNA variation was present, but no population structure was recognized.

Several females from otherwise thelytokous populations were uninfected and/or heterozygous for one or more microsatellite loci. No infected females were found in any of the arrhenotokous population. This suggests occasional migration from arrhenotokous to thelytokous populations, but not vice versa.

This is the first example from the field in which nuclear genetic variation in a PI-*Wolbachia*-infected host is due to occasional sex and not horizontal transmission of *Wolbachia*.

Introduction

Wolbachia are intracellular, symbiotic bacteria belonging to the order Rickettsiales within the α-Proteobacteria. It has been estimated that 66% of all insect species is (partly) infected with *Wolbachia* (Hilgenboecker *et al.* 2008). *Wolbachia* are maternally inherited, because a sperm cell contains too little cytoplasm to harbour the bacteria. Therefore, *Wolbachia* benefits from female-biased sex ratios in its hosts. To enhance its own transmission, *Wolbachia* can induce various alterations of the reproduction mechanism of its host, such as cytoplasmic incompatibility, feminization, male-killing and parthenogenesis induction (PI). *Wolbachia*-induced parthenogenesis is most commonly found in haplodiploid organisms, such as Hymenoptera (Werren 1997, Stouthamer *et al.* 1999, Werren *et al.* 2008). In uninfected haplodiploid organisms, fertilized eggs develop into diploid daughters and unfertilized eggs develop into haploid sons (arrhenotoky). In haplodiploids, PI-*Wolbachia* cause diploidization of the haploid eggs by alteration of meiotic and/or mitotic processes (Stouthamer & Kazmer 1994, Huigens & Stouthamer 2003, Pannebakker *et al.* 2004a) resulting in the production of daughters from unfertilized eggs (thelytoky).

Because *Wolbachia* are maternally inherited, vertical transmission (from mother to daughter) is the main transmission mode of *Wolbachia* within established hosts. However, horizontal transmission plays a major role in the spread of *Wolbachia* in(to) novel hosts. Horizontal transmission has been achieved experimentally both within and between species (Grenier *et al.* 1998, Heath *et al.* 1999, Huigens *et al.* 2000, 2004) and incongruence between the phylogenies of *Wolbachia* and their hosts (O'Neill *et al.* 1992, Rousset *et al.* 1992, Werren *et al.* 1995, Schilthuizen & Stouthamer 1997, Vavre *et al.* 1999) suggests widespread horizontal transmission of *Wolbachia* between hosts. Moreover, two recent field studies (Kraaijeveld *et al.* 2011a, chapter 6, Reumer *et al.* 2012) found evidence for horizontal transmission during the early stages of *Wolbachia* infection within the parasitoid wasps *Leptopilina clavipes* and *Asobara japonica*, respectively. This suggests that horizontal transmission not only plays a role in the spread of *Wolbachia* from one species to another, but also between individuals within a newly infected species. In this paper, we investigate whether horizontal transmission has played a role in the spread of a parthenogenesis-inducing-*Wolbachia* through populations of the parasitoid wasp *Tetrastichus coeruleus*.

Several scenarios for the spread of parthenogenesis-inducing-*Wolbachia* through

a novel host population are possible. Each scenario makes different predictions with regard to the patterns of genetic variation to be observed in various components of the host (meta)genome. When multiple females become infected independently by different strains of *Wolbachia*, variation in *Wolbachia* DNA and in the nuclear and mitochondrial DNA of the host would be expected. When several females become infected with the same strain of *Wolbachia* through horizontal transmission, there will be no variation in *Wolbachia* DNA, but nuclear and mitochondrial DNA should show congruent patterns of genetic variance (Kraaijeveld *et al.* 2011a, Kremer & Huigens 2011). A third possibility is that one female becomes infected and transfers the infection to her daughters through vertical transmission. No variation in the mitochondrial DNA would be expected under this scenario. However, variation in the nuclear DNA could still be observed when infected daughters occasionally reproduce sexually, so that variation in the nuclear DNA is introduced into the mitochondrial clonal lineage. Alternatively, DNA variation can arise due to novel mutations. In this case, a long period of time is necessary to accumulate mutations to give rise to considerable genetic variation and both nuclear and mitochondrial DNA should show genetic variation.

The parasitoid wasp *Tetrastichus coeruleus* (Hymenoptera: Eulophidae) forms an ideal model to investigate the spread of a parthenogenesis-inducing-*Wolbachia* infection, because it has both *Wolbachia*-infected and uninfected populations (chapter 2, Reumer *et al.* 2010). *T. coeruleus* is an egg-larval parasitoid of the common asparagus beetle (*Crioceris asparagi*). *C. asparagi* lives on the asparagus plant (*Asparagus officinalis*), which grows on sandy soils, such as coastal dune areas, and as a crop in monoculture on agricultural fields. Like all Hymenoptera, *T. coeruleus* is haplodiploid. Populations from the Dutch coastal dune areas are infected with *Wolbachia* and reproduce parthenogenetically (thelytoky), while populations from the Dutch agricultural fields are not infected and reproduce sexually (arrhenotoky) (chapter 2, Reumer *et al.* 2010). All infected populations harbour the same *Wolbachia* strain; no variation is present in *Wolbachia* DNA (chapter 2, Reumer *et al.* 2010). Infected and uninfected populations occur in close proximity, opening the possibility of inter-population dispersal and gene flow.

In this paper, we investigate the transmission mode with which the *Wolbachia* infection has spread through the populations of *T. coeruleus*. We determined the genetic variation in the nuclear and mitochondrial DNA of several populations of *T. coeruleus*. In addition, we looked for evidence for recent gene flow between the populations of *T. coeruleus*. Therefore, we paid special attention to individuals of *T. coeruleus* from all populations with unexpected characteristics, such as uninfected females or males in otherwise thelytokous populations.

Materials and Methods

Sampling locations

In the spring and summer of 2007 and 2008, eleven populations of *Tetrastichus coeruleus* were sampled throughout The Netherlands and Belgium (chapter 2, Reumer *et al.* 2010). Five thelytokous populations were sampled in coastal dune areas: Noord-Hollandse Duinen (NHD), Kennemerduinen (KD), Meijendel (MD), Voornse Duinen (VD) and Goeree Overflakkee (GO); five arrhenotokous populations were sampled from agricultural fields: Drenthe (DR), Gelderland (GL), Brabant (BR), Limburg (LB) and Antwerpen (AW); and one arrhenotokous population was sampled in a private allotment garden in Alphen aan den Rijn (AR). In addition, in the summer of 2006 a thelytokous population of *T. coeruleus* was sampled in a natural area in the Camargue, southern France (FR), and in the spring of 2008 a thelytokous population was sampled from agricultural fields and from road sides in Massachusetts, USA (MA) (chapter 2, Reumer *et al.* 2010). More details about the sampled locations can be found in chapter 2, Reumer *et al.* (2010). Although most individuals from thelytokous populations were *Wolbachia*-infected females, four uninfected females were sampled from otherwise thelytokous populations: one from the Noord-Hollandse Duinen, one from Meijendel, one from the Voornse Duinen and one from Goeree Overflakkee (chapter 2, Reumer *et al.* 2010). Also, one (uninfected) male was sampled from the Voornse Duinen (chapter 2, Reumer *et al.* 2010). From all arrhenotokous populations, except Antwerpen, both males and females were sampled, which were all uninfected (chapter 2, Reumer *et al.* 2010). All wasps were stored individually in 70% ethanol in a 1.5 ml Eppendorf tube and kept at -20°C.

DNA extraction

DNA extractions were performed using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol, using mini spin columns. Before starting the DNA extraction, each wasp was transferred to a new 1.5 ml Eppendorf tube. After evaporation of remaining ethanol, tissue lysis buffer (ATL) was added to the tube and the wasp was crushed using a plastic pestle. The tissue was incubated overnight in proteinase K at 56°C. The DNA was dissolved in 100 µl elution buffer (AE).

Table 3.1: Polymorphic microsatellite loci used for genotyping *Tetrastichus coeruleus*. For each locus, the forward and reverse primer sequences with the specific annealing temperature T_a (°C), the tandem repeat sequence and the range of allele sizes (bp) are given.

Locus	Forward primer (5' → 3')	Reverse primer (5' → 3')	T_a	Repeat	Allele size
A12a	TCG CAC TGG CAA ACT ACT G	GAC GGA TAA CTC GCA CGA C	55	GA_n	172-240
A105	AAA AAG TTC GCC GAC GAG	TTT CCA AGT GCC AAG TGC	62	GT_n	280-306
A110	CTG CAA AGT TTC AGC TTC GTA G	TTT AGG AGA ACC ATG TTC ATG C	55	AC_n	220-272
A121	CGA GAA CAC TAT CAG GAA CCA	CGT CGC TGT AGG TCA AAT C	62	$AC_n GC_n AC_n$	150-164
A122	CAA GTT TTC GTC GCT GTT AG	GTC GTT ATT TCT CCC GTC AC	54	GT_n	218-240
B106	ACA CGG ATA CAA GAG AGG AGC	CTC GGG TAG ATG AAG CAA TG	55	ATT_n	222-228
B107	GGT GGA GTT TAG GGG GTA G	AAG GAT TAG GGG GTG AGA G	59	TTA_n	302-308
B119	CGC TCA GGA TAG GGA GAG	GCG TTT ATT GGA CCT TTT TC	61	AAT_n	248-251
E4	AAA AAC GAC GAA TAC GAC TGC	GGC AAA GAA AGC CGA GAA	53	CTT_n	248-254
E9	TGC ATA CAC ACT CGA CTA CTC G	CAG GCG AAA AAT CTA TTC AAA G	56	TTC_n	149-161
E114	CGA CGA AAC ACT TCT TCA CC	TTC TCT CTG CTC GCT CAC TT	56	GAA_n	159-180
E119	GCT CCC TCT ACC AAT TTC G	GCG CTA CAT CAA GTT CAA GA	59	GAA_n	213-243

Microsatellite analysis

A *Tetrastichus coeruleus* genomic library enriched for microsatellites was constructed by Genetic Identification Services (Chatsworth, California, USA). Sequences were obtained for 68 insert containing clones. Primers were designed for 45 of these microsatellite containing DNA fragments. We tested and optimized polymerase chain reactions (PCRs) for 22 of these primers. These primer sequences have been submitted to the GenBank database (accession numbers: JN226579-JN226600). We selected 12 polymorphic microsatellite loci for genotyping (Table 3.1).

PCRs were performed in a total volume of 20.0 µl, containing 1x PCR-buffer (Qiagen), 200 µM dNTPs, 1 unit Taq polymerase, 150 nM forward primer with M13 extension, 150 nM reverse primer, 150 nM M13 primer with a fluorescent label and 2.0 µl DNA template. Forward primers were extended with the M13 universal primer at the 5'-end and M13 primers were labeled with a fluorescent dye (FAM, TAMRA or HEX) at the 5'-end (Boutin-Ganache *et al.* 2001). For microsatellite primer sequences and annealing temperatures see Table 3.1. A PTC-200 DNA Engine Thermal Cycler PCR machine (MJ Research, Waltham, MA, USA) was used for all PCRs. PCR conditions for all loci were as follows: 5 min at 94°C, then 40 cycles of 1 min at 94°C, 1 min at specific annealing temperature and 1 min at 72°C, and finally 10 min at 72°C. PCR products were checked by running them on a 2% agarose gel and visualizing them using ethidium bromide staining.

PCR fragment sizes were visualized by electrophoresis on a MegaBACE 1000 DNA Analysis System (Amersham Biosciences, Uppsala, Sweden) and scored manually using MegaBACE Fragment Profiler software (version 1.2; Amersham Biosciences). To minimise scoring errors, scoring was performed at least twice.

Deviations from the Hardy-Weinberg equilibrium and linkage disequilibrium were tested using GENEPOP software (version 4.1; Raymond & Rousset 1995, Rousset 2008). To test for Hardy-Weinberg equilibrium, we performed a probability test for each locus and each population. Weir & Cockerham's (1984) F_{IS} is reported. To test for linkage disequilibrium, we analysed pairwise associations between all loci. This analysis was repeated for each population as well as for the overall dataset. For both tests we used 10000 dememorization steps, 100 batches and 5000 iterations per batch. Males are hemizygous for each microsatellite locus and almost all females from the thelytokous populations were homozygous for all microsatellite loci. Therefore, we only used the females from the six arrhenotokous populations for these two tests.

Data were organized using GenAlEx software (version 6.4.1; Peakall & Smouse 2006), from where they were exported to other file formats for downstream analyses. GenAlEx was used to determine allele frequencies for each microsatellite locus.

We defined the number of populations or genetic clusters using STRUCTURE software (version 2.3.3; Pritchard *et al.* 2000). STRUCTURE uses a Bayesian model based clustering method to assign individual genotypes to a predefined number of genetic clusters (K). An admixture model with correlated allele frequencies was run,

using a burn-in period of 100000 steps followed by 100000 Markov Chain Monte Carlo (MCMC) steps. The model was run 20 times for each K, which was pre-defined from 1 to 17 genetic clusters (14 sampled locations + 3 extra). From the output, we determined the statistic ΔK using the method of Evanno *et al.* (2005). STRUCTURE only recognizes the uppermost hierarchical level of population structure (Evanno *et al.* 2005). Therefore, when populations were clustered within two or more genetic clusters, a new model was run for each cluster to define further structuring.

A neighbour joining tree was constructed using MEGA software (version 5; Tamura *et al.* 2011).

Weir & Cockerham's (1984) F_{ST} values were calculated using GENEPOP software (version 4.1; Raymond & Rousset 1995, Rousset 2008). Significance of F_{ST} values was tested using FSTAT software (version 2.9.3.2; Goudet 1995). Bonferroni corrections were applied to the obtained *p*-values to give table-wide significance levels of $p = 0.05$.

Mitochondrial DNA analysis

We sequenced part of the mitochondrial DNA of *T. coeruleus* from all populations. We sequenced part of the *NADH 1 dehydrogenase* (*ND1*) gene, using two different forward primers *ND1*-F1 (Smith & Kambhampati 1999) and *ND1*-F2 (Smith *et al.* 1999), both in conjunction with the same reverse primer *ND1*-R (Smith *et al.* 1999), with *ND1*-F1/*ND1*-R producing a 447 bp amplicon and *ND1*-F2/*ND1*-R producing a 430 bp amplicon. We also sequenced part of the *cytochrome oxidase 1* (*CO1*) gene, using the primers *CO1*-1775F/*CO1*-2413R and *CO1*-2222F/*CO1*-2773R (Scheffer & Grissell 2003), together producing a 991 bp amplicon.

PCRs were performed in a total volume of 25.0 µl, containing 1x PCR-buffer (Qiagen), 750 µM (extra) $MgCl_2$, 200 µM dNTPs, 1.25 units Taq polymerase, 320 nM forward primer, 320 nM reverse primer and 2.5 µl DNA template. A PTC-200 DNA Engine Thermal Cycler PCR machine (MJ Research) was used for all PCRs. PCR conditions for both genes were as follows: 5 min at 94°C, then 40 cycles of 1 min at 94°C, 1 min at 50°C and 1 min at 72°C, and finally 10 min at 72°C. All PCR products were run on a 2% agarose gel and visualized using ethidium bromide staining.

Sequencing was performed by Macrogen Inc. (Seoul, Korea). Sequences were checked with Sequencher software (version 4.2; Gene Codes, Ann Arbor, MI, USA) and aligned with BioEdit software (version 7.0.9; Hall 1999).

Haplotype diversity and nucleotide diversity were calculated using DnaSP software (version 5.10; Librado & Rozas 2009). The haplotype diversity (H_d), the nucleotide diversity (π), the average number of synonymous substitutions per synonymous site between populations (k_s) and the average number of nucleotide substitutions per site between populations (D_{xy}) were determined. Median joining

haplotype networks were drawn using Network software (version 4.6.0.0; Bandelt *et al.* 1999).

To calculate the divergence time between populations, we used the estimates for the *CO1* gene mutation rate in the parasitoid wasp *Nasonia* (Hymenoptera: Pteromalidae), described in Raychoudhury *et al.* (2010). However, mutation rates can vary considerably between species. Therefore, the calculated divergence times are only very rough estimates and must be interpreted with caution. The mitochondrial mutation rate in *Nasonia* was estimated to be 3.5 to 13 times higher than in *Drosophila melanogaster* (6.2 x 10^{-8} mutations per site per generation, Haag-Liautard *et al.* 2008), i.e. 2.2 x 10^{-7} and 8.1 x 10^{-7} mutations per site per generation (Raychoudhury *et al.* 2010). The divergence time (in generations) between two populations can be calculated by dividing k_s (the average number of synonymous substitutions per synonymous site between populations) by this mutation rate (the number of mutations per site per generation).

Comparison between microsatellites and mitochondrial DNA

We tested whether the variance in nuclear and mitochondrial DNA corresponded to each other. Two pairwise matrices were constructed: one for the nuclear microsatellite markers with the F_{ST} values as a measure of genetic distance and one for the mitochondrial DNA with the number of nucleotide differences between sequences. The two matrices were compared with each other using a Mantel test from the EcoDist package in R statistical software (version 2.12.1; R Developmental Core Team, 2010).

Results

Microsatellite analysis

In total, 255 females from 14 different locations were genotyped at 12 microsatellite loci (Table 3.2). In addition, 32 males from 6 locations were genotyped. Because males are haploid, they are hemizygous for each locus. Most females from the thelytokous populations were homozygous for all loci. Six females from the thelytokous populations were heterozygous for one or more loci (Table 3.3): three *Wolbachia*-infected females and three uninfected females. The uninfected female from the Noord-Hollandse Duinen (NHD9) was homozygous for all loci (Table 3.3). All females from the arrhenotokous populations were heterozygous for at least one locus (Fig. 3.1).

Within several arrhenotokous populations, several loci deviated significantly from the Hardy-Weinberg equilibrium: A121 in Drenthe ($F_{IS} = 0.4947$, $p = 0.0268$), E4 in Limburg ($F_{IS} = 0.2845$, $p = 0.0225$), E114 in Alphen aan den Rijn ($F_{IS} = 0.3988$, $p = 0.0296$) and E119 in Drenthe ($F_{IS} = 0.0426$, $p = 0.0453$). The locus B119 deviated from the Hardy-Weinberg equilibrium in all arrhenotokous populations (all $F_{IS} > 0.79$, all p-values < 0.001; overall χ^2 goes to infinity, $df = 12$, $p < 0.0001$).

Table 3.2: The number of genotyped females and males of *Tetrastichus coeruleus* per location used for microsatellite analysis. The number of *Wolbachia*-infected individuals is given between brackets. For a map of the locations see chapter 2, Reumer *et al.* (2010).

Sampled location	Females	Males
Noord-Hollandse Duinen	12 (11)	0 (0)
Kennemerduinen	18 (18)	0 (0)
Meijendel	21 (20)	0 (0)
Voornse Duinen	21 (20)	1 (0)
Goeree Overflakkee	15 (14)	0 (0)
Drenthe	20 (0)	6 (0)
Gelderland	20 (0)	8 (0)
Brabant	20 (0)	9 (0)
Limburg	20 (0)	6 (0)
Antwerpen	20 (0)	0 (0)
Alphen aan den Rijn	14 (0)	2 (0)
Camargue (FR)	20 (20)	0 (0)
Massachusetts roads (USA)	19 (19)	0 (0)
Massachusetts fields (USA)	15 (15)	0 (0)
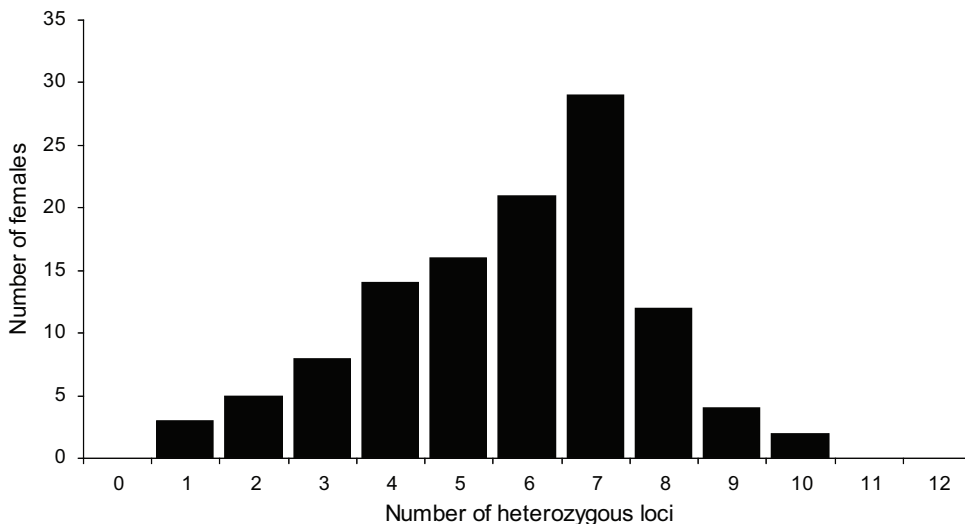
Figure 3.1: Frequency of number of heterozygous microsatellite loci for all females in six arrhenotokous populations of *Tetrastichus coeruleus*.

Table 3.3: Females from otherwise thelytokous populations of *Tetrastichus coeruleus* with unexpected genetic characteristics. Code = the individual code, Sampled location = the location of origin, *Wolbachia* = *Wolbachia* infection (yes or no), Heterozygous loci = when heterozygous for one or more microsatellite loci, the heterozygous loci are given, Cluster assignment = assignment by STRUCTURE to a specific genetic cluster (thelytokous or arrhenotokous), with the assignment probability between brackets.

Code	Sampled location	*Wolbachia*	Heterozygous loci	Cluster assignment
Expected		Yes	None	Thelytokous
NHD9	Noord-Hollandse Duinen	No	None (homozygous)	Thelytokous (99.50%)
KD18	Kemmerduinen	Yes	A12a, A105, A110, B106	Thelytokous (80.22%)
MD16	Meijendel	No	A105, A110, A121, B106, B107	Arrhenotokous (96.60%)
VD11	Voornse Duinen	No	A105, A110, A122	Arrhenotokous (98.87%)
GO1	Goeree Overflakkee	Yes	A105	Thelytokous (92.28%)
GO2	Goeree Overflakkee	No	A105, B106, E9, E119	Thelytokous (58.43%)
GO13	Goeree Overflakkee	Yes	A110, A121, A122, B106, B107, E114	Thelytokous (57.16%)

Overall, only in locus B119 was the deviation from the Hardy-Weinberg equilibrium severe, perhaps due to the presence of null alleles. Therefore, we discarded the locus B119 from all further analyses.

Within several arrhenotokous populations, several locus pairs were significantly associated to each other (Drenthe: A122 & B107, A122 & E4, B119 & E114; Gelderland: A122 & B107, A121 & B119; Brabant: A110 & A122, A110 & B107, A122 & B107, B106 & E119; Limburg: A110 & A122, A122 & B107, A105 & E4; Antwerpen: A121 & B107, A122 & B107, A121 & E9; Alphen aan den Rijn: A110 & A122, A122 & B107, A12a & B119; all *p*-values < 0.05). Across all arrhenotokous populations, three locus pairs were associated significantly to each other: A110 & A122 ($\chi^2 = 27.8673$, *df* = 10, *p* = 0.0019), A110 & B107 ($\chi^2 = 27.3817$, *df* = 10, *p* = 0.0023) and A122 & B107 (χ^2 goes to infinity, *df* = 12, *p* < 0.0001). Overall, the three loci A110, A122 and B107 were consistently linked to each other. Because the locus pair A122 & B107 gave the most significant association, we discarded both A122 and B107 from all further analyses.

STRUCTURE analysis showed that there were two genetic clusters at the uppermost hierarchical level ($\Delta K = 843.50$; Fig. 3.2A). One cluster was formed by the six arrhenotokous populations, while the other cluster was formed by the eight thelytokous populations. Most females were assigned to the expected cluster with more than 90% probability. However, eight females were assigned to the expected cluster with lower probabilities (Table 3.3; to the thelytokous cluster: three *Wolbachia*-infected females: KD18 with 80.22%, GO13 with 57.16% and FR13 with 80.70%, and one uninfected female: GO2 with 58.43%; to the arrhenotokous cluster: four uninfected females: GL14 with 89.33%, BR3 with 65.91%, LB1 with 77.08% and AW5 with 88.39%). Also, two uninfected females from thelytokous populations were assigned to the arrhenotokous cluster (Table 3.3; MD16 with 96.60% and VD11 with 98.87% probability). The uninfected female from the Noord-Hollandse Duinen (NHD9) was assigned with 99.50% probability to the thelytokous cluster (Table 3.3). The heterozygous *Wolbachia*-infected female from Goeree Overflakkee (GO1) was assigned with 92.28% probability to the thelytokous cluster (Table 3.3). Within the thelytokous cluster, four genetic clusters could be distinguished ($\Delta K = 42.84$; Fig. 3.2B). Within the arrhenotokous cluster, no further structuring was recognized (highest $\Delta K = 6.48$ at $K = 3$; Fig. 3.2C). When the STRUCTURE model was run a second time, similar results were obtained. Allele frequencies for each locus within the two genetic clusters (thelytokous and arrhenotokous) are given in Fig. 3.3 and Fig. 3.4.

The genetic clusters as recognized by STRUCTURE corresponded to the graphical representation of the data as an unrooted neighbour joining tree by MEGA (for clarity the tree was divided in two, Fig. 3.5 & Fig. 3.6). In the thelytokous part of tree (Fig. 3.5), females from the same location clustered together, while in the arrhenotokous part of the tree (Fig. 3.6) they did not. According to MEGA, three uninfected females from arrhenotokous populations (BR15, BR19 and LB1)
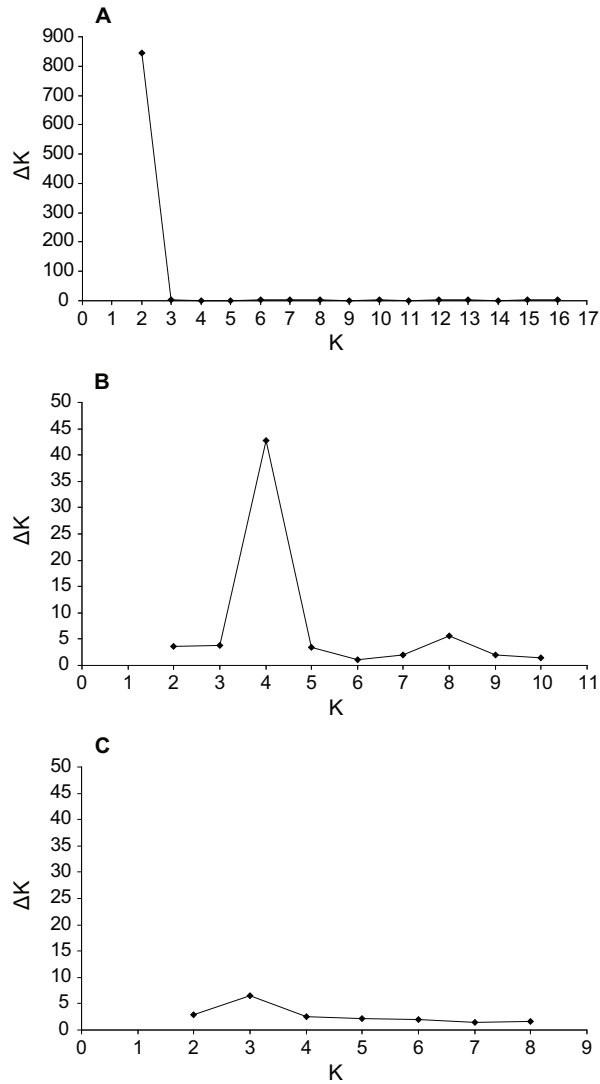
Figure 3.2: STRUCTURE results for three models. The ad hoc statistic ΔK estimates the number of genetic clusters (K). The modal value of K indicates the number of genetic clusters. A: ΔK for each of 1 to 17 predefined populations (K) for all females from 14 arrhenotokous and thelytokous populations of *Tetrastichus coeruleus*. B: ΔK for each of 1 to 11 predefined populations (K) for all females from 8 thelytokous populations of *T. coeruleus*. C: ΔK for each of 1 to 9 predefined populations (K) for all females from 6 arrhenotokous populations of *T. coeruleus*.

were more related to individuals from thelytokous populations than to individuals from their own populations. STRUCTURE however, assigned all three females to the arrhenotokous cluster (BR15 with 92.19%, BR19 with 94.78% and LB1 with 77.08% probability). According to MEGA, two uninfected females from thelytokous populations (MD16 and VD11) were more related to individuals from arrhenotokous populations than to individuals from their own populations, which corresponded to the cluster assignment by STRUCTURE (Table 3.3). Also, according to MEGA, the other two uninfected females from thelytokous populations (NHD9 and GO2) were closely related to individuals from their own sampling location, which corresponded to the assignment results by STRUCTURE (Table 3.3).

The two genetic clusters (thelytokous and arrhenotokous) as recognized by STRUCTURE differed significantly from each other ($F_{ST} = 0.3109$, $p < 0.001$). All pairwise population F_{ST} values and their significance are shown in Table 3.4. Within the thelytokous cluster, most populations differed significantly from each other ($F_{ST} = 0.5482$). However, there were no significant differences between the populations from the Voornse Duinen and Goeree Overflakkee and between the two populations from Massachusetts (USA). Within the arrhenotokous cluster, few differences were found between populations ($F_{ST} = 0.0332$). However, the population from Drenthe differed significantly from all populations, but not from Gelderland. Also, there were significant differences between the populations from Brabant and Limburg and between the populations from Antwerpen and Alphen aan den Rijn.

Most arrhenotokous males exhibited alleles for all microsatellite loci that were also present in the females from their own sampling location. Twelve males had alleles for one or two loci that were not present within their own sampling location. From eleven males, these alleles were present within one or more of the other arrhenotokous populations. However, two males had a new allele that was not present in any other population: one male from Drenthe had a new allele for the marker A121 with a length of 162 bp and one male from Gelderland had a new allele for the marker A12a with a length of 218 bp. Only seven microsatellite loci could be genotyped for the male from the thelytokous population from the Voornse Duinen (A12a: 200 bp, A110: 228 bp, A122: 218 bp, B119: 248 bp, E4: 251 bp, E9: 149 bp, E114: 159 bp). The allele of marker B119 with a length of 248 bp was also present in the females from the Voornse Duinen. The other six alleles were not present in the Voornse Duinen or in any other thelytokous population. The alleles of the markers A12a, A110 and E4 were present in one or more of the arrhenotokous populations. The alleles of the markers A122, E9 and E114 were new for all populations.
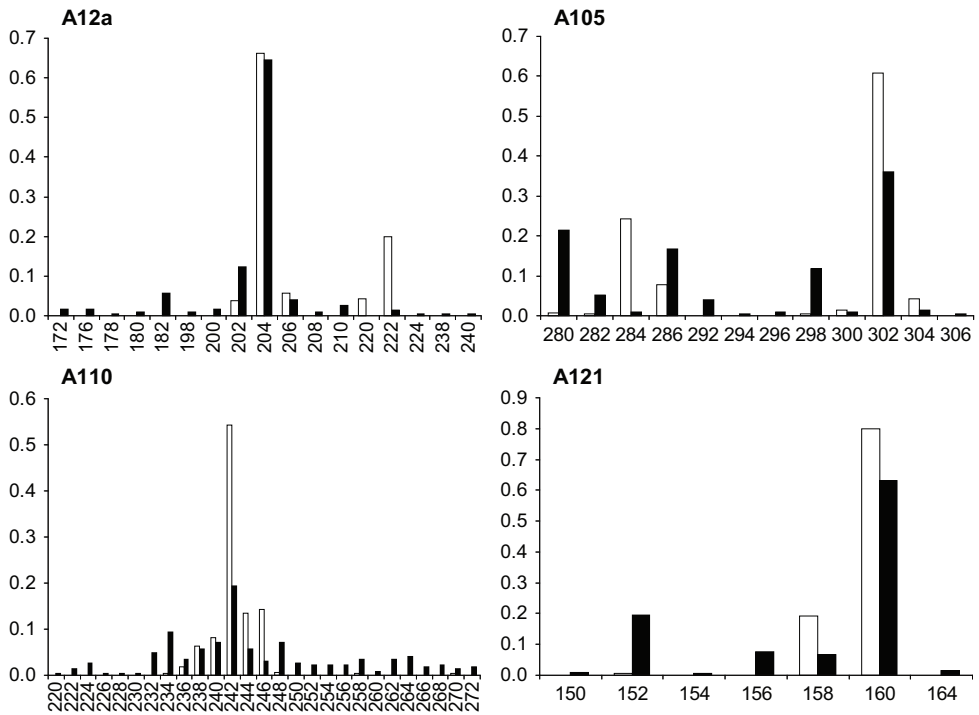
Figure 3.3: Allele frequencies of four of the nine polymorphic microsatellite markers used for genotyping *Tetrastichus coeruleus*. White bars represent allele frequencies in eight thelytokous populations; black bars represent allele frequencies in six arrhenotokous populations. Allele sizes are indicated at the x-axis for each microsatellite locus.
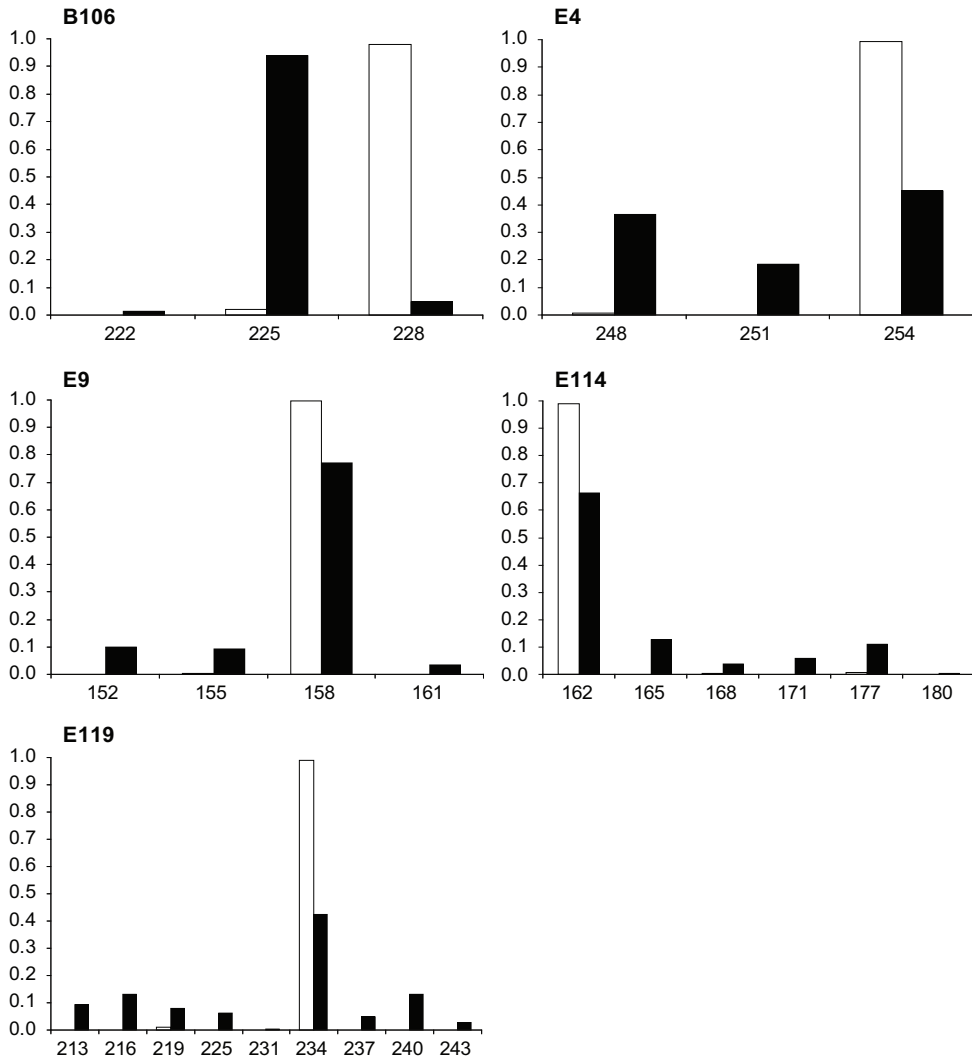
Figure 3.4: Allele frequencies of five of the nine polymorphic microsatellite markers used for genotyping *Tetrastichus coeruleus*. White bars represent allele frequencies in eight thelytokous populations; black bars represent allele frequencies in six arrhenotokous populations. Allele sizes are indicated at the x-axis for each microsatellite locus.
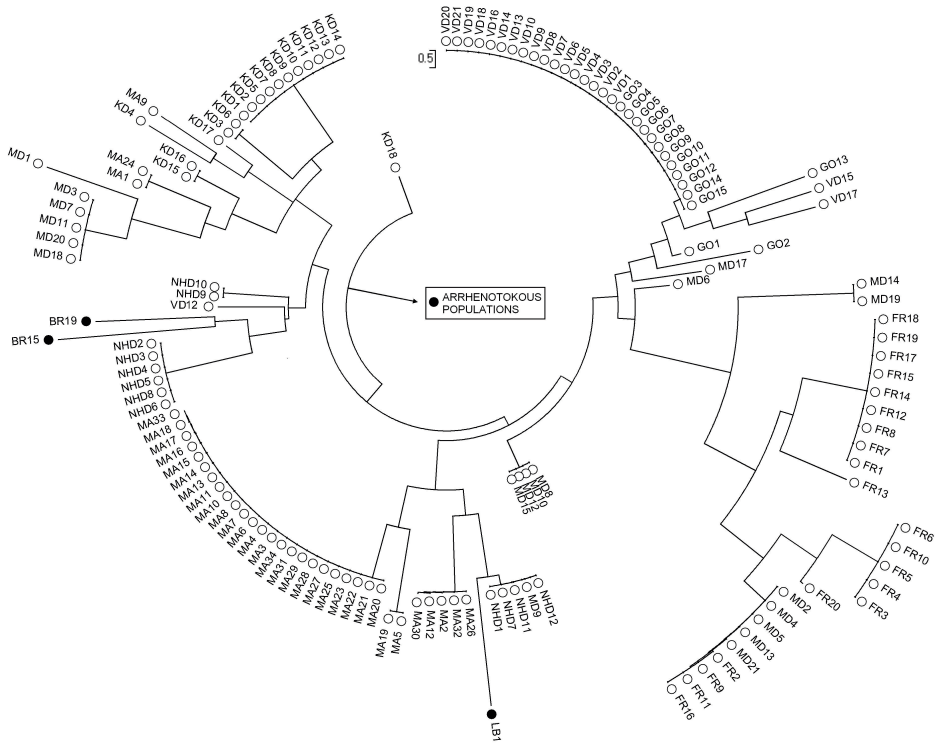
Figure 3.5: Thelytokous genetic cluster of *Tetrastichus coeruleus*. Unrooted neighbour joining tree based on nine polymorphic microsatellite markers. Each circle represents one female. White circles indicate individuals from thelytokous populations (NHD = Noord-Hollandse Duinen, KD = Kennemerduinen, MD = Meijendel, VD = Voornse Duinen, GO = Goeree Overflakkee, FR = Camargue (France), MA = Massachusetts (USA)); black circles indicate individuals from arrhenotokous populations (BR = Brabant, LB = Limburg). The length of the branches indicates genetic distance between individuals.
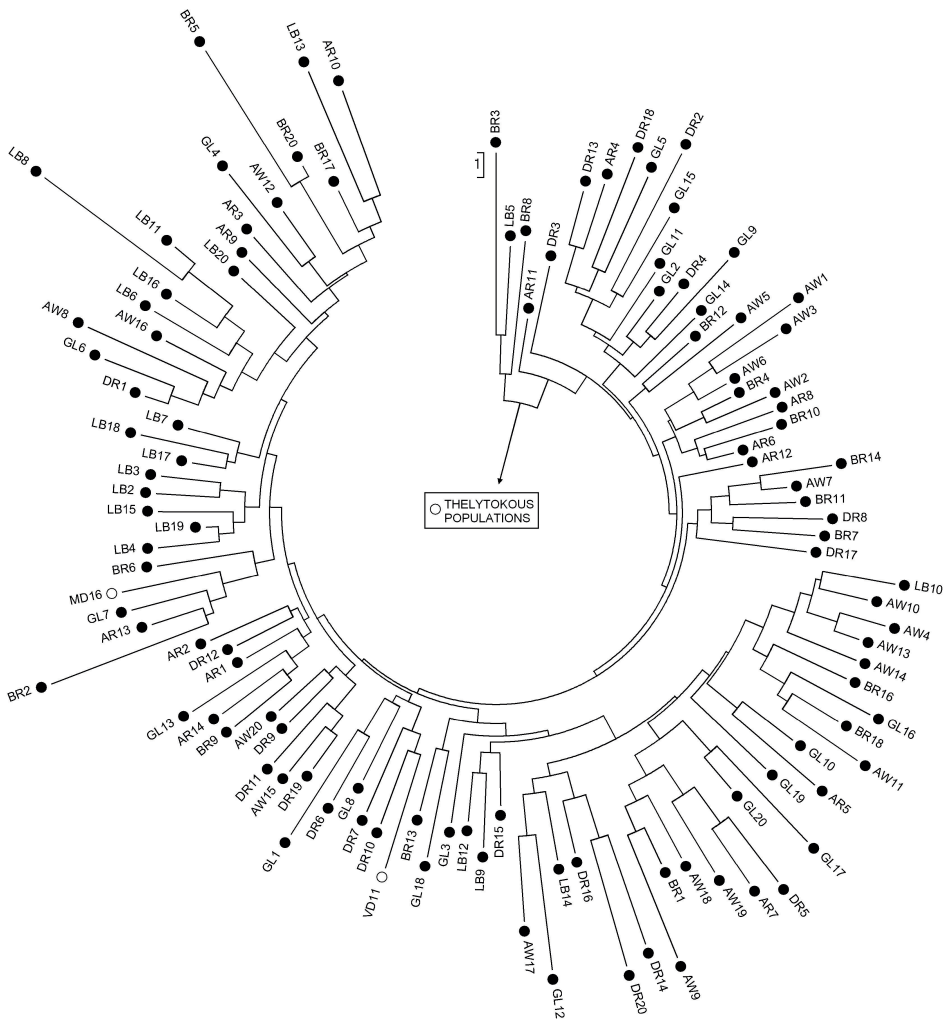
Figure 3.6: Arrhenotokous genetic cluster of *Tetrastichus coeruleus*. Unrooted neighbour joining tree based on nine polymorphic microsatellite markers. Each circle represents one female. Black circles indicate individuals from arrhenotokous populations (DR = Drenthe, GL = Gelderland, BR = Brabant, LB = Limburg, AW = Antwerpen, AR = Alphen aan den Rijn); white circles indicate individuals from thelytokous populations (MD = Meijendel, VD = Voornse Duinen). The length of the branches indicates genetic distance between individuals.

Table 3.4: Matrix of pairwise genetic differentiation between all populations of *Tetrastichus coeruleus* (NHD = Noord-Hollandse Duinen, KD = Kennemerduinen, MD = Meijendel, VD = Voornse Duinen, GO = Goeree Overflakkee, FR = Camargue (France), MA1 = Massachusetts (USA) roads, MA2 = Massachusetts (USA) fields, DR = Drenthe, GL = Gelderland, BR = Brabant, LB = Limburg, AW = Antwerpen, AR = Alphen aan den Rijn). F_{ST} values are shown in the lower triangle; significance of these F_{ST} values after Bonferroni correction are shown in the upper triangle (*** corresponds to significance at the 0.1% nominal level, ** corresponds to significance at the 1% nominal level and * corresponds to significance at the 5% nominal level, NS stands for not significant).

|	NHD	KD	MD	VD	GO	FR	MA1	MA2	DR	GL	BR	LB	AW	AR
NHD	-	***	*	***	***	***	***	***	***	***	***	***	***	***
KD	0.3839	-	**	***	***	***	***	***	***	***	***	***	***	***
MD	0.1765	0.2083	-	***	***	***	***	***	***	***	***	***	***	***
VD	0.5067	0.631	0.3783	-	NS	***	***	***	***	***	***	***	***	***
GO	0.5358	0.6702	0.3858	0	-	***	***	***	***	***	***	***	***	***
FR	0.5952	0.5484	0.3131	0.7249	0.7446	-	***	***	***	***	***	***	***	***
MA1	0.5389	0.6298	0.353	0.6973	0.7241	0.7449	-	NS	***	***	***	***	***	***
MA2	0.563	0.6555	0.3509	0.7313	0.7674	0.7667	0	-	***	***	***	***	***	***
DR	0.3367	0.375	0.3017	0.4502	0.4313	0.4651	0.4551	0.4439	-	NS	***	***	***	**
GL	0.3416	0.37	0.2931	0.3968	0.4322	0.4265	0.4764	0.4643	0.0055	-	NS	*	NS	***
BR	0.2769	0.3578	0.27	0.45	0.3716	0.4236	0.4205	0.4089	0.0489	0.0304	-	NS	NS	***
LB	0.3478	0.4179	0.3278	0.4461	0.4274	0.4864	0.4954	0.4855	0.0744	0.054	0.0135	-	NS	***
AW	0.3465	0.4255	0.3253		0.4215	0.4803	0.495	0.4848	0.0572	0.0275	0.0105	0.0245	-	**
AR	0.3345	0.4285	0.305	0.5007	0.4787	0.4836	0.5077	0.4957	0.0326	0.0218	0.0124	0.0419	0.0315	-

Mitochondrial DNA analysis

We sequenced part of the mitochondrial DNA of 13 females, representing seven thelytokous and six arrhenotokous populations of *T. coeruleus*. The primers *ND1*-F1/*ND1*-R amplified DNA from all arrhenotokous populations, while the primers *ND1*-F2/*ND1*-R amplified DNA from all thelytokous populations. For further analyses therefore, we used the sequences obtained with *ND1*-F1/*ND1*-R for all arrhenotokous populations and the sequences obtained with *ND1*-F2/*ND1*-R for all thelytokous populations. The primers *CO1*-1775F/*CO1*-2413R and *CO1*-2222F/*CO1*-2773R yielded sequences for all populations. The sequences have been submitted to the GenBank database (*CO1* accession numbers: JN226601-JN226613; *ND1* accession numbers: JN226614-JN226626).

Based on the combined *CO1* and *ND1* gene sequences, we found seven unique mitochondrial haplotypes ($H_d = 0.7949$; Fig. 3.7). The seven haplotypes contained 60 polymorphic sites ($\pi = 0.0181$; 57 synonymous and 4 non-synonymous mutations). Arrhenotokous and thelytokous populations did not share haplotypes. Among the arrhenotokous populations we found five haplotypes ($H_d = 0.9333$) with 26 polymorphic sites ($\pi = 0.0070$; 26 synonymous and 1 non-synonymous mutations). The two females from the populations from Brabant and Antwerpen shared the same haplotype, while all four females from the other arrhenotokous populations exhibited unique haplotypes. Among the thelytokous populations, we found two haplotypes ($H_d = 0.2857$) with only one polymorphic site ($\pi = 0.0002$; 1 synonymous mutation). The female from the population from the Voornse Duinen exhibited a unique haplotype, while all females from the other thelytokous populations shared the same haplotype. There were on average 44 nucleotide differences between the arrhenotokous and thelytokous populations ($k_s = 0.1470$; $D_{xy} = 0.0310$).

Within the *CO1* gene sequence, the seven haplotypes could be distinguished ($H_d = 0.7949$). The *CO1* gene contained 48 polymorphic sites ($\pi = 0.0206$; 46 synonymous and 3 non-synonymous mutations). Among the arrhenotokous populations, we found five haplotypes ($H_d = 0.9333$) with 21 polymorphic sites ($\pi = 0.0084$; 22 synonymous mutations), while among the thelytokous populations, we found two haplotypes ($H_d = 0.2857$) with one polymorphic site ($\pi = 0.0003$; 1 synonymous mutation). There were on average 35 nucleotide differences between the arrhenotokous and thelytokous populations ($k_s = 0.1635$; $D_{xy} = 0.0351$).

Within the *ND1* gene sequence, only four haplotypes were found ($H_d = 0.6539$). The *ND1* gene contained 12 polymorphic sites ($\pi = 0.0122$; 11 synonymous and 1 non-synonymous mutations). Among the arrhenotokous populations, we found three haplotypes ($H_d = 0.6000$) with five polymorphic sites ($\pi = 0.0039$; 4 synonymous and 1 non-synonymous mutations). The four females from the populations from Gelderland, Brabant, Limburg and Antwerpen shared the same haplotype, while the two female from the populations from Drenthe and Alphen aan den Rijn both exhibited unique haplotypes. All females from the thelytokous populations shared
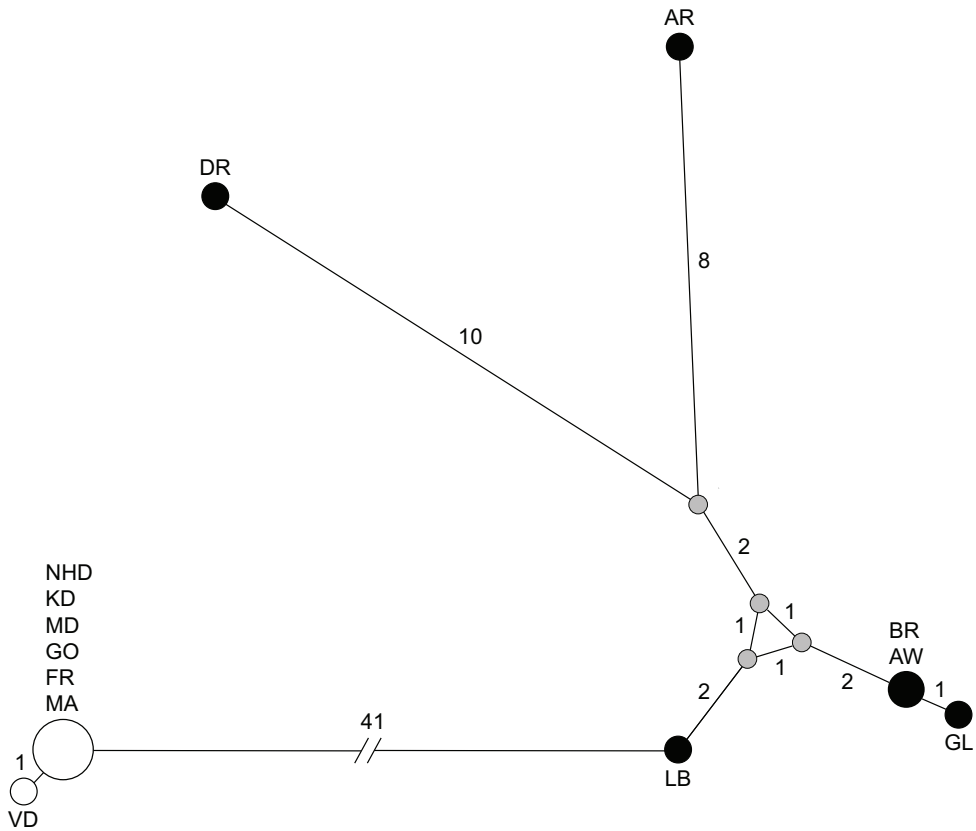
Figure 3.7: Haplotype network based on 991 bp of the mitochondrial *CO1* gene and 430 bp of the mitochondrial *ND1* gene of *Tetrastichus coeruleus*. Each circle represents one haplotype. The size of the circle represents the haplotype frequency. White circles indicate haplotypes found in thelytokous populations; black circles indicate haplotypes found in arrhenotokous populations; grey circles indicate intermediate haplotypes that were not sampled. Population names are given for the haplotype in which they were found (NHD = Noord-Hollandse Duinen, KD = Kennemerduinen, MD = Meijendel, VD = Voornse Duinen, GO = Goeree Overflakkee, FR = Camargue (France), MA = Massachusetts (USA), DR = Drenthe, GL = Gelderland, BR = Brabant, LB = Limburg, AW = Antwerpen, AR = Alphen aan den Rijn). Lines show mutational routes between haplotypes. The length of the lines and the numbers on the lines indicate the number of mutations between haplotypes.

the same haplotype ($H_d = 0.0000$); there were no nucleotide differences ($\pi = 0.0000$). There were on average 9 nucleotide differences between the arrhenotokous and thelytokous populations ($k_s = 0.1083$; $D_{xy} = 0.0213$).

The mean divergence time, based only on the *CO1* gene, between the arrhenotokous and thelytokous populations ($k_s = 0.1635$) was estimated to be between 7.5 x 10^5 and 2.0 x 10^5 generations ago.

Comparison between microsatellites and mitochondrial DNA

There was no significant correlation between the pairwise F_{ST} values in the nuclear microsatellite matrix and the pairwise nucleotide differences in the mitochondrial DNA matrix (Mantel $r = 0.0589$, $p = 0.5740$). Also, there was no significant correlation between the two matrices among the thelytokous populations (Mantel $r = 0.0324$, $p = 1.0000$) and among the arrhenotokous populations (Mantel $r = 0.2981$, $p = 0.3600$).

Discussion

We investigated the transmission mode with which the *Wolbachia* infection has spread through the populations of *T. coeruleus*. Also, we searched for evidence of gene flow between arrhenotokous and thelytokous populations of *T. coeruleus*.

The results showed the presence of two major genetic clusters. Both nuclear microsatellite markers and mitochondrial DNA showed a clear distinction between the thelytokous and arrhenotokous populations. The mean divergence time between the arrhenotokous and thelytokous populations was estimated to be between 7.5 x 10^5 and 2.0 x 10^5 generations ago. Almost all females from thelytokous populations were homozygous for all microsatellite loci, which was expected from the known cellular mechanisms of *Wolbachia*-induced parthenogenesis (Stouthamer & Kazmer 1994, Huigens & Stouthamer 2003, Pannebakker *et al.* 2004a). All females from the arrhenotokous cluster were heterozygous for at least one locus.

Within the thelytokous cluster, two mitochondrial haplotypes could be recognized. All thelytokous populations exhibited the same haplotype, except the population from the Voornse Duinen, which differed by one base pair from the rest. The microsatellites showed no nuclear genetic distinction between the populations from the Voornse Duinen and Goeree Overflakkee, suggesting that the difference in mitochondrial DNA arose as a novel mutation. Because mitochondrial DNA is maternally inherited, the absence of mitochondrial DNA variation suggests that a single female was the ancestor of all thelytokous populations we examined. Because almost all females from thelytokous populations are infected with *Wolbachia* (chapter 2, Reumer *et al.* 2010), this ancestral female must also have been infected. Together with the mitochondrial DNA, the *Wolbachia* infection has spread through the populations from mothers to daughters. Under the horizontal transmission scenario, we would have expected to find several distinct mitochondrial clones, as is

the case in the parasitoid wasps *Leptopilina clavipes* (Kraaijeveld *et al.* 2011a) and *Asobara japonica* (chapter 6, Reumer *et al.* 2012).

Although there was no variation in mitochondrial DNA among the thelytokous populations, there was considerable variation in nuclear DNA. Within the thelytokous genetic cluster, individuals from the same population were more closely related to each other than to individuals from other thelytokous populations and all thelytokous populations differed significantly from each other (except the populations from the Voornse Duinen and Goeree Overflakkee, and the two populations from Massachusetts). The genetic variation in nuclear DNA suggests that infected females mated with males during the spread of the *Wolbachia* infection, introducing nuclear DNA variation into the mitochondrial clonal lineage. Different nuclear clones may subsequently have become dominant in each population. In principle, females may still occasionally reproduce sexually. The differences in nuclear DNA between the different populations may be the result of extensive recent backcrossing of infected females with genetically distinct arrhenotokous males. However, this seems unlikely given that the extant arrhenotokous populations showed no genetic substructuring and no differences between thelytokous populations would be expected. Also, most of the females from thelytokous populations were homozygous, suggesting that they did not mate with genetically different males. Moreover, thelytokous females can not be induced to mate with arrhenotokous males in the lab (chapter 5, Reumer *et al.* in prep). Also, within each thelytokous population the nuclear genetic variation is small. Alternatively, the differences in nuclear DNA could be the result of novel mutations. However, it seems unlikely that all variation is due to novel mutations because there is no congruence between the nuclear and mitochondrial DNA variation. We conclude that while the *Wolbachia* infection was spreading through the populations of *T. coeruleus*, infected females mated with males, so that nuclear DNA variation was introduced into the mitochondrial clonal lineage.

Occasional or historical sex by *Wolbachia*-infected females raises the possibility of the spread of 'functional virginity mutations' as proposed by Stouthamer *et al.* (2010). When a parthenogenesis-inducing endosymbiont enters a population the population sex ratio will become more female-biased. Males will have a higher fitness than before, because there are more females to mate with. Some of these females will be infected, while others are not. Females that produce more males will have a higher fitness than females that produce fewer males. Because males develop from unfertilized haploid eggs, females that do not fertilize their eggs will thus have a higher fitness than females that do fertilize their eggs. Mutations that cause females not to fertilize their eggs, for example by not mating or not using the sperm, will therefore have an initial selective advantage. These so called 'virginity mutations' paradoxically must spread through the population via sexual reproduction from males to both infected and uninfected females. In the end, this will result in a population fixed for the *Wolbachia* infection (i.e. all females are infected) and the

virginity mutation (i.e. none of the females fertilizes her eggs when she mates with a male). The nuclear DNA variation in infected *T. coeruleus* populations suggests that females mated with males, thereby facilitating the possible spread of 'virginity mutations'. The reluctance of thelytokous *T. coeruleus* females to mate (chapter 5, Reumer *et al.* in prep) may be the result of such mutations.

Within the thelytokous genetic cluster, several individuals exhibited unexpected characteristics. Four females from four otherwise thelytokous populations were not infected with *Wolbachia*. These females may be uninfected either because the *Wolbachia* transmission from mother to daughter is not always 100% or because they immigrated from an arrhenotokous population. We found evidence for both scenarios. Six females from four thelytokous populations were heterozygous for one or more microsatellite loci, while all other females from thelytokous populations were homozygous for all loci. Two of these females (MD16 and VD11) were uninfected and assigned with high probabilities to the arrhenotokous cluster. The third uninfected, heterozygous female (GO2) was assigned to the thelytokous genetic cluster. However, the assignment probability was low. Therefore, we conclude that these three female migrated from an arrhenotokous population to the thelytokous populations in which they were found. The fourth uninfected female (NHD9) was homozygous for all loci and was assigned with high probability to the thelytokous cluster. This female probably is uninfected due to inefficient transmission of *Wolbachia* from mother to daughter. The last three heterozygous females were infected with *Wolbachia* and were assigned to the thelytokous genetic cluster. One of these females was heterozygous for only one locus and was assigned with high probability. In this case, the heterozygous genotype was probably due to a novel mutation. However, the other two infected, heterozygous females were heterozygous for four and six loci, respectively, and the assignment probabilities for the thelytokous cluster were low. These females may be the offspring of an infected female that had mated with a genetically different male and fertilized her eggs. Such a male may have migrated from an arrhenotokous population. This possibility is strengthened by the observation of a male in the thelytokous population from the Voorne Duinen, which exhibited alleles that were not present in any of the thelytokous populations, but that were found in the arrhenotokous populations. This male also harboured alleles that were new to all populations. This male must have migrated from an arrhenotokous population to the Voorne Duinen. No infected females were found in any of the arrhenotokous populations. Overall, there is very occasional migration from arrhenotokous populations to thelytokous populations, but not vice versa. This might explain why the *Wolbachia* infection, until now, never has arrived in the Dutch agricultural fields.

Although both the mitochondrial and nuclear DNA showed a clear distinction between the thelytokous and arrhenotokous populations, there was no correlation between the two data sets. Within the thelytokous genetic cluster, there was no

variation in mitochondrial DNA, but there was variation in nuclear DNA between populations. This incongruence not only explains the lack of correlation between mitochondrial and nuclear DNA among the thelytokous populations, but also among all populations. Nuclear and mitochondrial DNA variation were also uncorrelated in the arrhenotokous cluster, despite extensive variation in both mitochondrial and nuclear DNA. This probably is caused by the lack of population structure within the arrhenotokous cluster.

Based on the nuclear and mitochondrial DNA, we conclude that in the early stages of infection *Wolbachia* has spread through vertical transmission and not through horizontal transmission between individuals and populations of *T. coeruleus*. During this period, females mated with males, so that nuclear DNA variation was introduced into the mitochondrial clonal lineage. The early spread of *Wolbachia* may have been facilitated by 'virginity mutations'. Now, there is occasional migration from arrhenotokous populations to thelytokous populations, but not vice versa. Although infected *T. coeruleus* females can not be induced to mate in the lab, we presented evidence for two possible cases of recent matings between thelytokous females and arrhenotokous males. This is the first example from the field in which nuclear genetic variation in a PI-*Wolbachia*-infected host is due to occasional sex and not horizontal transmission of *Wolbachia*.

Acknowledgements

Chapter 4

Life history traits in populations of *Tetrastichus coeruleus* (Hymenoptera: Eulophidae) that differ in ecology, *Wolbachia* infection frequency and mode of reproduction

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Manuscript

Life history strategies are characterized by trade-offs, in particular between investment in reproduction and survival. Differences in life history traits between populations may reflect adaptive strategies that optimize reproduction and survival in relation to their respective environments. Furthermore, life history strategies may vary with the mode of reproduction or may be manipulated by parasites. Quantifying differences in life history traits between populations that differ in these aspects is an important first step in identifying the roles of these factors in shaping life history variation.

The parasitoid wasp *Tetrastichus coeruleus* occurs both in natural and agricultural environments. Populations in natural areas are infected with parthenogenesis-inducing *Wolbachia* bacteria and reproduce asexually, whereas populations from agricultural fields are not infected and reproduce sexually.

In this paper, we describe differences in life history traits between two populations of *T. coeruleus*, one from a natural (Meijendel) and one from an agricultural (Brabant) environment. We quantified clutch size, life span, female weight and female nutrient concentrations (proteins, lipids, sugars and glycogen).

Females from Brabant laid larger clutches, had longer life spans and were heavier than females from Meijendel. There was no difference in the relative amounts of proteins, lipids or sugars, but females from Meijendel had relatively more glycogen

than females from Brabant.

The two populations therefore exhibit markedly different life history strategies. Females from Brabant invest relatively more in survival, body size and clutch size, whereas females from Meijendel seem to be more active or fly longer distances. Further studies are needed to determine if and how these life history differences are related to differences in ecology, mode of reproduction or *Wolbachia* infection.

Introduction

Life history traits are involved in the timing and duration of key events in an organism's life, in particular reproduction and survival. Important life history traits are development time, (reproductive) life span, number and size of offspring, adult size and mortality rate (Roff 1992, 2002, Stearns 1992). In order to maximize fitness, an organism needs to optimize each of these parameters. However, as resources can be used only once, it is not possible to maximize reproduction and survival at the same time. Therefore, life history traits are subject to trade-offs, such as between longevity and fecundity or between number and size of offspring (Roff 1992, 2002, Stearns 1992). The allocation of resources is thus an important aspect in life history trade-offs.

There are three essential nutrient resources: proteins, lipids and carbohydrates. In parasitoid wasps, proteins are mainly used for egg production, but also for maintenance, carbohydrates are mainly used for maintenance and lipids can be used for both (Jervis & Kidd 1986, Heimpel & Collier 1996, Rivero & Casas 1999). For example, in the parasitoid wasp *Asobara tabida* lipids are used for metabolic maintenance (survival), flight and egg production (Ellers 1996, Ellers & van Alphen 1997, Ellers *et al.* 1998). Of these three nutrients, proteins can be obtained through host feeding as an adult or carried over from the host during the larval stage. Carbohydrates can be obtained from sugar meals, such as nectar or honey, and are stored as glycogen. Lipids, stored as triglycerides, provide more energy per unit weight and take up less storage space than carbohydrates (Rivero & Casas 1999, Visser & Ellers 2008). However, since most parasitoid wasps cannot biosynthesize lipids (Visser *et al.* 2010), lipid reserves need to be carried over from the host during the larval stage. Therefore, carbohydrates provide a first, short-term energy supply, whereas lipids provide a long-term energy supply and are used when carbohydrate reserves are low (Rivero & Casas 1999, Visser & Ellers 2008).

Differences in life history strategies between populations may result from differences in ecology, mode of reproduction or from manipulation by parasites (Roff 1992, 2002, Stearns 1992). Differences in ecology between populations are probably the most obvious cause of differences in life history strategies. For example, the parasitoid wasp *Leptopilina boulardi* exhibits differences in egg load, life span, activity and energy reserves (amount of lipids, sugars and glycogen) between populations that differ in climatic factors and host distribution (Moiroux *et al.* 2010, Seyahooei

2010). The parasitoid wasp *Asobara tabida* exhibits differences in virulence against its host, diapause, survival, adult size, egg load and fat reserves, which are caused by differences in food- and host-availability between geographically different populations (Kraaijeveld & van der Wel 1994, Kraaijeveld & van Alphen 1994, 1995, Ellers & van Alphen 1997, Kraaijeveld & Godfray 1999).

However, different modes of reproduction may also result in different life history strategies. Individuals from sexual populations are less closely related to each other than individuals from asexual populations (e.g. chapter 3, Reumer *et al.* in prep). The level of competition for resources between individuals may therefore be different between sexual and asexual populations.

Alternatively, the presence or absence of a microbial infection may also cause differences in life history traits (Gross *et al.* 2009). For example, the parasitoid wasp *Leptopilina boulardi* is infected with a viral symbiont that manipulates the superparasitism behaviour of its host in order to enhance its own horizontal transmission (Varaldi *et al.* 2003). *Wolbachia* bacteria also have been shown to affect life history traits in several of its hosts. For example, *Wolbachia*-infected *Drosophila melanogaster* females had a longer life span and increased competitiveness compared to uninfected females (Alexandrov *et al.* 2007), *Wolbachia* had a positive effect on fecundity of *D. melanogaster* during periods of nutritional stress (Brownlie *et al.* 2009) and female longevity and fecundity of the psocid *Liposcelis tricolor* decreased dramatically when they were cured from their *Wolbachia* infection (Jia *et al.* 2009). Moreover, the parasitoid wasp *Asobara tabida* is dependent on *Wolbachia* for completing oogenesis (Dedeine *et al.* 2001, Kremer *et al.* 2010).

The parasitoid wasp *Tetrastichus coeruleus* (Hymenoptera: Eulophidae) is a gregarious, synovigenic, egg-larval parasitoid of the common asparagus beetle (*Crioceris asparagi*). *T. coeruleus* both feeds on and parasitizes the eggs of *C. asparagi* (Capinera & Lilly 1975a, van Alphen 1980). *C. asparagi* lives on the asparagus plant (*Asparagus officinalis*), which grows on sandy soils, such as coastal dune areas, and as a crop in monoculture on agricultural fields. Interestingly, populations of *T. coeruleus* from the Dutch coastal dune areas are highly female-biased (more than 92% females), while populations from the Dutch agricultural fields have nearly equal sex ratios (55% females) (chapter 2, Reumer *et al.* 2010). The populations from the Dutch coastal dune areas are infected with *Wolbachia* bacteria that induce parthenogenesis, whereas populations from the Dutch agricultural fields are not infected and reproduce sexually (chapter 2, Reumer *et al.* 2010).

In this paper, we compare life history traits between two populations of *T. coeruleus* that differ in ecology, *Wolbachia* infection and mode of reproduction as part of a larger study into the interplay between these three factors. We measured clutch sizes (the number of emerged wasps per host pupa), life span, female weight and the amounts of four nutrient resources (proteins, lipids, sugars and glycogen) in females at the time of emergence from the host pupa. We discuss possible explana-

tions for differences in life history traits between the two populations.

Materials and Methods

Tetrastichus coeruleus populations

Two populations of the parasitoid wasp *Tetrastichus coeruleus* (Hymenoptera: Eulophidae) were used in all experiments. One population was sampled from agricultural fields in Brabant (BR), The Netherlands. This population was not infected with *Wolbachia* and reproduced sexually (chapter 2, Reumer *et al.* 2010). Both males and females were collected from this population (ca. 43% males; chapter 2, Reumer *et al.* 2010). The other population was sampled from the natural dune area Meijendel (MD), The Netherlands. This population was infected with *Wolbachia* and reproduced parthenogenetically (chapter 2, Reumer *et al.* 2010). However, a small percentage of males was produced in this population (ca. 7% males; chapter 2, Reumer *et al.* 2010). More details about the sampled locations can be found in chapter 2, Reumer *et al.* (2010). In the spring and summer of 2009 and 2010 individuals from both populations were obtained from the field by collecting larvae of the asparagus beetle *Crioceris asparagi*, the host of *T. coeruleus*. These larvae were reared in the lab at 20°C, light:dark 16:8, 65% relative humidity. Some of these had been parasitized by *T. coeruleus*, in which case wasps instead of beetles would emerge from the pupae. The sex of each wasp was determined by ascertaining under a binocular the presence of a groove on the ventral side of the abdomen which envelopes the ovipositor. In males this groove is absent.

Clutch size

To test for differences in clutch size between pupae from Brabant and Meijendel, we scored how many wasps emerged from each host pupa (collection years 2009 and 2010, which were also used for the life span and physiology experiments, respectively).

Life span

To test for differences in life span between individuals from Brabant and Meijendel, we measured the life span of all males and females that emerged from the host pupae (collection year 2009). After emergence, the wasps were transferred to a glass tube (2.5 x 8.0 cm) with a medium of agar and a foam stopper with a drop of honey and kept individually at 20°C, light:dark 16:8, 65% relative humidity. Deaths were recorded daily, until all individuals were dead.

Physiology

To test for physiological differences between females from Brabant and Meijendel, we used unrelated zero-to-one-day-old virgin females (collection year 2010), which were stored individually in a 1.5 ml Eppendorf tube and kept at -80°C until mea-

surement. During measurement, samples were kept on ice at all times. Each female was weighted to the nearest µg before other measurements were taken. The measured amounts of proteins, lipids, sugars and glycogen were corrected for the total weight of the female.

The amounts of proteins, lipids, sugars and glycogen were quantified following the method described by Van Handel (1985a, 1985b) and Van Handel & Day (1988) and modified by Giron *et al.* (2002). Individual females were crushed with a plastic pestle in 300 µl methanol and centrifugated at 180 g for 15 minutes.

For the protein analysis, 100 µl of the supernatant was transferred to a new 1.5 ml Eppendorf tube and 700 µl physiological water and 200 µl filtered Bradford reagent (Bio-Rad, Hercules, CA, USA) were added. After 45 minutes at room temperature, the absorbance was measured at 595 nm using an Ultrospec II spectrophotometer (Pharmacia LKB, Uppsala, Sweden).

We then added 100 µl methanol, 150 µl chloroform and 60 µl 2% sodium sulphate to the original sample (200 µl supernatant and precipitate). After centrifugation at 180 g for 15 minutes, lipids and sugars remain dissolved in the supernatant, while the precipitate contains the glycogen. The supernatant was transferred to a new 1.5 ml Eppendorf tube and both the supernatant and precipitate were stored on ice.

For the lipid analysis, 50 µl of the supernatant was transferred to a new 1.5 ml Eppendorf tube and heated at 90°C until near complete evaporation. Then, 40 µl 98% sulphuric acid was added and the sample was reheated at 90°C for two minutes and cooled on ice for 5 minutes, after which 960 µl vanillin reagent was added. After 20 minutes at room temperature, the absorbance was measured at 525 nm using a spectrophotometer.

For the sugar analysis, 80 µl of the supernatant was transferred to a new 1.5 ml Eppendorf tube and heated at 90°C until near complete evaporation. Then, 1 ml anthrone reagent was added and the sample was left at room temperature for 15 minutes, after which it was reheated at 90°C for 15 minutes. After cooling on ice for 5 minutes, the absorbance was measured at 625 nm using a spectrophotometer.

For the glycogen analysis, the precipitate was washed twice by adding 400 µl 80% methanol and centrifugating it at 180 g for 5 minutes, after which the supernatant was removed. Then, 1 ml anthrone reagent was added and the sample was left at room temperature for 15 minutes, after which it was reheated at 90°C for 15 minutes. After cooling on ice for 5 minutes, the absorbance was measured at 625 nm using a spectrophotometer.

A fresh control sample and a blank sample were measured during each round of absorbance measurements. Amounts of proteins, lipids, sugars and glycogen were calculated using standard calibration curves of known concentrations of bovine serum albumin for proteins, sunflower oil for lipids and glucose for sugars and glycogen.

Statistical analysis

Statistical analyses were performed in R (version 2.13.1; R Developmental Core Team, 2011).

Analyses of variance (anova) were used to test for differences in clutch size between pupae from Brabant and Meijendel. Significance of explanatory variables (population and collection year) was tested by dropping (interactions between) explanatory variables from the model and comparing the resulting change in variance using an F-test. Clutch size means and their standard errors are reported.

Survival analyses (in the 'survival' package) were used to test for differences in life span between individuals from Brabant and Meijendel and between females and males within populations. Significance of explanatory variables (population, sex and clutch size) was examined in a cox proportional hazard model using a score log rank test. Significance of interactions between explanatory variables was tested by dropping the interactions from the model and comparing the resulting change in deviance to a χ^2-distribution. Life span means and their standard errors are reported.

Analyses of covariance (ancova) were used to test for differences in weight and amounts of proteins, lipids, sugars and glycogen between females from Brabant and Meijendel. Significance of explanatory variables (population, clutch size and female weight) was tested by dropping (interactions between) explanatory variables from the model and comparing the resulting change in variance using an F-test. Means and their standard errors are reported.

Results

Clutch size

We scored the clutch sizes of 683 parasitized host pupae: 551 pupae from Brabant (330 collected in 2009 and 221 in 2010) and 132 pupae from Meijendel (43 collected in 2009 and 89 in 2010).

Clutch sizes in Brabant ranged between 1 and 22 wasps per pupa in 2009, with 7 wasps per pupa as the most frequently observed clutch size (Fig. 4.1), while in 2010 clutch sizes ranged between 1 and 19 wasps per pupa, with 6 wasps per pupa as the most frequently observed clutch size (Fig. 4.2). Clutch sizes in Meijendel ranged between 1 and 13 wasps per pupa in 2009, with 2 and 8 wasps per pupa as the most frequently observed clutch sizes (Fig. 4.1), while in 2010 clutch sizes ranged between 1 and 9 wasps per pupa, with 6 wasps per pupa as the most frequently observed clutch size (Fig. 4.2).

Overall, pupae from Brabant had a significantly larger clutch size than pupae from Meijendel (mean clutch size = 6.64 ± 0.15 and 4.79 ± 0.22 wasps per pupa, respectively) and the clutch sizes from pupae collected in 2009 were significantly larger than the clutch sizes from pupae collected in 2010 (mean clutch size = 6.77 ± 0.18 and 5.70 ± 0.19 wasps per pupa, respectively) (overall: $F_{3,679} = 14.45$, $p <$
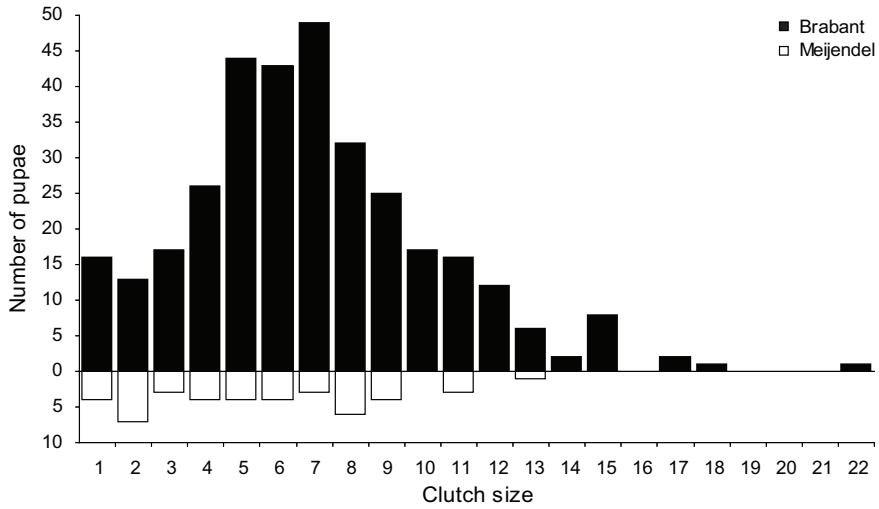
Figure 4.1: Frequency of clutch sizes (number of wasps per host pupa) of *Tetrastichus coeruleus* for pupae from Brabant and Meijendel in collection year 2009.
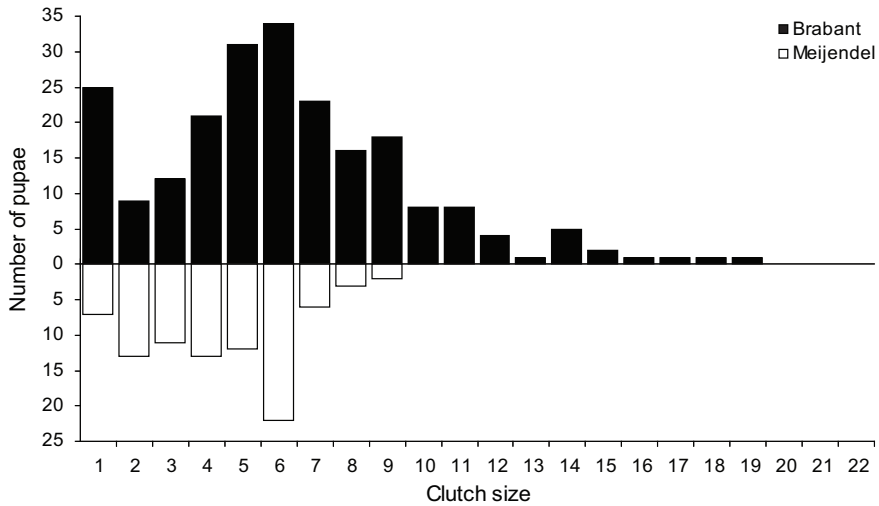
Figure 4.2: Frequency of clutch sizes (number of wasps per host pupa) of *Tetrastichus coeruleus* for pupae from Brabant and Meijendel in collection year 2010.

0.0001; population: $F_{1,681} = 33.28$, $p < 0.0001$; year: $F_{1,681} = 17.45$, $p < 0.0001$). There was no significant effect of the interaction between population and collection year ($F_{1,679} = 0.31$, $p = 0.58$).

Both within 2009 and 2010, pupae from Brabant had a significantly larger clutch size than pupae from Meijendel (mean clutch size 2009 $= 6.94 \pm 0.19$ and 5.53 ± 0.49 wasps per pupa, respectively; $F_{1,371} = 6.49$, $p = 0.01$; Fig. 4.1; mean clutch size 2010 $= 6.21 \pm 0.24$ and 4.43 ± 0.21 wasps per pupa, respectively; $F_{1,308} = 19.81$, $p < 0.0001$; Fig. 4.2).

Also, both within Brabant and Meijendel, the clutch sizes from pupae collected in 2009 were significantly larger than the clutch sizes from pupae collected in 2010 (mean clutch size Brabant $= 6.94 \pm 0.19$ and 6.21 ± 0.24 wasps per pupa, respectively; $F_{1,549} = 5.83$, $p = 0.02$; mean clutch size Meijendel $= 5.53 \pm 0.49$ and 4.43 ± 0.21 wasps per pupa, respectively; $F_{1,130} = 5.79$, $p = 0.02$).

Life span

We measured the life span of 2424 individuals: 1270 females and 926 males from Brabant and 217 females and 11 males from Meijendel.

Females from Brabant had significantly longer life spans than females from Meijendel (Fig. 4.3; mean survival $= 33.03 \pm 0.31$ and 29.24 ± 0.65 days, respectively; overall: $Z = 31.86$, $df = 3$, $p < 0.0001$; population: $Z = 31.21$, $df = 1$, $p < 0.0001$). There was no significant effect of clutch size or the interaction between population and clutch size on female life span (clutch size: $Z = 1.02$, $df = 1$, $p = 0.31$; interaction: $\chi^2 = 0.09$, $df = 1$, $p = 0.77$).

There was no significant difference in life span between males from Brabant and Meijendel (mean survival $= 30.69 \pm 0.37$ and 32.45 ± 2.89 days, respectively; overall: $Z = 3.81$, $df = 3$, $p = 0.28$; population: $Z = 0.01$, $df = 1$, $p = 0.92$). Also, there was no significant effect of clutch size or the interaction between population and clutch size on male life span (clutch size: $Z = 3.79$, $df = 1$, $p = 0.052$; interaction: $\chi^2 = 0.02$, $df = 1$, $p = 0.88$).

Within Brabant, females had significantly longer life spans than males (Fig. 4.4; mean survival $= 33.03 \pm 0.31$ and 30.69 ± 0.37 days, respectively; overall: $Z = 18.56$, $df = 3$, $p = 0.0003$; sex: $Z = 13.97$, $df = 1$, $p = 0.0002$). There was no significant effect of clutch size or the interaction between sex and clutch size on life span (clutch size: $Z = 2.03$, $df = 1$, $p = 0.15$; interaction: $\chi^2 = 3.20$, $df = 1$, $p = 0.07$).

Within Meijendel, there was no significant difference in life span between females and males (mean survival $= 29.24 \pm 0.65$ and 32.45 ± 2.89 days, respectively; overall: $Z = 0.79$, $df = 3$, $p = 0.85$; sex: $Z = 0.72$, $df = 1$, $p = 0.39$). Also, there was no significant effect of clutch size or the interaction between sex and clutch size on life span (clutch size: $Z = 0.05$, $df = 1$, $p = 0.82$; interaction: $\chi^2 = 0.001$, $df = 1$, $p = 0.97$).
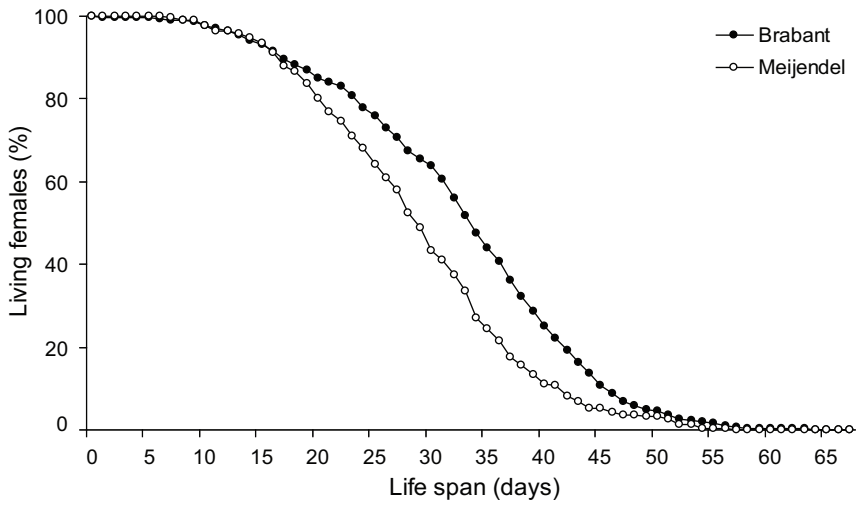
Figure 4.3: Survival probability of *Tetrastichus coeruleus* females from Brabant (black dots) and Meijendel (white dots).
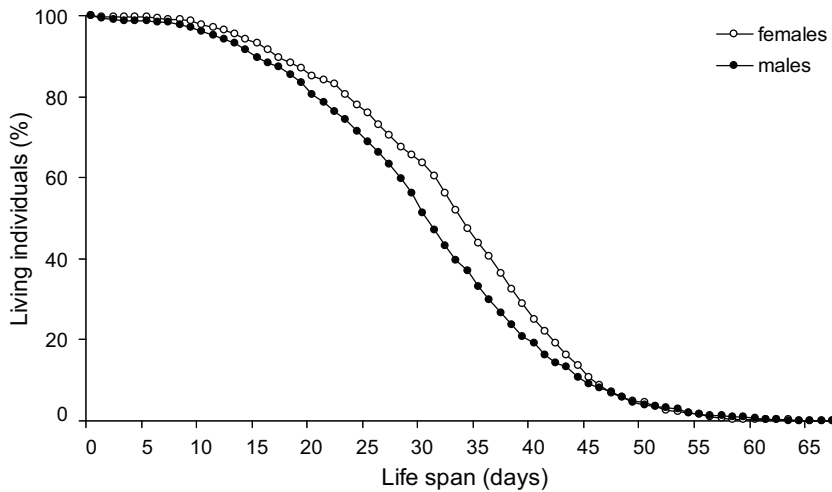
Figure 4.4: Survival probability of *Tetrastichus coeruleus* females (white dots) and males (black dots) from Brabant.

Physiology

In total, 97 individual females were tested: 49 females from Brabant and 48 females from Meijendel. All females were weighed and amounts of proteins ($n_{BR} = 49$, $n_{MD} = 48$), lipids ($n_{BR} = 48$, $n_{MD} = 48$), sugars ($n_{BR} = 48$, $n_{MD} = 48$) and glycogen ($n_{BR} = 49$, $n_{MD} = 48$) were measured.
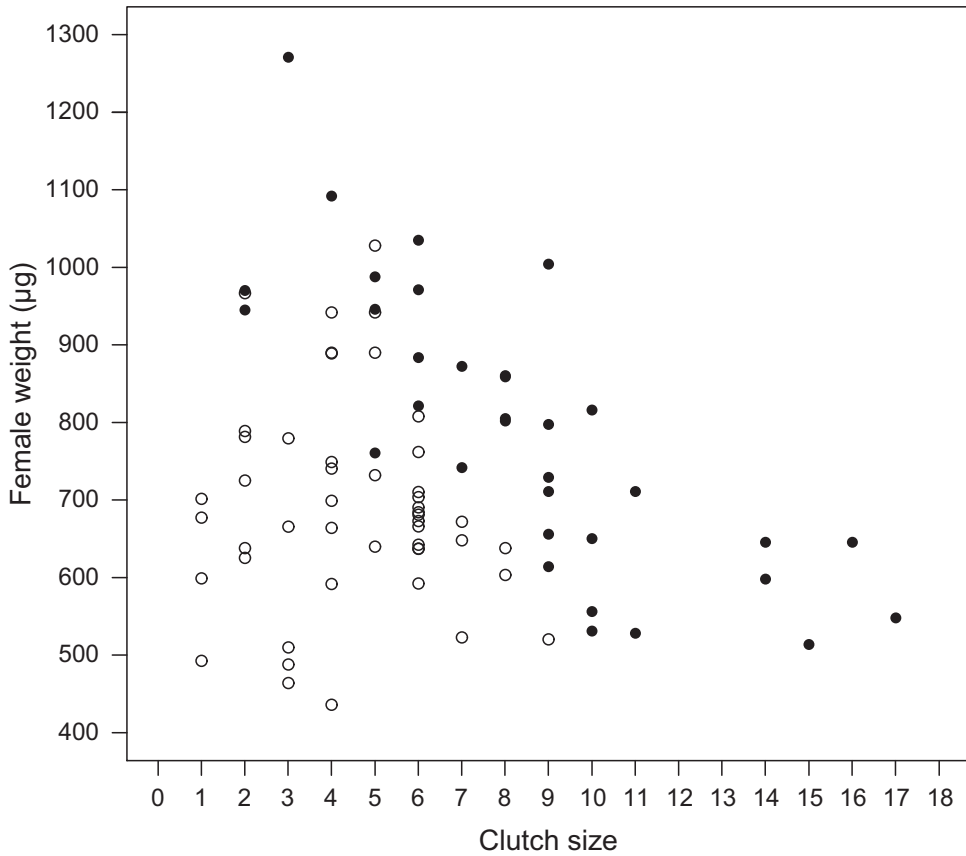
Females from Brabant were significantly heavier than females from Meijendel (Fig. 4.5; mean weight $= 799.66 \pm 24.50$ and 692.30 ± 19.25 µg, respectively; $F_{1,95} = 11.81$, $p = 0.0009$). There was a significant effect of the interaction between population and clutch size on female weight ($F_{1,78} = 9.60$, $p = 0.003$). Within Brabant, clutch size had a significant effect on female weight: females from smaller clutches were heavier than females from larger clutches (Fig. 4.5; $F_{1,32} = 49.92$, $p < 0.0001$). Within Meijendel, there was no significant effect of clutch size on female weight (Fig. 4.5; $F_{1,46} = 0.22$, $p = 0.64$).

Females from Brabant had significantly more proteins than females from Meijendel (Fig. 4.6; mean $= 5.85 \pm 0.23$ and 5.14 ± 0.22 µg, respectively; $F_{1,95} = 4.88$, $p = 0.03$). However, this was due to the significant effect of female weight on the amount of proteins (overall: $F_{3,93} = 51.93$, $p < 0.0001$; weight: $F_{1,95} = 153.36$, $p < 0.0001$). There was no significant difference in the relative amount of proteins between the populations (Fig. 4.6; $F_{1,94} = 0.45$, $p = 0.50$). The interaction between female population and female weight had no significant effect on the amount of proteins ($F_{1,93} = 1.71$, $p = 0.19$). Removing outliers did not change the results.

There was no significant difference in the amount of lipids between females from Brabant and Meijendel (Fig. 4.7; mean $= 68.65 \pm 4.65$ and 62.35 ± 3.84 µg, respectively; $F_{1,94} = 1.09$, $p = 0.30$), but there was a significant effect of female weight on the amount of lipids (overall: $F_{3,92} = 33.50$, $p < 0.0001$; weight: $F_{1,94} = 94.43$, $p < 0.0001$). Also, there was no significant difference in the relative amount of lipids between the populations (Fig. 4.7; $F_{1,93} = 3.94$, $p = 0.05$). The interaction between female population and female weight had no significant effect on the amount of lipids ($F_{1,92} = 0.12$, $p = 0.73$). Removing outliers did not change the results.

There was no significant difference in the amount of sugars between females from Brabant and Meijendel (Fig. 4.8; mean $= 5.02 \pm 1.46$ and 4.43 ± 1.03 µg, respectively; $F_{1,94} = 0.11$, $p = 0.74$) and there was no significant effect of female weight on the amount of sugars (overall: $F_{3,92} = 0.40$, $p = 0.76$; weight: $F_{1,94} = 0.06$, $p = 0.81$). Also, there was no significant difference in the relative amount of sugars between the populations (Fig. 4.8; $F_{1,93} = 0.19$, $p = 0.66$). The interaction between female population and female weight had no significant effect on the amount of sugars ($F_{1,92} = 0.94$, $p = 0.34$). Removing outliers did not change the results.

There was no significant difference in the amount of glycogen between females from Brabant and Meijendel (Fig. 4.9; mean $= 27.83 \pm 1.61$ and 28.27 ± 1.19 µg, respectively; $F_{1,95} = 0.05$, $p = 0.83$), but there was a significant effect of female weight on the amount of glycogen (overall: $F_{3,93} = 11.29$, $p < 0.0001$; weight:
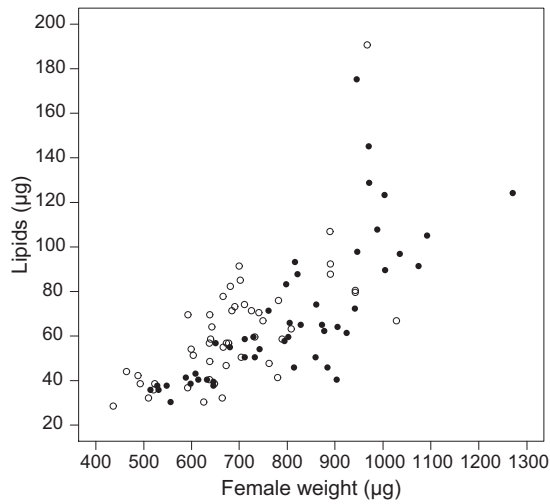
Figure 4.5: Relation between female weight (µg) and clutch size (number of wasps per host pupa) for *Tetrastichus coeruleus* females from Brabant (black dots) and Meijendel (white dots).

$F_{1,95} = 28.29$, $p < 0.0001$). Due to the significant effect of female weight, there was a significant difference in the relative amount of glycogen between the populations (Fig. 4.9; $F_{1,94} = 4.75$, $p = 0.03$); females from Meijendel had relatively more glycogen than females from Brabant. The interaction between female population and female weight had no significant effect on the amount of glycogen ($F_{1,93} = 0.06$, $p = 0.81$). Removing outliers did not change the results.
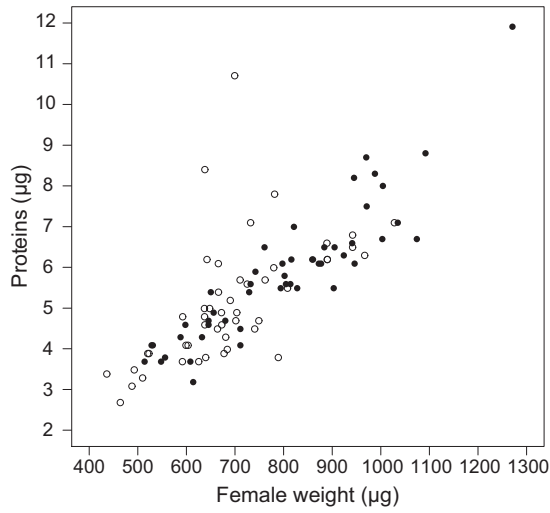
Figure 4.6: Relation between amount of proteins (µg) and female weight (µg), for *Tetrastichus coeruleus* females from Brabant (black dots) and Meijendel (white dots).

Figure 4.7: Relation between amount of lipids (µg) and female weight (µg), for *Tetrastichus coeruleus* females from Brabant (black dots) and Meijendel (white dots).
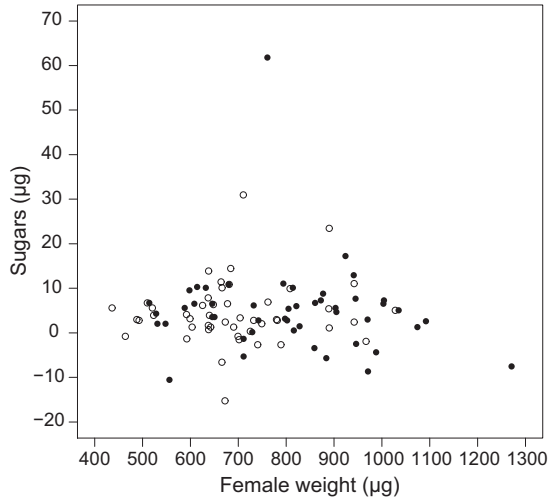
Figure 4.8: Relation between amount of sugars (µg) and female weight (µg), for *Tetrastichus coeruleus* females from Brabant (black dots) and Meijendel (white dots).
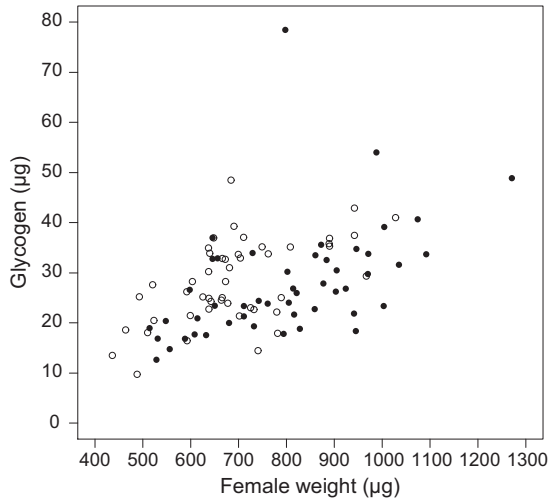
Figure 4.9: Relation between amount of glycogen (µg) and female weight (µg), for *Tetrastichus coeruleus* females from Brabant (black dots) and Meijendel (white dots).

Discussion

We compared life history traits between two populations of *T. coeruleus* that differ in ecology, *Wolbachia* infection and mode of reproduction. Females from Brabant laid larger clutches, had longer life spans and were heavier than females from Meijendel. Furthermore, females from Brabant had lower levels of glycogen per unit body weight than females from Meijendel.

The finding that clutch sizes are larger and female body weight is higher in Brabant than in Meijendel seems paradoxical. Given that individuals from larger clutches have to share the same amount of resources with more siblings, parasitoid wasps emerging from larger clutches are expected to be smaller than those emerging from small clutches. Indeed, we found a negative correlation between clutch size and female weight in Brabant, although not in Meijendel. A possible explanation for the larger clutches and heavier females is that hosts may be larger in Brabant than in Meijendel. Larger hosts will have more resources available and either more or larger parasitoid wasps may emerge from one host pupa. Unfortunately, we have no data on host size in these populations. Alternatively, wasps from Brabant may be more efficient in extracting resources from their host than wasps from Meijendel. Regardless of the cause of the difference in body weight, larger individuals are expected to have a longer life span than smaller ones, because they have more resources available. In agreement with this, females from Brabant were heavier and lived longer than females from Meijendel.

We found a difference in resource allocation only for glycogen. Females from Meijendel contained higher relative glycogen levels than females from Brabant. Levels of other resources (lipids, proteins and sugars) did not differ between females from Brabant and Meijendel after correcting for body weight. Glycogen is used for maintenance, in particular short-term activities (Rivero & Casas 1999). The higher glycogen levels suggest that females from Meijendel invest more in short-term activities, such as flight (Rivero & Casas 1999).

Overall, the two populations appear to employ markedly different life history strategies. Females from Brabant invest more heavily in survival, body size and clutch size, whereas females from Meijendel seem to be more active. The populations from Brabant and Meijendel differ in ecology, *Wolbachia* infection and mode of reproduction. Each of these differences may potentially account for the differences in life history traits we observed. For example, the spatial and temporal availability of hosts differs between Brabant and Meijendel. On agricultural fields in Brabant, asparagus plants occur close together and in high numbers. While hosts may sometimes be abundant, their distribution is rendered unpredictable due to spraying of the asparagus plants with insecticides. This may select for a life history strategy in which females grow large, so they can live longer and exploit available hosts optimally when they are encountered. In natural areas, asparagus plants occur in lower numbers and densities, so females may need to fly more between plants in

search of hosts. However, this is only one of multiple scenarios that may explain the observed differences in life history traits. Furthermore, the direction of causality, if any, cannot be ascertained based on our current data. It is possible that the ecology in Brabant selects for a life history strategy that is less compatible with parthenogenesis than with sexual reproduction, giving uninfected wasps a competitive advantage. Further sampling and experimentation are needed to determine if and how the observed life history differences are related to differences in ecology, mode of reproduction or *Wolbachia* infection.

Acknowledgements

Chapter 5

Sexual functionality of parthenogenetic *Tetrastichus coeruleus* (Hymenoptera: Eulophidae)

Barbara M. Reumer, Jacques J.M. van Alphen & Ken Kraaijeveld
Manuscript

Wolbachia are endosymbiotic bacteria known to manipulate the reproduction of their hosts, for example by inducing parthenogenesis. In most cases of *Wolbachia*-induced parthenogenesis, the infection is fixed and the entire host population consists of females. In the absence of males and sexual reproduction, genes involved in sexual reproduction are not actively maintained by selection. Accumulation of neutral mutations or selection against the maintenance of such traits may lead to their loss or deterioration. In addition, females may loose the ability to reproduce sexually due to 'functional virginity mutations' that may spread concomitantly with the *Wolbachia* infection through a population. The parasitoid wasp *Tetrastichus coeruleus* forms an ideal model to study the decay of sexual functionality, because it has both *Wolbachia*-infected populations that reproduce parthenogenetically and uninfected, sexual populations. We compared the sexual functionality of arrhenotokous (sexual) and thelytokous (parthenogenetic) *T. coeruleus* females.

Mated females had a shorter life span than virgin females, showing that either mating or sperm storage was costly. Several sexual traits of thelytokous females have degraded compared to arrhenotokous females. Arrhenotokous and thelytokous females were equally attractive to males, but thelytokous females were unreceptive to males. Furthermore, there was a clear difference in spermathecal morphology between arrhenotokous and thelytokous females. Selection against the maintenance of costly sexual traits appears to have resulted in the degradation of receptivity and spermathecal morphology of thelytokous females. However, we cannot exclude that these traits have degraded due to functional virginity mutations or accumulation of neutral mutations.

Introduction

Wolbachia are maternally inherited, intracellular, symbiotic bacteria belonging to the order Rickettsiales within the α-Proteobacteria. It has been estimated that 66% of all insect species are (partly) infected with *Wolbachia* (Hilgenboecker *et al.* 2008). To enhance its own transmission, *Wolbachia* can induce various alterations of the reproduction of its host, such as cytoplasmic incompatibility, feminization, male-killing and parthenogenesis induction (PI). *Wolbachia*-induced parthenogenesis is most commonly found in haplodiploid organisms, such as Hymenoptera (Werren 1997, Stouthamer *et al.* 1999, Werren *et al.* 2008). In uninfected haplodiploid organisms, fertilized eggs develop into diploid daughters and unfertilized eggs develop into haploid sons (arrhenotoky). In haplodiploids, PI-*Wolbachia* cause diploidization of the haploid eggs by alteration of meiotic and/or mitotic processes (Stouthamer & Kazmer 1994, Huigens & Stouthamer 2003, Pannebakker *et al.* 2004a) resulting in the production of daughters from unfertilized eggs (thelytoky).

In most cases of *Wolbachia*-induced parthenogenesis, the infection is fixed and the entire host population consists of females (Huigens & Stouthamer 2003). In the absence of males and sexual reproduction, genes involved in sexual reproduction are not actively maintained by selection. Accumulation of neutral mutations or selection against the maintenance of costly sexual traits may lead to their loss or deterioration (Carson *et al.* 1982, Pijls *et al.* 1996, Pannebakker *et al.* 2005, Kraaijeveld *et al.* 2009). Because male traits are not expressed in parthenogenetic populations, they are likely to degenerate due to neutral mutations. Female traits, which are expressed but not used, may also be selected against when they are costly to maintain (Pijls *et al.* 1996). In addition, females may loose the ability to reproduce sexually due to 'functional virginity mutations' that may spread concomitantly with the *Wolbachia* infection through a population (Stouthamer *et al.* 2010, King & Hurst 2010). Virginity mutations arise during the early stages of PI-*Wolbachia* infection and affect traits in females involved in sexual reproduction, e.g. mating or egg fertilization. Accumulation of neutral mutations or selection against the maintenance of costly traits arise after a longer period of parthenogenetic reproduction and can potentially affect all traits involved in sexual reproduction, both in males and females, e.g. courtship behaviour or pheromone production.

Sexual traits may evolve to be costly when the reproductive interests of males and females are different. Sexual conflict may result in sexually antagonistic coevolution, in which males evolve adaptations that manipulate female behaviour and females evolve resistance to male manipulation (Rice 1996, Chapman *et al.* 2003, Arnqvist & Rowe 2005). In the absence of males, selection on manipulative male traits and costly female resistance traits will be absent. Adaptations evolved under sexually antagonistic coevolution are released from selection and alleles that were favoured can be replaced by others due to accumulation of random mutations (Carson *et al.* 1982, Pijls *et al.* 1996) or antagonistic pleiotropy (Pijls *et al.* 1996, Pannebakker *et*

al. 2005). Therefore, thelytokous females may be either more receptive to mating when no males are present, which is the case in the parasitoid wasp *Leptopilina clavipes* (Kraaijeveld *et al.* 2009), or not receptive at all when a few males are still present.

Many insect species with female-only populations exhibit deterioration or loss of female sexual traits, as expected by theory. In the parasitoid wasp *Apoanagyrus diversicornis*, arrhenotokous and thelytokous females were equally attractive, but thelytokous females mated less often than arrhenotokous females (Pijls *et al.* 1996). Similarly, asexual *Drosophila mercatorum* females mated less often than sexual females (Carson *et al.* 1982). Thelytokous females of the parasitoid wasp *Muscidifurax uniraptor* were attractive to males, but never mated with them (Gottlieb & Zchori-Fein 2001). In contrast, thelytokous females of the parasitoid wasp *Leptopilina clavipes* mated more often than arrhenotokous females and copulations with thelytokous females lasted longer (Kraaijeveld *et al.* 2009). Differences between thelytokous and arrhenotokous females could also be found in the morphology of the sexual organs. Spermathecae are organs in which females store sperm after mating until oviposition. In the parasitoid wasp *L. clavipes*, there was a clear difference in spermathecal morphology (Kraaijeveld *et al.* 2009). Moreover, the spermathecae of thelytokous females were less capable of storing sperm than the ones of arrhenotokous females (Kraaijeveld *et al.* 2009). In the parasitoid wasp *M. uniraptor*, thelytokous females lacked a major muscle in their spermathecae, which were therefore considered to be vestigial and not capable of storing and transporting sperm (Gottlieb & Zchori-Fein 2001).

The parasitoid wasp *Tetrastichus coeruleus* (Hymenoptera: Eulophidae) forms an ideal model to study the decay of sexual functionality, because it has both arrhenotokous and thelytokous populations (chapter 2, Reumer *et al.* 2010). *T. coeruleus* is an egg-larval parasitoid of the common asparagus beetle (*Crioceris asparagi*). *C. asparagi* lives on the asparagus plant (*Asparagus officinalis*), which grows on sandy soils, such as coastal dune areas, and as a crop in monoculture on agricultural fields. Like all Hymenoptera, *T. coeruleus* is haplodiploid. Populations from the Dutch coastal dune areas are infected with *Wolbachia* and reproduce parthenogenetically (thelytoky), while populations from the Dutch agricultural fields are not infected and reproduce sexually (arrhenotoky) (chapter 2, Reumer *et al.* 2010). The arrhenotokous and thelytokous populations diverged between 7.5×10^5 and 2.0×10^5 generations ago (chapter 3, Reumer *et al.* in prep). The *Wolbachia* infection and thelytokous reproductive mode are probably as old as this divergence. This has given all three mutation accumulation possibilities (neutral mutation accumulation, selection against the maintenance of costly traits and functional virginity mutations) time to act on traits involved in sexual reproduction in the thelytokous populations.

In this paper, we compared the sexual functionality of arrhenotokous and thelytokous *T. coeruleus* females. We predicted that several sexual traits should have

degraded in thelytokous females. First, we examined whether mating is costly to females by measuring the life span of mated and virgin females. Second, we tested whether arrhenotokous and thelytokous females were equally attractive and receptive to males. Last, we studied the morphology of the spermathecae of arrhenotokous and thelytokous females.

Materials and Methods

Tetrastichus coeruleus populations
Two populations of the parasitoid wasp *Tetrastichus coeruleus* (Hymenoptera: Eulophidae) were used in all experiments. One population was sampled from agricultural fields in Brabant (BR), The Netherlands. This population is not infected with *Wolbachia* and reproduces arrhenotokously (chapter 2, Reumer *et al.* 2010). The other population was sampled from the natural dune area Meijendel (MD), The Netherlands. This population is infected with *Wolbachia* and reproduces thelytokously (chapter 2, Reumer *et al.* 2010). More details about the sampled locations can be found in chapter 2, Reumer *et al.* (2010). In the spring and summer of 2010 individuals from both populations were obtained from the field by collecting larvae of the asparagus beetle *Crioceris asparagi*, the host of *T. coeruleus*. These larvae were reared in the lab at 20°C, light:dark 16:8, 65% relative humidity. Some of these had been parasitized by *T. coeruleus*, in which case wasps in stead of beetles would emerge from the pupae. The sex of each wasp was determined by looking under a binocular for the presence of a groove on the ventral side of the abdomen which envelopes the ovipositor. In males this groove is absent.

Cost of mating
To test whether mating is costly to females, we measured the life span of all females from the mating experiment (described below) that mated with a male. After mating, the female was transferred to a glass tube (2.5 x 8.0 cm) with a medium of agar and a foam stopper with a drop of honey. As a control we measured the life span of virgin females that never were exposed to male courtship or mating. All females were kept at 20°C, light:dark 16:8, 65% relative humidity. Female deaths were scored daily, until all females were dead.

Courtship and mating
To test whether arrhenotokous and thelytokous females differ in attractiveness and/or receptiveness towards males, we performed a mating experiment. Zero-to-four-day-old virgin arrhenotokous and thelytokous females were put individually in plastic tubes (1.0 x 5.0 cm), with small-mesh gauze on one side and a rubber stopper on the other side. One male from the arrhenotokous population was introduced into each tube and courtship behaviour and mating were scored. After one hour or after mating, whichever came first, the trial was stopped. Because the

first element of the male courtship sequence is wing fanning, we used male wing fanning as a measure of female attractiveness. Because females may be attractive but not receptive to males, we used mating as a measure of female receptiveness. Both traits (attractiveness and receptiveness) were scored as dichotomous variables (yes/no).

Spermathecae

To examine differences in spermathecal morphology, we visualized the spermathecae of zero-to-ten-day-old virgin arrhenotokous and thelytokous females. The females were dissected in a drop of demineralized water, and the spermathecae visualized using a Nikon Labophot binocular microscope (Nikon Corporation, Tokyo, Japan) and photographed at 40x magnification. Subsequently, the length and width of the spermathecae were measured by analysing the photos using Adobe Photoshop CS4 (version 11.0; Adobe Systems, San Jose, CA, USA). To examine spermathecal shape, we used the ratio between length and width of the spermathecae for the analysis.

Statistical analysis

Statistical analyses were performed in R (version 2.12.1; R Developmental Core Team, 2010).

Survival analysis was used to test for differences in life span between mated and virgin females. Significance of the explanatory variable (mated or not) was examined in a cox proportional hazard model using a score log rank test.

Generalized linear models (glm) with a binomial error distribution were used to test for differences in male wing fanning and mating between arrhenotokous and thelytokous females. In the wing fanning model, the number of trials with males that showed wing fanning was used as the response variable and the total number of trials as the binomial denominator. In the mating model, the number of trials that ended with a mating was used as the response variable and the total number of trials as the binomial denominator. Significance of explanatory variables (female population and female age) was tested by dropping (interactions between) explanatory variables from the model and comparing the resulting change in deviance to a χ^2-distribution.

Analysis of covariance (ancova) was used to test for differences in the ratio between length and width (elongation) of the spermathecae between arrhenotokous and thelytokous females. Significance of explanatory variables (female population and female age) was tested by dropping (interactions between) explanatory variables from the model and comparing the resulting change in variance using an F-test.

Results

Cost of mating

We measured the life span of 20 females from the mating experiment that had mated with a male and that of 20 virgin females. All females were from the arrhenotokous population. Virgin females (mean survival = 30.95 days) had significantly longer life spans than females that had mated with a male (mean survival = 25.15 days) (Fig. 5.1; $Z = 3.92$, $df = 1$, $p = 0.048$).
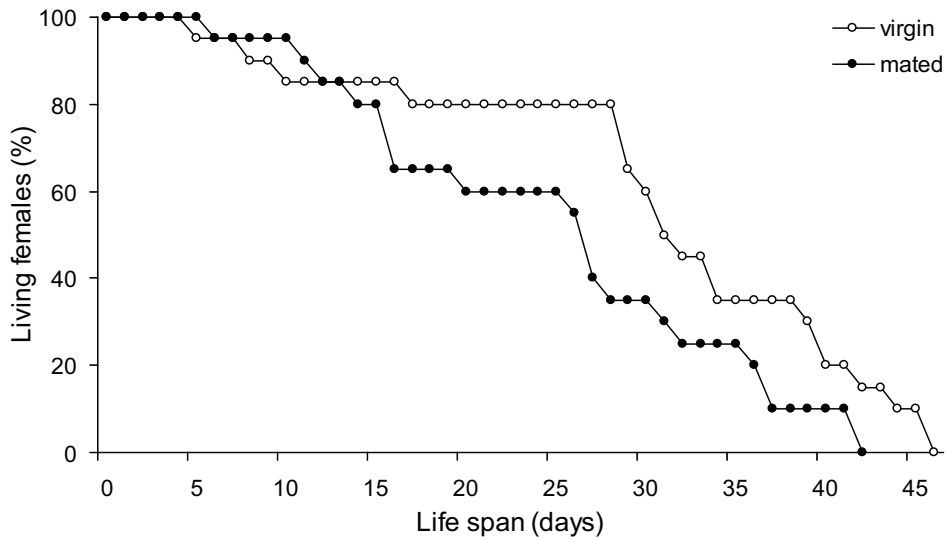
Figure 5.1: Survival probability of arrhenotokous *Tetrastichus coeruleus* females. Open circles: virgin females, solid circles: mated females.

Courtship and mating

In total, 96 individual females were tested: 50 arrhenotokous females and 46 thelytokous females. Males showed wing fanning in 31 of the 50 trials (62%) with arrhenotokous females and in 30 of the 46 trials (65%) with thelytokous females. There were no significant differences in wing fanning frequency between female populations, female age or their interaction (Fig. 5.2; overall: $\Delta_{deviance} = 3.92$, $df = 3$, $p = 0.27$; interaction: $\Delta_{deviance} = 0.0001$, $df = 1$, $p = 0.99$; female population: $\Delta_{deviance} = 0.11$, $df = 1$, $p = 0.74$; female age: $\Delta_{deviance} = 3.82$, $df = 1$, $p = 0.051$).

Twenty of the 50 trials (40%) with arrhenotokous females ended with a mating, while none of the thelytokous females mated with a male. All of the matings were preceded by male wing fanning behaviour. Arrhenotokous and thelytokous females

differed significantly in mating frequency (Fig. 5.2; overall: $\Delta_{deviance} = 31.23$, $df = 3$, $p < 0.0001$; female population: $\Delta_{deviance} = 30.95$, $df = 1$, $p < 0.0001$). There were no significant effects of female age or the interaction between female age and female population on mating frequency (interaction: $\Delta_{deviance} < 0.0001$, $df = 1$, $p = 1.00$; female age: $\Delta_{deviance} = 0.22$, $df = 1$, $p = 0.64$).
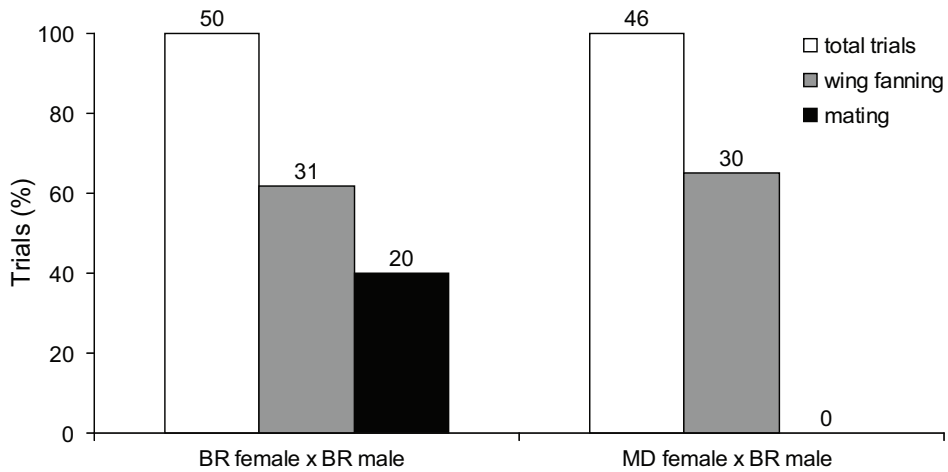
Figure 5.2: Percentage of trials in which arrhenotokous (BR) *Tetrastichus coeruleus* males exhibited wing fanning behaviour and percentage of trials that ended with a mating, for arrhenotokous (BR) and thelytokous (MD) *T. coeruleus* females. White bars: total number of trials, grey bars: percentage of trials in which males showed wing fanning behaviour, black bars: percentage of trials that ended with a mating. Number of trials are indicated above the bars.

Spermathecae

In total, we visualized and measured the spermathecae of 55 females: 30 from arrhenotokous females and 25 from thelytokous females. All spermathecae from arrhenotokous females were circular with a smooth surface (Fig. 5.3A). Five of the spermathecae from thelytokous females were similar to those from arrhenotokous females, while the other 20 spermathecae were more elongated and often had bulges (Fig. 5.3B-D). The spermathecae of arrhenotokous and thelytokous females differed significantly in the ratio between length and width of the spermathecae (overall: $F_{3,51} = 7.70$, $p = 0.0002$; female population: $F_{1,53} = 23.46$, $p < 0.0001$). There were no significant effects of female age or the interaction between female age and female population on the ratio between length and width of the spermathecae (interaction: $F_{1,51} = 0.14$, $p = 0.71$; female age: $F_{1,53} = 0.83$, $p = 0.37$).
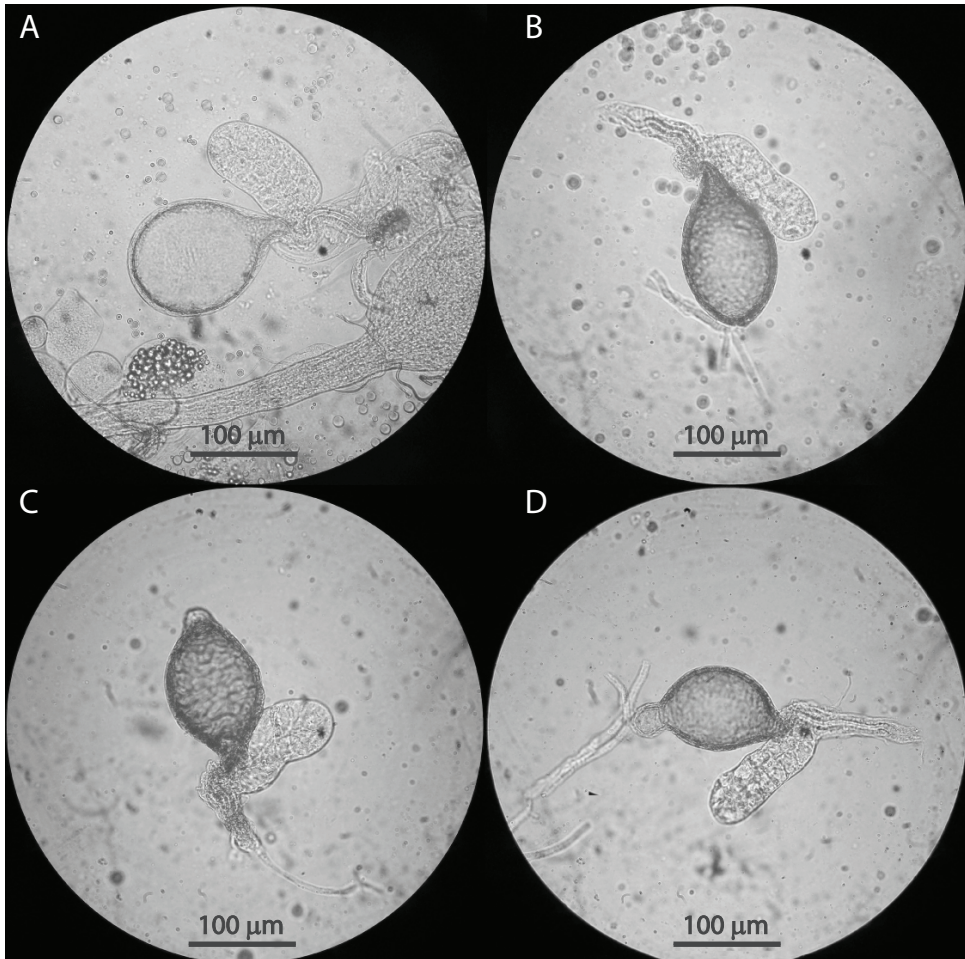
Figure 5.3: Spermathecae of arrhenotokous (A) and thelytokous (B-D) *Tetrastichus coeruleus* females. The spermatheca in B was slightly more elongated than the one in A. The spermathecae in C and D exhibited small and large, respectively, bulges as seen in many spermathecae of thelytokous females, but never in arrhenotokous females.

Discussion

Sexual traits may evolve to be costly when the reproductive interests of males and females are different. We therefore examined whether mating is costly to *T. coeruleus* females. This was only tested for arrhenotokous females, because thelytokous females never mated with males. The results showed that mated females had a shorter life span than virgin females. Next, we compared the sexual functionality of arrhenotokous and thelytokous *T. coeruleus* females. The results showed that arrhenotokous and thelytokous females were equally attractive to males, but that thelytokous females never mated with males. Furthermore, there was a clear difference between spermathecae from arrhenotokous and thelytokous females. Arrhenotokous females had circular spermathecae with a smooth surface, whereas the spermathecae of thelytokous females were more elongated and often had bulges.

Thelytokous *T. coeruleus* females were equally attractive to males as arrhenotokous females. Therefore, male courtship does not seem to be costly to females, as is the case in the field cricket *Gryllus bimaculatus* and the parasitoid wasp *Leptopilina clavipes* (Bateman *et al.* 2006, Reumer *et al.* 2007, respectively).

Similar to many other insects, mating was costly to *T. coeruleus* females. Many male insects are known to manipulate females through proteins that they transfer to females during mating along with the seminal fluid (Chapman *et al.* 1995, Wigby & Chapman 2005, South & Lewis 2011). Such manipulative male proteins may cause a reduction in female receptivity, an increase in female fecundity and the removal of sperm of previous mates, but it can also have substantial side-effects, such as mating-induced reduction in female life span (e.g. Fowler & Partridge 1989, Chapman *et al.* 1995, Rice 1996, Garcia-Gonzalez & Simmons 2010, South & Lewis 2011, Xu & Wang 2011). Females are expected to evolve counter-adaptations to reduce the harmful effects of male manipulation (Chapman *et al.* 2003, Arnqvist & Rowe 2005). However, when no males are present in a population, which is the case in most PI-*Wolbachia*-infected populations (Huigens & Stouthamer 2003), female adaptations against male manipulation may be released from such selection. Therefore, thelytokous females may be more receptive to mating when they have evolved without males for a long period of time. This seems to be the case in the parasitoid wasp *L. clavipes*, where thelytokous females mated more frequently with males than arrhenotokous females and copulations lasted longer (Kraaijeveld *et al.* 2009), although in this species mating does not result in a shortened female life span (Reumer *et al.* 2007). However, when males occur at low frequency in a thelytokous population, females may still be selected to avoid the cost of mating, while they no longer need to mate to fertilize their eggs. In such cases, thelytokous females may be less receptive or not receptive at all to mating. In agreement with this, males are found in low frequencies in thelytokous populations of *T. coeruleus* (chapter 2, Reumer *et al.* 2010) and thelytokous *T. coeruleus* females never mated with males. Although thelytokous *T. coeruleus* females never mated in the lab, Reumer *et al.*

(chapter 3, in prep) found clues for occasional matings between males and thely-tokous females in the field. Because of these sporadic matings, females may still be exposed to the cost of mating and adaptations of females against male manipulations keep evolving. Alternatively, the unreceptiveness of thelytokous females may be due to 'functional virginity mutations' that may spread concomitantly with the *Wolbachia* infection through a population (Stouthamer *et al.* 2010, King & Hurst 2010).

However, the longevity cost may also have been due to a cost of sperm storage. If this is true, then there would be selection against traits involved in sperm storage. Arrhenotokous females need to be able to store and transport sperm in order to produce female offspring. Thelytokous females however, do not need sperm to reproduce and traits involved in sperm storage may be released from selection in order to maintain them. In agreement with this, the spermathecae of arrhenotokous and thelytokous *T. coeruleus* females differed in their morphology. All spermathecae of arrhenotokous females were circular with a smooth surface, whereas the spermathecae of thelytokous females were more elongated, often had bulges and differed between individuals. This suggests that thelytokous females no longer use their spermathecae, which have become vestigial. In addition, the unreceptiveness of thelytokous females may be a response to the possible cost of sperm storage in stead of a cost of copulation. Alternatively, the spermathecae may have degraded due to neutral mutation accumulation, because these organs are no longer used by thelytokous females.

In conclusion, we observed a longevity cost of mating or sperm storage in *T. coeruleus* females. Selection against the maintenance of costly sexual traits appears to have resulted in the degradation of receptivity and spermathecal morphology of thelytokous females. However, we cannot exclude that these traits have degraded due to functional virginity mutations or accumulation of neutral mutations.

Acknowledgements

Chapter 6

Occasional males in parthenogenetic populations of *Asobara japonica* (Hymenoptera: Braconidae): low *Wolbachia* titer or incomplete co-adaptation?

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Wolbachia are endosymbiotic bacteria known to manipulate the reproduction of their hosts. Some populations of the parasitoid wasp *Asobara japonica* are infected with *Wolbachia* and reproduce parthenogenetically, while other populations are not infected and reproduce sexually. *Wolbachia*-infected *A. japonica* females regularly produce small numbers of male offspring. Because all females in the field are infected and infected females are not capable of sexual reproduction, male production seems to be maladaptive. We investigated why these females nevertheless produce males.

We tested three hypotheses: high rearing temperatures could result in higher offspring sex ratios (more males), low *Wolbachia* titer of the mother could lead to higher offspring sex ratios and/or the *Wolbachia* infection is of relatively recent origin and not enough time has passed to allow complete co-adaptation between *Wolbachia* and host.

Thirty-three percent of the *Wolbachia*-infected females produced males and 56% of these males were also infected with *Wolbachia*. Neither offspring sex ratio nor male infection frequency were significantly affected by rearing temperature or *Wolbachia* concentration of the mother. The mitochondrial DNA sequence of one of the uninfected populations was identical to that of two of the infected populations. Therefore, the initial *Wolbachia* infection of *A. japonica* must have occurred recently. Mitochondrial sequence variation among the infected populations suggests that the spread of *Wolbachia* through the host populations involved horizontal transmission.

We conclude that the occasional male production by *Wolbachia*-infected females is most likely a maladaptive side-effect of incomplete co-evolution between symbiont and host in this relatively young infection.

Introduction

Wolbachia are maternally inherited, intracellular, symbiotic bacteria belonging to the order Rickettsiales within the α-Proteobacteria. It has been estimated that *Wolbachia* infect about 66% of all insect species, and either some or all individuals per species (Hilgenboecker *et al.* 2008). To enhance its own transmission, *Wolbachia* can induce various alterations of the reproduction of its host, such as cytoplasmic incompatibility, feminization, male-killing and parthenogenesis induction (PI). *Wolbachia*-induced parthenogenesis is most commonly found in haplodiploid organisms, such as Hymenoptera (Werren 1997, Stouthamer *et al.* 1999, Werren *et al.* 2008). In uninfected haplodiploid organisms, fertilized eggs develop into diploid daughters and unfertilized eggs develop into haploid sons (arrhenotoky). In haplodiploids, PI-*Wolbachia* cause diploidization of the haploid eggs by alteration of meiotic and/or mitotic processes (Huigens & Stouthamer 2003, Pannebakker *et al.* 2004a) resulting in the production of daughters from unfertilized eggs (thelytoky). In most cases of *Wolbachia*-induced parthenogenesis, the infection is fixed and the whole host population consists of females (Huigens & Stouthamer 2003). Males tend to be absent or rare in such populations. Here, we identify an exception to this pattern and examine its cause.

Asobara japonica (Hymenoptera: Braconidae) is a larval-pupal parasitoid of drosophilid flies and naturally occurs in Japan. Populations of *A. japonica* on the main islands of Japan exhibit highly female biased sex ratios (92.7% - 99.2% females), whereas population sex ratios on the smaller southern islands are not biased (Mitsui *et al.* 2007). The populations on the main islands are infected with parthenogenesis-inducing *Wolbachia*, while the populations on the smaller southern islands are not (Kremer *et al.* 2009). Also, during routine culturing in our lab, *Wolbachia*-infected *A. japonica* females regularly produce small numbers of male offspring and in rare cases even male-biased sex ratios. The production of males in parthenogenetic populations of *A. japonica* seems to be maladaptive, because parthenogenetic females are not capable of sexual reproduction (Kremer *et al.* 2009).

The occasional male production suggests that transmission of *Wolbachia* from mother to daughter is not always 100% efficient. In several hosts, infected females loose their *Wolbachia* when exposed to high temperatures (e.g. Pijls *et al.* 1996, Clancy & Hoffmann 1998, Pintureau *et al.* 1999, Hurst *et al.* 2000). Eggs laid at high temperatures would then contain low *Wolbachia* concentrations, which may cause the effect of *Wolbachia* to be reduced or lost (Clancy & Hoffmann 1998, Hurst *et al.* 2000). In PI-*Wolbachia*-infected females this would lead to male offspring production. A similar effect would be predicted if *Wolbachia* concentrations are

reduced for reasons other than temperature. In contrast, Mouton *et al.* (2006) found increased *Wolbachia* densities in the parasitoid wasp *Leptopilina heterotoma* when reared at high temperatures, but this did not influence the effect of *Wolbachia* on its host.

An alternative explanation for inefficient transmission of *Wolbachia* might be that the *Wolbachia* infection is relatively young. While vertical transmission is the main transmission mode of *Wolbachia* within established hosts, horizontal transmission plays a major role in the spread of *Wolbachia* in(to) novel hosts (Hurst *et al.* 1992, O'Neill *et al.* 1992, Rousset *et al.* 1992, Werren *et al.* 1995, Vavre *et al.* 1999, Huigens *et al.* 2000, 2004, Kraaijeveld *et al.* 2011a). This predicts that upon invasion of a new host population, *Wolbachia* would initially be selected for efficient horizontal transmission, as well as efficient vertical transmission. This would be followed by adaptation to vertical transmission only once most of the host population is infected. For example, the parasitoid wasp *Leptopilina boulardi* is infected with a symbiont that manipulates the superparasitism behaviour of its host in order to enhance its own horizontal transmission (Varaldi *et al.* 2003). Successful experimental horizontal transmission of *Wolbachia* often leads to unstable infections in the new host and reduced or altered expression of the reproductive manipulation (Grenier *et al.* 1998, Heath *et al.* 1999, Huigens *et al.* 2004, Jaenike 2007). An explanation for such poor vertical transmission might be residual incompatibilities or asynchronies between *Wolbachia* and the new host (Heath *et al.* 1999).

In this paper, we investigate why PI-*Wolbachia*-infected *A. japonica* females produce males. First, we quantified how often and in what numbers male offspring are produced by infected females and whether male production is affected by rearing temperature. We predicted that higher rearing temperatures would result in higher offspring sex ratios (more males). Second, we examined whether male offspring production is influenced by *Wolbachia* titer of the mother. Quantitative PCR was used to measure *Wolbachia* concentrations of *A. japonica* females. We predicted that lower *Wolbachia* concentrations would lead to higher offspring sex ratios. Last, we used mitochondrial DNA sequences to date the initial infection of *A. japonica* with *Wolbachia*. We hypothesized that the *Wolbachia* infection in *A. japonica* is relatively young.

As far as we know, this is the first example from the field of a possible relation between incomplete *Wolbachia*-host co-adaptation and a relatively recent *Wolbachia* infection.

Materials and Methods

Asobara japonica strains

Five *Wolbachia*-infected thelytokous strains of the parasitoid wasp *Asobara japonica* (Hymenoptera: Braconidae) were used in all experiments: Sapporo, Hirosaki, Sendai, Tokyo and Kagoshima. In addition, two uninfected arrhenotokous strains were used for the mitochondrial DNA analysis: Amami and Iriomote. The strains were kindly provided by M.T. Kimura from cultures derived from the field samples, collected along the entire length of Japan, described in Mitsui *et al.* (2007) and Murata *et al.* (2009). Maps of the sampled locations can be found in both papers. These strains were subsequently maintained in our lab under a partial inbreeding regime. Each generation, three females per strain were allowed to parasitize about 100-200 two-day-old (first or second instar) larvae of *Drosophila melanogaster* in glass jars with a medium of agar covered by a layer of 2 ml baker's yeast suspension and kept at 25°C, light:dark 16:8, 65% relative humidity.

Temperature experiment

To test whether temperature affected male production, we examined the offspring sex ratio of *Wolbachia*-infected thelytokous strains of *A. japonica* at two temperatures. One-week-old females from five thelytokous strains (Sapporo, Hirosaki, Sendai, Tokyo and Kagoshima) were placed individually in a glass jar with a medium of agar covered by a layer of 2 ml baker's yeast suspension, in which they were allowed to parasitize about 100-200 two-day-old (first or second instar) larvae of *D. melanogaster*. Because thelytokous *A. japonica* females are not capable of sexual reproduction (Kremer *et al.* 2009), we assumed that all females used in the sex ratio experiment were virgins. For each strain, half of the females were kept at 25°C, light:dark 16:8, 65% relative humidity, while the other half were kept at 20°C, light:dark 16:8, 65% relative humidity. We chose the normal rearing temperature as the highest temperature, because we previously observed that males are produced at that temperature. After 10-13 days, mothers were removed from the jars. The number of male and female offspring was counted several times a week for the next seven weeks. To count the offspring, wasps were anaesthetized with CO_2. Females could be distinguished from males by their ovipositor, which permanently and prominently protrudes from the posterior abdomen. Mothers and offspring were stored separately in 70% ethanol in 1.5 ml Eppendorf tubes until DNA extraction.

DNA extraction

DNA extractions were performed using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol, using mini spin columns. Before starting the DNA extraction, each wasp was transferred to a new 1.5 ml Eppendorf tube. After evaporation of remaining ethanol, tissue lysis buffer (ATL) was added to the tube and the wasp was crushed using a plastic pestle. The

tissue was incubated overnight in proteinase K at 56°C. The DNA was dissolved in 100 μl elution buffer (AE).

Wolbachia detection

All male offspring were tested for *Wolbachia* infection by amplifying the *Wolbachia*-specific *wsp* and *ftsZ* genes, using the primers *wsp*-81F/*wsp*-691R (Braig *et al.* 1998, Zhou *et al.* 1998) and *ftsZ*-F/*ftsZ*-R (Holden *et al.* 1993, Sinkins *et al.* 1995), respectively. Polymerase Chain Reactions (PCR) were performed in a total volume of 20.0 μl, containing 1x PCR-buffer (Qiagen), 62.5 μM dNTPs, 1 unit Taq polymerase, 250 nM forward primer, 250 nM reverse primer and 2.0 μl DNA template. A PTC-200 DNA Engine Thermal Cycler PCR machine (MJ Research, Waltham, MA, USA) was used for all PCRs. PCR conditions for the *wsp* gene were as follows: 3 min at 94°C, then 35 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C, and finally 5 min at 72°C. PCR conditions for the *ftsZ* gene were as follows: 3 min at 94°C, then 35 cycles of 45 sec at 94°C, 1 min at 55°C and 1 min at 72°C and finally 5 min at 72°C. All PCR products were run on a 2% agarose gel and visualized using ethidium bromide staining.

Quantitative PCR analysis

To test whether offspring sex ratios produced by *Wolbachia*-infected females were affected by *Wolbachia* titer, we measured the *Wolbachia* concentration of the mothers from the temperature experiment using quantitative Polymerase Chain Reactions (qPCR). We also tested whether the *Wolbachia* concentration of the mother affected the percentage of *Wolbachia*-infected males among her offspring.

qPCRs were performed for the *Wolbachia*-specific *wsp* and *gatB* genes, with the nuclear *ITS2* gene as a control. To optimize the qPCRs, we designed new primers for all three genes. Primer sequences for *wsp* and *gatB* were based on the sequences for *wAjap* described in chapter 7, Kraaijeveld *et al.* (2011b), while the primer sequences for *ITS2* were based on the sequence for *A. japonica* described in Kremer *et al.* (2009). The following primers were used: *wsp-wAjap*-F: 5'- GAG GCA AAA TTT ACG CCA GA -3' and *wsp-wAjap*-R: 5'- AAC TAG CCC TGA AAT TGC TGT TA -3', producing a 60 bp amplicon; *gatB-wAjap*-F: 5'- GAA GCA AAG AGG ATG CAA GC -3' and *gatB-wAjap*-R: 5'- TCC TGG CTT ACC TCA ACA GG -3', producing a 73 bp amplicon; *ITS2-Ajap*-F: 5'- GGC AAG CAC AAT CAA GGT CT -3' and *ITS2-Ajap*-R: 5'- ACA AAA ACA AAT TTT GCG GC -3', producing a 93 bp amplicon. Each qPCR was performed in a total volume 10.0 μl, containing 1x SybrGreen Mastermix (Roche, Penzberg, Germany), 300 nM forward primer, 300 nM reverse primer and 2.0 μl DNA template. A LightCycler 480 Real-Time PCR System (Roche) was used for all qPCRs. qPCR conditions for all genes were as follows: 10 min at 95°C, then 45 cycles of 10 sec at 95°C, 30 sec at 60°C and 20 sec at 72°C, and finally 5 min at 72°C. Each qPCR was performed in triplicate.

The resulting Ct values were used to calculate the relative quantity of the focal gene:

relative gene quantity = mean PCR efficiency $^{\text{mean overall Ct} - \text{mean sample Ct}}$

To correct for the total amount of DNA, we calculated the ratio between the *Wolbachia* gene and the control gene:

ratio = relative quantity *Wolbachia* gene / relative quantity control gene

Mitochondrial DNA analysis

To estimate the age of the *Wolbachia* infection, we sequenced part of the mitochondrial DNA of *A. japonica* from both infected and uninfected strains. We sequenced part of the *NADH 1 dehydrogenase* (*ND1*) gene, using the primers *ND1*-F (Smith & Kambhampati 1999) and *ND1*-R (Smith *et al.* 1999), producing a 447 bp amplicon, and the *cytochrome oxidase 1* (*CO1*) gene, using the primers *CO1*-1775F/*CO1*-2413R and *CO1*-2222F/*CO1*-2773R (Scheffer & Grissell 2003), together producing a 997 bp amplicon. PCRs were performed in a total volume of 25.0 µl, containing 1x PCR-buffer (Qiagen), 750 µM (extra) MgCl$_2$, 200 µM dNTPs, 1.25 units Taq polymerase, 320 nM forward primer, 320 nM reverse primer and 2.5 µl DNA template. A PTC-200 DNA Engine Thermal Cycler PCR machine (MJ Research) was used for all PCRs. PCR conditions for both genes were as follows: 5 min at 94°C, then 40 cycles of 1 min at 94°C, 1 min at 50°C and 1 min at 72°C, and finally 10 min at 72°C. All PCR products were run on a 2% agarose gel and visualized using ethidium bromide staining.

Sequencing was performed by Macrogen Inc. (Seoul, Korea). Sequences were checked with Sequencher software (version 4.2; Gene Codes, Ann Arbor, MI, USA) and aligned with BioEdit software (version 7.0.9; Hall 1999).

Haplotype diversity and nucleotide diversity were calculated using DnaSP software (version 5.10; Librado & Rozas 2009). The haplotype diversity (H_d), the nucleotide diversity (π), the average number of synonymous substitutions per synonymous site between populations (k_s) and the average number of nucleotide substitutions per site between populations (D_{xy}) were determined. Median joining haplotype networks were drawn using Network software (version 4.6.0.0; Bandelt *et al.* 1999).

To calculate the divergence time between strains, we used the estimates for the *CO1* gene mutation rate in the parasitoid wasp *Nasonia* (Hymenoptera: Pteromalidae), described in Raychoudhury *et al.* (2010). However, mutation rates can vary considerably between species. Therefore, the calculated divergence times are only very rough estimates and must be interpreted with caution. The mitochondrial mutation rate in *Nasonia* was estimated to be 3.5 to 13 times higher

than in *Drosophila melanogaster* (6.2×10^{-8} mutations per site per generation; Haag-Liautard *et al.* 2008), i.e. 2.2×10^{-7} to 8.1×10^{-7} mutations per site per generation (Raychoudhury *et al.* 2010). The divergence time (in generations) between two populations can be calculated by dividing k_s (the average number of synonymous substitutions per synonymous site between populations) by this mutation rate (the number of mutations per site per generation).

Statistical analysis

Statistical analyses were performed in R software (version 2.12.1; R Developmental Core Team 2010). Generalized linear models (glm) with a binomial error distribution and an empirically estimated scale parameter were used to test for differences in sex ratio and male infection frequency. In the sex ratio model, the number of males was used as the response variable and clutch size as the binomial denominator. In the male infection frequency model, the number of infected males was used as the response variable and the total number of males as the binomial denominator. Significance of explanatory variables (*Wolbachia* concentration of the mother, rearing temperature and *A. japonica* strain) was tested by dropping (interactions between) explanatory variables from the model and comparing the resulting change in deviance using an *F*-test. Because the *Wolbachia* concentration of the mother was measured twice, using two different genes for *Wolbachia*, the correlation between the two estimates was calculated using the Pearson's product moment correlation method.

Results

Temperature experiment

In total, 100 individual females were used: 20 females from each of the five thelytokous strains, of which ten were kept at 25°C and ten at 20°C. All females produced offspring (ranging from 5 to 133 individuals). Sixty-seven of these females produced only daughters, while 33 females produced both daughters and sons (ranging from 1 to 69 sons per clutch). The mean sex ratio was 2%, ranging from 0% to 60% per clutch. Most of the male producing females produced small numbers of males, with sex ratios ranging from 1% to 11%. Only two females produced higher sex ratios (Sapporo at 25°C: clutch size = 121, 57% males and Tokyo at 20°C: clutch size = 45, 60% males). There were no significant differences in sex ratio between rearing temperatures, strains, or their interaction (Fig. 6.1; overall: $F_{9,90} = 1.77$, $p = 0.09$; interaction: $F_{4,90} = 1.18$, $p = 0.33$; temperature: $F_{1,98} = 1.08$, $p = 0.30$; strain: $F_{4,95} = 1.46$, $p = 0.22$).
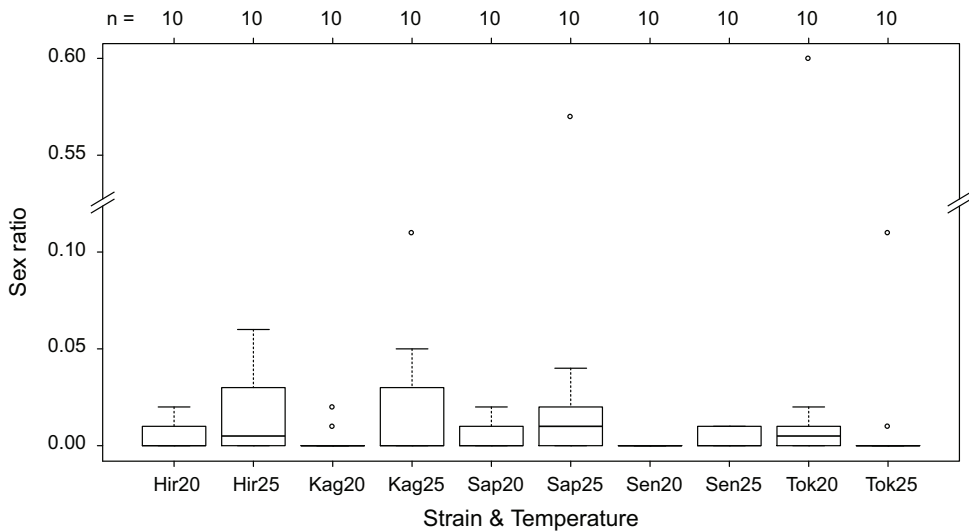
Figure 6.1: Sex ratio (proportion males) of *Asobara japonica* per strain (Hir = Hirosaki, Kag = Kagoshima, Sap = Sapporo, Sen = Sendai, Tok = Tokyo) and rearing temperature (20°C and 25°C). Number of clutches (n = sample size) are indicated above the graph. The horizontal dark lines represent the median sex ratios, the bottom and top of the boxes indicate the 25th and 75th percentiles, the whiskers show up to 1.5 times the interquartile range and the dots represent outliers.

Wolbachia detection

The two *Wolbachia*-specific genes used in the qPCRs were amplified in all samples. Therefore, all mothers were infected with *Wolbachia*.

In total, 177 sons were produced by 33 thelytokous females. Of these, 100 (56%, produced by 31 females) were infected with *Wolbachia*. The male infection frequency per clutch ranged from 0% to 100% (mean 88%). There was a significant interaction effect between rearing temperatures and strains on male infection frequency (Fig. 6.2; overall: $F_{8,24} = 5.99$, $p < 0.001$; interaction: $F_{3,24} = 4.05$, $p = 0.02$). However, this effect appeared to be spurious. When we removed two outlying data points with extreme sex ratios, there were no significant differences in male infection frequency between rearing temperatures, strains or their interaction (Fig. 6.2; overall: $F_{8,22} = 1.41$, $p = 0.25$; interaction: $F_{3,22} = 2.16$, $p = 0.12$; temperature: $F_{1,29} = 0.58$, $p = 0.45$; strain: $F_{4,26} = 0.71$, $p = 0.59$).

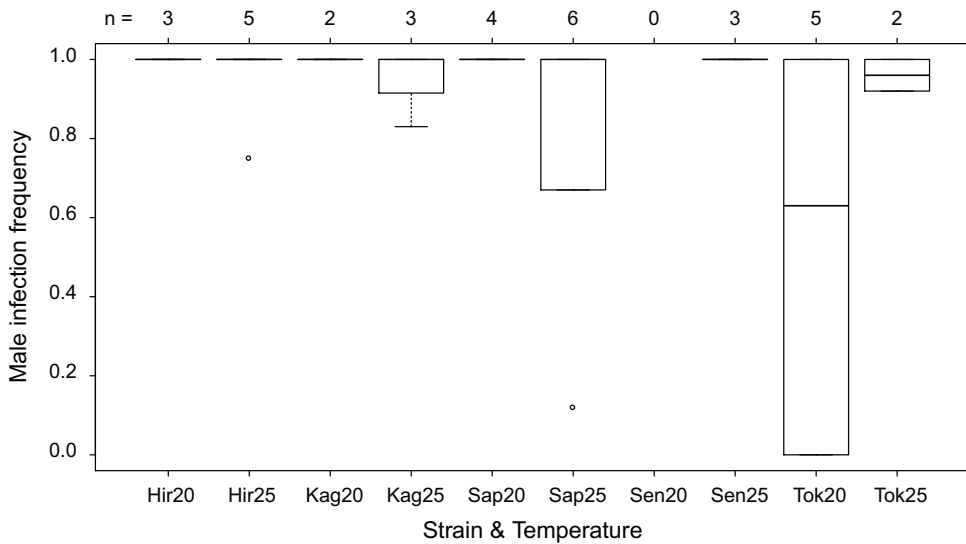**Figure 6.2:** Male infection frequency (proportion infected males) of *Asobara japonica* per strain (Hir = Hirosaki, Kag = Kagoshima, Sap = Sapporo, Sen = Sendai, Tok = Tokyo) and rearing temperature (20°C and 25°C). Number of clutches (n = sample size) are indicated above the graph. The horizontal dark lines represent the median male infection frequencies, the bottom and top of the boxes indicate the 25th and 75th percentiles, the whiskers show up to 1.5 times the interquartile range and the dots represent outliers.

Quantitative PCR analysis

The *Wolbachia* concentration of 64 females from the temperature experiment was measured. Half of these females produced both daughters and sons, while the other half produced only daughters (*gatB*: $n_{withsons} = 32$, $n_{withoutsons} = 32$; *wsp*: $n_{withsons} = 30$, $n_{withoutsons} = 31$). The two estimates for *Wolbachia* concentration (*gatB/ITS2* and *wsp/ITS2*) correlated significantly with each other ($r = 0.92$, $t = 17.90$, $df = 59$, $p < 0.0001$).

There were no significant relations between sex ratio and *Wolbachia* concentration of the mother, rearing temperatures, strains or their interactions (*gatB*: Fig. 6.3; overall: $F_{7,56} = 0.08$, $p = 0.99$; *Wolbachia*: $F_{1,62} = 1.20$, $p = 0.28$; temperature: $F_{1,62} = 0.75$, $p = 0.39$; strain: $F_{1,62} = 0.15$, $p = 0.70$; none of the interactions between explanatory variables were significant; *wsp*: overall: $F_{7,53} = 0.30$, $p = 0.95$; *Wolbachia*: $F_{1,59} = 0.32$, $p = 0.58$; temperature: $F_{1,59} = 0.47$, $p = 0.50$; strain: $F_{1,59} = 0.04$, $p = 0.85$; none of the interactions between explanatory variables were significant).
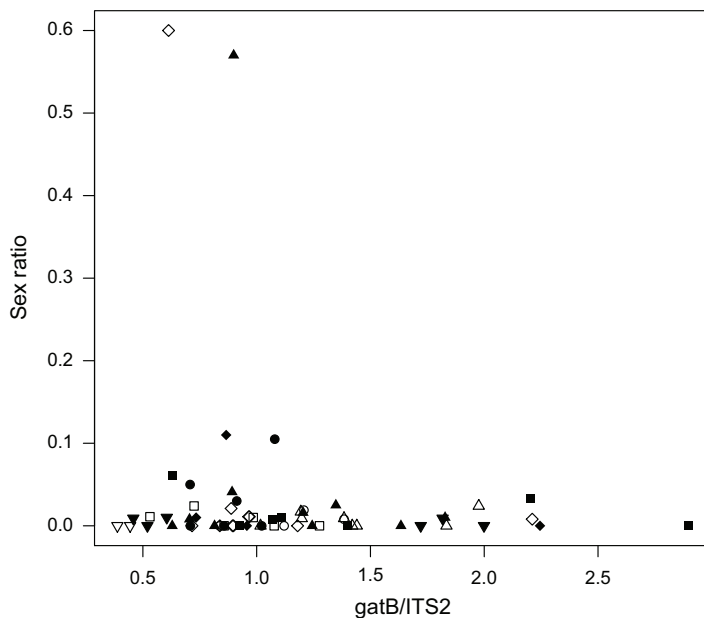
Figure 6.3: Relation between sex ratio (proportion males) and *Wolbachia* concentration (measured as the ratio between the *Wolbachia*-specific gene *gatB* and the control gene *ITS2*) of *Asobara japonica* per rearing temperature (white symbols: 20°C, black symbols: 25°C) and strain (squares: Hirosaki, circles: Kagoshima, upward triangles: Sapporo, downward triangles: Sendai, diamonds: Tokyo).

Also, there were no significant relations between male infection frequency and *Wolbachia* concentration of the mother, rearing temperatures, strains or their interactions (*gatB*: Fig. 6.4; overall: $F_{7,24} = 1.05$, $p = 0.42$; *Wolbachia*: $F_{1,30} = 1.17$, $p = 0.29$; temperature: $F_{1,30} = 2.20$, $p = 0.15$; strain: $F_{1,30} = 0.03$, $p = 0.86$; none of the interactions between explanatory variables were significant; *wsp*: overall: $F_{7,22} = 1.24$, $p = 0.33$; *Wolbachia*: $F_{1,28} = 0.01$, $p = 0.91$; temperature: $F_{1,28} = 3.73$, $p = 0.06$; strain: $F_{1,28} = 0.53$, $p = 0.47$; none of the interactions between explanatory variables were significant).
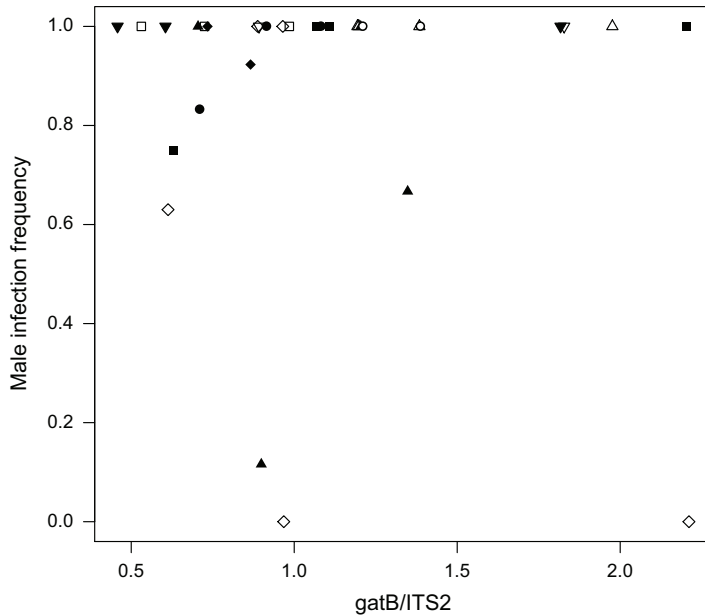
Figure 6.4: Relation between male infection frequency (proportion infected males) and *Wolbachia* concentration (measured as the ratio between the *Wolbachia*-specific gene *gatB* and the control gene *ITS2*) of *Asobara japonica* per rearing temperature (white symbols: 20°C, black symbols: 25°C) and strain (squares: Hirosaki, circles: Kagoshima, upward triangles: Sapporo, downward triangles: Sendai, diamonds: Tokyo).

Mitochondrial DNA analysis

We sequenced part of the mitochondrial DNA of seven females, representing five thelytokous and two arrhenotokous strains of *A. japonica*. The sequences have been submitted to the GenBank database (*CO1* accession numbers: JF430425-JF430431; *ND1* accession numbers: JF430432-JF430438).

We found five unique mitochondrial haplotypes ($H_d = 0.8571$). Two thelytokous strains (Hirosaki and Sendai) and the arrhenotokous strain Amami exhibited the same DNA sequence and thus shared the same haplotype, while the other four
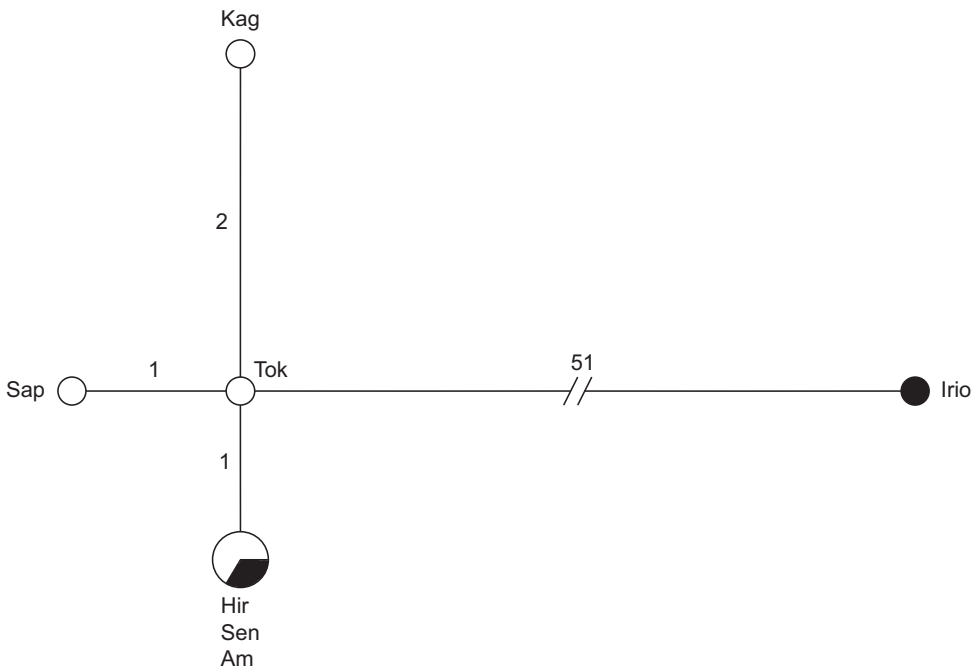
Figure 6.5: Haplotype network based on 997 bp of the mitochondrial *CO1* gene and 447 bp of the mitochondrial *ND1* gene of *Asobara japonica*. Each circle represents one haplotype. The size of the circle represents the haplotype frequency. White circles indicate haplotypes found in thelytokous strains, black circles indicate haplotypes found in arrhenotokous strains, mixed black and white circles indicate haplotypes shared by thelytokous and arrhenotokous strains. Strain names are given for the haplotype in which they were found (Sap = Sapporo, Hir = Hirosaki, Sen = Sendai, Tok = Tokyo, Kag = Kagoshima, Am = Amami, Irio = Iriomote). Lines show mutational routes between haplotypes. The length of the lines and the numbers on the lines indicate the number of mutations between haplotypes.

strains (Sapporo, Tokyo, Kagoshima and Iriomote) all exhibited unique haplotypes (Fig. 6.5). These five haplotypes contained 55 polymorphic sites ($\pi = 0.0111$), of which 50 were synonymous and five were non-synonymous mutations. The arrhenotokous strain Iriomote differed with 51 to 53 mutations from all the other haplotypes ($k_s = 0.1741$; $D_{xy} = 0.0360$). Within these other four haplotypes there were zero to three mutations between strains and four polymorphic sites ($\pi = 0.0011$; 3 synonymous and 1 non-synonymous mutations).

The five haplotypes could be distinguished within the *CO1* gene sequence ($H_d = 0.8571$). Within the *CO1* gene, the five haplotypes contained 40 polymorphic sites ($\pi = 0.0118$; 37 synonymous and 3 non-synonymous mutations). The arrhenotokous strain Iriomote differed with 36 to 38 mutations from all the other haplotypes ($k_s = 0.1788$; $D_{xy} = 0.0371$). Within these other four haplotypes there were zero to three mutations between strains and four polymorphic sites ($\pi = 0.0016$; 3 synonymous and 1 non-synonymous mutations). Only two haplotypes were found for the *ND1* gene sequence ($H_d = 0.2857$). All thelytokous strains (Sapporo, Hirosaki, Sendai, Tokyo and Kagoshima) and the arrhenotokous strain Amami exhibited the same *ND1* haplotype, while the second *ND1* haplotype only was represented by the arrhenotokous strain Iriomote. The two *ND1* haplotypes differed by 15 polymorphic sites ($\pi = 0.0096$; 13 synonymous and 2 non-synonymous mutations; $k_s = 0.1625$; $D_{xy} = 0.0336$).

The mean divergence time, based only on the *CO1* gene, between the arrhenotokous strain Iriomote and the other six strains ($k_s = 0.1788$) was estimated to be between 8.2×10^5 and 2.2×10^5 generations ago. Because the arrhenotokous strain from Amami exhibited the same mitochondrial haplotype as two thelytokous strains, we concluded that these strains have not (yet) diverged in their mtDNA.

Discussion

PI-*Wolbachia*-infected *A. japonica* females regularly produced small numbers of male offspring and rarely male-biased offspring sex ratios. Slightly more than half of these males were infected with *Wolbachia*. Neither offspring sex ratio nor male infection frequency were affected by strain, rearing temperature or *Wolbachia* concentration of the mother.

Within seven strains of *A. japonica*, we found five unique mitochondrial haplotypes. Four of these haplotypes, represented by six strains, were closely related, while the uninfected arrhenotokous strain from Iriomote was very different from the rest. The uninfected arrhenotokous strain from Amami exhibited the same haplotype as two thelytokous strains from Hirosaki and Sendai. More or less the same pattern, based on 645 bp of the mitochondrial *CO1* gene, was found by Murata *et al.* (2009).

Fertile crosses between males and females from Iriomote and Amami and between (natural and cured) males from Kagoshima and females from Amami indicate that

individuals from these strains belong to the same species (Murata *et al.* 2009, chapter 7, Kraaijeveld *et al.* 2011b). However, Murata *et al.* (2009) also found indications for weak asymmetrical sexual isolation, suggesting that Iriomote and Amami have been geographically isolated for a long time. Based on the mitochondrial DNA sequences, we estimated that the divergence time between Iriomote and the other strains was between 8.2×10^5 and 2.2×10^5 generations ago. However, given that these estimates are extrapolated from mutation rates in a different species (*Nasonia*), they should be interpreted with caution.

The uninfected arrhenotokous strain from Amami exhibited the same mitochondrial haplotype as two thelytokous strains. There also was very little mitochondrial variation between Amami and the other three thelytokous strains. Moreover, there is no variation between the *Wolbachia* strains in the five thelytokous *A. japonica* strains (chapter 7, Kraaijeveld *et al.* 2011b). This suggests that the *Wolbachia* infection in *A. japonica* is relatively young. An alternative possibility might be that the strain from Amami was infected with *Wolbachia* before, but has lost its infection. However, this seems unlikely because thelytokous females that have been cured from their *Wolbachia* infection with antibiotics are not capable of sexual reproduction (Kremer *et al.* 2009).

New bacterial symbiont infections can spread rapidly in host populations. Invasions of *Wolbachia* in *Drosophila simulans* in California and *Rickettsia* in *Bemisia tabaci* in Arizona have been reported in which the infection frequency increased from 0% to near fixation in less than 100 generations (Turelli & Hoffmann 1991, Himler *et al.* 2011). The mitochondrial variation among the five thelytokous strains suggests that multiple infection events have occurred and that the *Wolbachia* infection has spread (partly) via horizontal transmission. The five infected thelytokous strains were collected from the two Japanese main islands, with 1580 km distance between the northernmost location Sapporo and the southernmost location Kagoshima (for maps of the locations see Mitsui *et al.* 2007 and Murata *et al.* 2009). The distance between the two main Japanese islands is very small (20 km) and the *Wolbachia* infection probably spread easily between the two islands. However, the distance between the island of Amami and the main islands of Japan may be too large (290 km) for the *Wolbachia* infection to invade Amami. No geographical gradient could be distinguished from the mitochondrial data. Although Kagoshima and Amami are geographically closest to each other (370 km), based on their mtDNA they are the least closely related within the 'thelytokous' population. Also, Hirosaki and Sendai are geographically distant from Amami (1680 km and 1520 km, respectively), but based on their mtDNA they are closely related.

The occasional male production by PI-*Wolbachia*-infected *A. japonica* females is most likely due to a relatively young *Wolbachia* infection. There may have been too little time to allow complete co-evolution between *Wolbachia* and *A. japonica*. Incomplete co-adaptation may be caused by remaining incompatibilities or asyn-

chronies between *Wolbachia* and its host (Heath *et al.* 1999). The incomplete adaptation of *Wolbachia* to its host *A. japonica* may lead to incomplete diploidization of the haploid eggs, so that part of the eggs remains haploid and develop into sons. Because thelytokous *A. japonica* females are not capable of sexual reproduction (Kremer *et al.* 2009), these males will have zero fitness.

The inability of thelytokous *A. japonica* females to reproduce sexually could be due to selection against the maintenance of costly sexual traits, or to accumulation of neutral mutations (Pijls *et al.* 1996). However, this interpretation seems at odds with the recent origin of the *Wolbachia* infection in this species. The spread of parthenogenesis-inducing *Wolbachia* may be facilitated by the concomitant spread of 'functional virginity mutations' (Stouthamer *et al.* 2010). Mutations that prevent females from fertilizing their eggs will have a selective advantage in the presence of PI-*Wolbachia*-infected females because they induce the bearer to produce more sons which will have many mating opportunities. Virginity mutations may thus explain both the rapid spread of PI-*Wolbachia* through the population of *A. japonica* and the inability of *A. japonica* females to reproduce sexually.

We conclude that the occasional male production by PI-*Wolbachia*-infected *A. japonica* females is not due to high rearing temperatures or low *Wolbachia* concentrations of the mother, but most likely is a maladaptive side-effect of the relatively young age of the *Wolbachia* infection. *Wolbachia* possibly is not (yet) fully adapted to its host *A. japonica*.

Chapter 7

Does a parthenogenesis-inducing *Wolbachia* induce vestigial cytoplasmic incompatibility?

Ken Kraaijeveld, Barbara M. Reumer, Laurence Mouton, Natacha Kremer, Fabrice Vavre & Jacques J.M. van Alphen

Wolbachia is a maternally inherited bacterium that manipulates the reproduction of its host. Recent studies have shown that male-killing strains can induce cytoplasmic incompatibility (CI) when introgressed into a resistant host. Phylogenetic studies suggest that transitions between CI and other *Wolbachia* phenotypes have also occurred frequently, raising the possibility that latent CI may be widespread among *Wolbachia*. Here, we investigate whether a parthenogenesis-inducing *Wolbachia* strain can also induce CI. Parthenogenetic females of the parasitoid wasp *Asobara japonica* regularly produce a small number of males that may be either infected or not. Uninfected males were further obtained through removal of the *Wolbachia* using antibiotics and from a naturally uninfected strain. Uninfected females that had mated with infected males produced a slightly, but significantly more male-biased sex ratio than uninfected females mated with uninfected males. This effect was strongest in females mated with males that had a relatively high *Wolbachia* titer. Quantitative PCR indicated that infected males did not show higher ratios of nuclear versus mitochondrial DNA content. *Wolbachia* therefore does not cause diploidization of cells in infected males. While these results are consistent with CI, other alternatives such as production of abnormal sperm by infected males cannot be completely ruled out. Overall, the effect was very small (9%), suggesting that if CI is involved it may have degenerated through the accumulation of mutations.

Introduction

Wolbachia is a cytoplasmically inherited bacterium, known for its ability to manipulate reproduction in its arthropod hosts (Stouthamer *et al.* 1999). These manipulations include the induction of parthenogenesis (PI), male-killing, feminization and cytoplasmic incompatibility (CI). The first three result in highly female-biased sex ratios, while CI decreases the offspring production of uninfected females by inducing sterility in crosses between infected males and uninfected females. In haplodiploids, only fertilized eggs, which normally develop into diploid females, suffer CI, while unfertilized eggs, which develop into haploid males, do not. Incompatible eggs either die or are converted into males. In both cases, CI results in a more male-biased sex ratio.

Phylogenetically, *Wolbachia* strains inducing different phenotypes do not form monophyletic clusters (Baldo *et al.* 2006), suggesting that switches between phenotypes have occurred repeatedly. Recurrent acquisition and loss of genes involved in reproductive manipulations in *Wolbachia* genomes may lead to rapid changes in the phenotype. Alternatively, all manipulations may be induced by the same *Wolbachia* strain, but their expression may depend on the host genetic background. In support of the latter, several recent studies have shown that some *Wolbachia* can switch rapidly between male-killing and CI (e.g. Jaenike 2007, Hornett *et al.* 2008). For example, Hornett *et al.* (2008) showed that a male-killing *Wolbachia* infecting the butterfly *Hypolimnas bolina* induces CI when introgressed into host strains that are resistant to male-killing, showing it has a latent ability to induce CI. Whether *Wolbachia* that normally induce parthenogenesis or feminization also have the ability to induce CI is unknown.

Hurst *et al.* (2002) showed theoretically that CI-only strains are highly susceptible to invasion by mutants that can manipulate host sex ratios while retaining their CI ability. When sex ratio distortion is complete (i.e. infected females produce no males), as is common for parthenogenesis-inducing strains, CI is not expressed anymore and the ability to cause CI is expected to degrade by selection and/or mutation, resulting in a strain that only distorts the sex ratio (Hurst *et al.* 2002).

In this study, we investigate whether the *Wolbachia* strain *wAjap* that normally induces parthenogenesis in its *Drosophila* parasitoid host *Asobara japonica* (Hymenoptera: Braconidae) can also induce CI. Phylogenetic analysis showed *wAjap* to be closely related to several strains that induce CI (Kremer *et al.* 2009), suggesting that transitions between CI and other phenotypes have occurred in this group. Populations of *A. japonica* on the main islands of Japan reproduce through *Wolbachia*-induced thelytokous parthenogenesis, in which females are produced from unfertilized eggs. Populations from the southern subtropical islands are uninfected and reproduce through arrhenotoky, in which females develop from fertilized, diploid eggs and males from unfertilized, haploid eggs (Mitsui *et al.* 2007, Kremer *et al.* 2009). During routine culturing, infected *A. japonica* females regularly produce

small numbers of males (rarely many), some of which are infected with *Wolbachia*, a situation rarely observed in species infected with PI-*Wolbachia*. This allowed us to investigate whether *wAjap* is able to induce CI.

Materials and Methods

Asobara japonica strains and antibiotic treatment
A. japonica strains were kindly provided by M.T. Kimura from cultures derived from the field samples described in Mitsui *et al.* (2007) and Murata *et al.* (2009). An infected, thelytokous population from Kagoshima and an uninfected, arrhenotokous population from Amami-Oshima were used in all experiments. Culturing and removal of *Wolbachia* were described in Kremer *et al.* (2009). Briefly, parasitoid eggs and larvae were exposed to antibiotics through the host's haemolymph. Most of the antibiotic-treated larvae developed as aposymbiotic females. These females produced only males when allowed to oviposit, indicating they were cured of their *Wolbachia* infection. Few antibiotic-treated larvae developed directly as aposymbiotic males. Hence, we used both males from the first generation, which had been exposed to the antibiotic, and from the second generation, of which only their mother had been exposed.

Mating experiments
To obtain virgin females from the arrhenotokous strain, pupae were transferred individually to PCR tubes just prior to emergence. These tubes were checked twice daily for emerged females, which were then transferred to a small glass tube (2.5 x 8.0 cm) containing a layer of agar. Each female was provided with a single male of one of five types (arrhenotokous and thelytokous refer to the line of origin): uninfected arrhenotokous, uninfected thelytokous antibiotics-exposed, uninfected thelytokous from antibiotics-exposed mother, naturally uninfected thelytokous, infected thelytokous. Males develop faster than females. To ensure that the males were virgin, the culture jars were checked every three hours around the time of emergence and virgin males were transferred to fresh jars and kept in single-sex groups. No males were collected from jars in which females had started emerging. The mating pair was kept together in the glass tube for two days, after which the female was allowed to parasitize about 200 *D. melanogaster* larvae. All males were checked for *Wolbachia* infection through PCR assay (see below). The numbers of male and female offspring emerging from these crosses were scored for the next four weeks.

DNA extraction

DNA extractions were performed using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol, using mini spin columns. Before starting the DNA extraction, each wasp was transferred to a new 1.5 ml Eppendorf tube. After evaporation of remaining ethanol, tissue lysis buffer (ATL) was added to the tube and the wasp was crushed using a plastic pestle. The tissue was incubated overnight in proteinase K at 56°C. The DNA was dissolved in 100 µl elution buffer (AE).

Wolbachia detection

Wasps were tested for *Wolbachia* infection by amplifying the *Wolbachia*-specific *wsp* gene, with primers *wsp*-81F and *wsp*-691R (Braig *et al.* 1998, Zhou *et al.* 1998) and the *Wolbachia*-specific *ftsZ* gene (Holden *et al.* 1993, Sinkins *et al.* 1995). Polymerase Chain Reactions (PCR) for both genes were performed in a total volume of 20.0 µl, containing 1x PCR-buffer (Qiagen), 62.5 µM dNTPs, 1 unit Taq polymerase, 250 nM forward primer, 250 nM reverse primer and 1.0 µl DNA template. A PTC-200 DNA Engine Thermal Cycler PCR machine (MJ Research, Waltham, MA, USA) was used for all PCRs. PCR conditions for the *wsp* gene were as follows: 3 min at 94°C, then 35 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C, and finally 5 min at 72°C. PCR conditions for the *ftsZ* gene were as follows: 3 min at 94°C, then 35 cycles of 45 sec at 94°C, 1 min at 55°C and 1 min at 72°C and finally 5 min at 72°C. All PCR products were run on a 2% agarose gel and visualized using ethidium bromide staining.

Real-time quantitative PCR

To test whether the sex ratios induced by infected males were affected by *Wolbachia* density, we conducted real-time quantitative PCR (qPCR) on the DNA samples from the mating experiment. We also compared *Wolbachia* density between infected males and infected females. *Wolbachia* density was assessed by quantifying the copy number of the *Wolbachia*-specific *wsp* gene, using the nuclear *18S* gene to control for DNA concentration. The number of *Wolbachia* cells was determined by using the generalist primers *wsp*-81F/*wsp*-691R which amplified the single-copy *wsp* gene as described in Mouton *et al.* (2004). The multicopy nuclear gene *18S*rRNA was amplified using the primers *18S*.lo1/NS58+2, as described in Mouton *et al.* (2009).

Furthermore, we tested whether *Wolbachia* infection resulted in diploidization of cells in males. We again quantified the nuclear *18S* gene and compared this to the quantity of the mitochondrial *CO1* (*Cytochrome Oxidase I subunit*) gene, which should not be affected by diploidization. In this test, we compared infected with uninfected males. We designed new primers from the alignment of the *CO1* sequences of 16 *A. japonica* strains (Murata *et al.* 2009) especially to optimize qPCR reactions: *Ajap-CO1*-159F (5'- ACC TGT AAT ATT AGG TGG ATT TGG

-3') and *Ajap-CO1*-289R (5'- CCA ACA CCT ACA TTT AAT ATT CCT CT -3'; amplified product 139bp). The PCR conditions consisted of 10 min at 95°C followed by 35 cycles, each consisting of denaturing for 10 sec at 95°C, annealing for 10 sec at 54°C and elongation for 10 sec at 72°C. qPCR reactions were performed on the LightCycler 480 Real-Time PCR System (Roche, Penzberg, Germany). The 10 µL reaction mix contained 200 nM of each primer, 5 µL of LightCycler 480 SYBR Green I Master (Roche), and 1 µL of template DNA.

MLST sequencing

In order to assess whether the *Wolbachia* infecting the male *A. japonica* was indeed *wAjap* (and not an additional strain that had escaped detection in infected females), we sequenced a set of five MLST genes (Multi Locus Sequence Typing) and the *wsp* gene for one infected male and one infected female for each of five thelytokous *A. japonica* strains (Kagoshima, Sapporo, Hirosaki, Sendai and Tokyo; Mitsui *et al.* 2007). The protocols for PCR and sequencing were described in Baldo *et al.* (2006).

Statistical analysis

The sex ratios of the offspring produced by females mated to different types of males were compared using generalized linear models (glm) with a binomial error distribution and an empirically estimated scale parameter. The number of males was the response variable and the total number of offspring the binomial denominator. Significance was assessed by removing explanatory variables from the model and comparing the change in deviance using an F-test. Clutch size was compared using analysis of variance (anova).

Since the data sets for the qPCR experiments did not follow normal distributions (Shapiro test), we used non-parametric Wilcoxon rank sum tests, with $\alpha = 0.05$.

All analyses were performed in R software (version 2.9.2; R Developmental Core Team 2006), except the power analysis, for which we used the sample size calculator available at: http://www.stat.ubc.ca/~rollin/stats/ssize/n2.html.

Results

Mating experiments

A total of 34 males were produced by infected mothers, of which 18 (53%) tested positive for *Wolbachia* in the PCR assay. The sex ratios produced by arrhenotokous females differed significantly, depending on the type of male they had mated with (Fig. 7.1; $F_{4,64} = 2.81$, $p = 0.03$). Lumping the uninfected male groups did not result in a significant change in deviance of the model (comparison of the model with five male types ($df = 4$) to a model with only infection status ($df = 1$): $F_{3,64} = 0.31$, $p = 0.82$). However, the infection status of the male had a significant effect on the sex ratio of the offspring ($F_{1,67} = 10.61$, $p = 0.002$). The difference

in sex ratio between infected and uninfected groups was 9% (infected: 37% male offspring, uninfected: 28% male offspring). Clutch size was highly variable, but did not differ between females mated to infected or uninfected males (infected males: mean = 114.39 ± 24.91 SD, uninfected males: mean = 104.96 ± 33.91 SD, $F_{1,67} = 1.16$, $p = 0.28$). However, the variability meant that we would have needed to sample about 100 females in each group to detect a 9% difference in clutch size (1-sided test, $\alpha = 0.05$, power = 0.8).
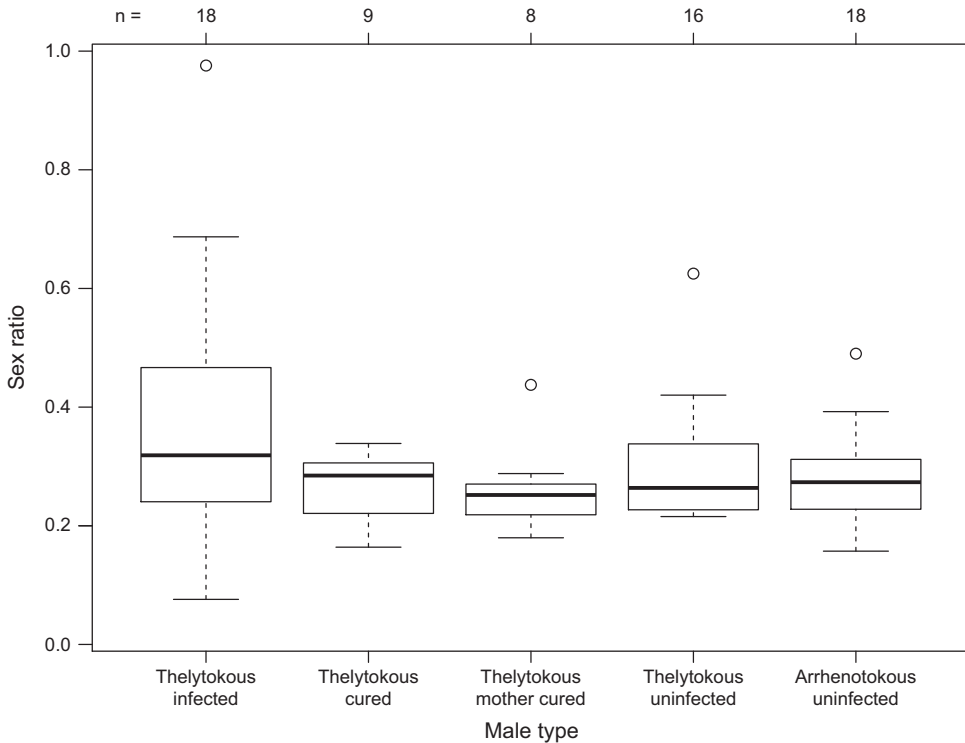
Figure 7.1: Sex ratio (proportion males) of offspring produced by uninfected, arrhenotokous *Asobara japonica* females that mated with various types of males. Sample sizes (n) are indicated above the graph. The horizontal dark lines represent the median sex ratios, the bottom and top of the boxes indicate the 25th and 75th percentiles, the whiskers show up to 1.5 times the interquartile range and the dots represent outliers.

Wolbachia density

Wolbachia density was significantly lower in infected males than in infected females (ratio *wsp/18S*: $n_{males} = 18$, $n_{females} = 20$, Wilcoxon $W = 360$, $p < 0.0001$). Among infected males, *Wolbachia* density correlated with the sex ratio produced by the females they mated with (Fig. 7.2; ratio *wsp/18S*: $F_{1,16} = 10.82$, $p = 0.005$). This correlation was also significant when *CO1* was used instead of *18S* to control for DNA content (ratio *wsp/CO1*: $F_{1,15} = 8.53$, $p = 0.01$). Males with higher *Wolbachia* densities induced more male-biased clutches than males with lower *Wolbachia* titers. There was no difference in the *18S/CO1* ratio between infected and uninfected males from the thelytokous strain, indicating that *Wolbachia* does not cause diploidization of cells in infected males ($n_{infected} = 17$, $n_{unifected} = 13$, Wilcoxon $W = 111$, $p = 0.99$).
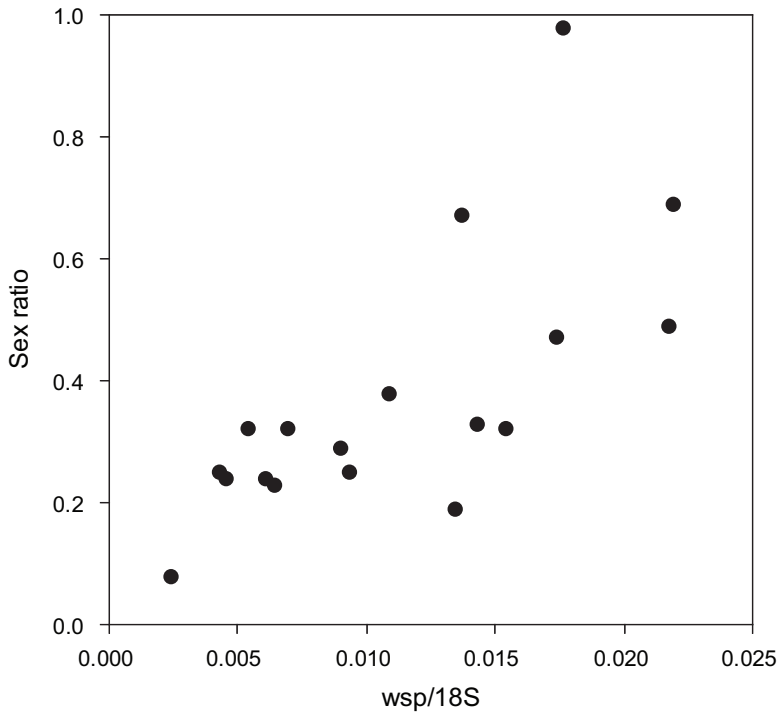
Figure 7.2: Sex ratio (proportion males) of offspring produced by uninfected, arrhenotokous *Asobara japonica* females that mated with males from a thelytokous strain in relation to the *Wolbachia* density in these males. *Wolbachia* density was measured as the ratio between the copy numbers of the *Wolbachia*-specific *wsp* gene and the nuclear *18S* gene.

MLST sequencing

The sequences of *Wolbachia* genes obtained from all males and all females of all strains were identical (GenBank accession numbers: HM241181-HM241186). There was thus no indication that strains other than *wAjap* are infecting *A. japonica*, or that infected thelytokous males harbour a different *Wolbachia* strain than the one that is normally inducing parthenogenesis in this species.

Discussion

Our results show a significant difference between the sex ratios produced by females that were mated to *Wolbachia*-infected males and those of females that were mated to uninfected males. This effect was strongest when the *Wolbachia* density in the infected male was relatively high. Uninfected thelytokous males did not show a reduction in fertilization capacity compared to arrhenotokous males, indicating normal spermatogenesis and absence of nuclear incompatibilities between individuals originating from thelytokous and arrhenotokous populations. Furthermore, *Wolbachia* did not cause (partial) diploidization of infected males. No other symbiont has been detected in *A. japonica* (Kremer *et al.* 2009) and MLST indicated that the *Wolbachia* strain that caused this effect was the same as the strain that induces parthenogenesis in the females. *Wolbachia* infection in males thus causes male-biased sex ratios when crossed with uninfected females.

Different hypotheses can be proposed for explaining this pattern. First, infected males could be mosaics of haploid and diploid cells as happens in *Nasonia* (Kamping *et al.* 2007), leading to production of some diploid sperm. However, infected males did not show an elevated ratio of nuclear versus mitochondrial DNA content compared to uninfected males as would be expected under diploidization. Furthermore, diploid sperm should lead to an increase in mortality in the offspring of these males, for which we find no evidence. Second, the presence of *Wolbachia* may result in a reduction in sperm production, leading to the observed pattern. However, this reduction needs to be substantial, because males used in this experiment were virgin and only had one female to mate with. The third hypothesis is that the parthenogenesis-inducing *Wolbachia* strain *wAjap* is able to induce CI when present in males. One way to test for this hypothesis would be to test for the rescue ability of *wAjap* in infected females. Unfortunately, females from thelytokous strains are not receptive to mating (Kremer *et al.* 2009), making this experiment impossible.

The mean difference in sex ratio induced by infected versus uninfected males was very small (9%) and the frequency distributions between the groups overlapped. If this effect is indeed due to CI expression, there are several potential explanations for the low degree of CI induced by the normally parthenogenesis-inducing *wAjap*. We show that the sex ratio produced by females is correlated to the *Wolbachia* titer in their mates. The males with the highest *Wolbachia* titers in our sample induced sex ratios up to 98% males, which approaches the sex ratio bias seen in incom-

patible crosses in other hymenopterans infected with CI-*Wolbachia*. For example, the difference in sex ratio between compatible and incompatible crosses was 35% in *A. tabida* (Dedeine *et al.* 2004) and 40% in *Trichopria cf. drosophilae* (Vavre *et al.* 2002). CI results in all-male broods in *Leptopilina heterotoma* and *Nasonia vitripennis* (Vavre *et al.* 2001, Bordenstein *et al.* 2003, respectively). Thus, *wAjap* may be able to induce full CI, but the overall low degree of CI that we saw may be due to low *Wolbachia* titers in most infected males. However, given that only one mating resulted in nearly complete sex bias, it is too early to rule out alternative explanations. *A. japonica* may be a host in which *Wolbachia* is not able to induce strong CI or *A. japonica* may be a competent host, but *wAjap* may not be able to induce strong CI. Hurst *et al.* (2002) predicted that an ancestral ability to induce CI would degrade when an invading mutant that distorts sex ratio approaches 100% efficiency. As far as is known, the infection with *wAjap* is fixed on the islands where it occurs. *wAjap* normally induces >97% parthenogenesis. In the field, infected males can not encounter uninfected females, since infected and uninfected populations are on different islands, and females from thelytokous populations do not elicit courtship by males. CI is thus not expressed in the field. In addition, any fitness cost imposed by the retention of CI ability will select for its loss. It is possible that the low level of CI induced by *wAjap* is partly due to this ability having degenerated by selection and/or neutral mutation accumulation.

Contrary to the situation observed in *A. japonica*, several male-killing *Wolbachia* induce complete CI when introgressed within a resistant host background. Why is low CI not seen in male-killing *Wolbachia*? The first possible explanation is related to bacterial load. Resistance to male-killing does not rely on the reduction of the *Wolbachia* titer, but to a direct resistance to the male-killing phenotype. On the contrary, we show here that infected males have low *Wolbachia* density in *A. japonica*, and this might be related to the fact that individuals with high density are diploidized and thus converted into females. This difference in the mechanisms leading to male production may structurally impose lower CI in PI-*Wolbachia* compared to male-killing *Wolbachia*. The other possibility is related to the dynamics of the co-evolutionary process between partners. Male-killing imposes strong selection for suppression in its host, because it kills a large proportion of offspring (Hurst *et al.* 2002). Thus, hosts infected by a male-killing *Wolbachia* may evolve resistance before sufficient time has passed to allow degradation of its ancestral CI ability (Hornett *et al.* 2008). In contrast, parthenogenesis is a very efficient mode of reproduction and selection for its suppression may be weak. In particular, as soon as parthenogenesis is fixed, as seems to be the case in *A. japonica*, there is no selection for resistance. Further understanding of the interplay between parthenogenesis- and CI-induction by *Wolbachia* might come from comparative studies. If the CI ability has indeed degraded in *wAjap*, it might be possible to find stronger CI in host species that have only recently become infected with a parthenogenesis-inducing *Wolbachia*.

Acknowledgements

Chapter 8

General Discussion

I studied the dynamics, causes and consequences of a PI-*Wolbachia* infection in two parasitoid wasps *Tetrastichus coeruleus* and *Asobara japonica*. Interestingly, both species have populations that are infected with *Wolbachia* and reproduce parthenogenetically (thelytoky) and populations that are not infected and reproduce sexually (arrhenotoky) (*T. coeruleus*: chapter 2, Reumer *et al.* 2010; *A. japonica*: Mitsui *et al.* 2007, Kremer *et al.* 2009).

Population genetics shows that different scenarios for the spread of a parthenogenesis-inducing *Wolbachia* infection may occur in different species. A recent study on the PI-*Wolbachia*-infected parasitoid wasp *Leptopilina clavipes*, was the first to show that horizontal transmission of *Wolbachia* plays a major role in the spread of *Wolbachia* between individuals and populations within a novel host (Kraaijeveld *et al.* 2011a, Kremer & Huigens 2011). Although sexual reproduction during the early stages of *Wolbachia* infection may explain the variation in nuclear DNA of *L. clavipes*, it can not explain the variation in mitochondrial DNA (Kraaijeveld *et al.* 2011a, Kremer & Huigens 2011). Similarly, variation in the mitochondrial DNA of *A. japonica* shows that after the initial infection, *Wolbachia* has spread via horizontal transmission (in addition to regular vertical transmission) through the *A. japonica* populations (chapter 6, Reumer *et al.* 2012). I do not have information about the nuclear DNA of *A. japonica*. In contrast, *T. coeruleus* did not exhibit mitochondrial DNA variation, indicating that after the initial infection *Wolbachia* has spread via vertical transmission through the populations and not via horizontal transmission (chapter 3, Reumer *et al.* in prep). Variation in the nuclear DNA of *T. coeruleus* suggests that females mated with males during the early stages of infection, when males were still present, and may still occasionally do so, although males are rare in thelytokous populations (chapter 3, Reumer *et al.* in prep). No variation in *Wolbachia* DNA was found in any of these three parasitoid wasp species (*L. clavipes*: Kraaijeveld *et al.* 2011a; *T. coeruleus*: chapter 2, Reumer *et al.* 2010; *A. japonica*: chapter 7, Kraaijeveld *et al.* 2011b). Therefore, all three species became infected with a single *Wolbachia* strain that has subsequently spread via different mechanisms through the populations of the parasitoid wasps.

Both *T. coeruleus* and *A. japonica* have populations that are infected with *Wolbachia* and populations that are not infected (*T. coeruleus*: chapter 2, Reumer *et al.* 2010; *A. japonica*: Mitsui *et al.* 2007, Kremer *et al.* 2009). The reason for this difference between populations seems to be different for the two species. New bacterial symbiont infections can spread rapidly in host populations. Invasions of *Wolbachia* in *Drosophila simulans* in California and *Rickettsia* (another endosymbiotic bacterium) in *Bemisia tabaci* in Arizona have been reported in which the infection frequency increased from 0% to near fixation in less than 100 generations (Turelli & Hoffmann 1991, Himler *et al.* 2011). However, when host populations occur allopatrically, the spread of a symbiont infection may not be so easy. Similar to *T. coeruleus* and *A. japonica*, the parasitoid wasp *L. clavipes* also has PI-*Wolbachia*-infected and uninfected populations (Pannebakker *et al.* 2004b, Kraaijeveld *et al.* 2011a). Infected populations occur in northern Europe, while uninfected populations occur in Spain. However, no populations were found in the region from the Pyrenees to the Massif Central in France, which seems to act as a geographical barrier preventing gene flow between the infected and uninfected populations (Pannebakker *et al.* 2004b, Kraaijeveld *et al.* 2011a). A geographical barrier also seems to be present between infected and uninfected populations of *A. japonica*. *Wolbachia*-infected populations of *A. japonica* occur on the two Japanese main islands, while uninfected populations occur on the smaller southern islands of Japan (Mitsui *et al.* 2007). The geographical distance between the islands probably is too large for *Wolbachia* to invade the southern islands of Japan (chapter 6, Reumer *et al.* 2012). In contrast, *Wolbachia*-infected and uninfected populations of *T. coeruleus* occur geographically close together within The Netherlands. However, these populations occur in different habitats: *Wolbachia*-infected populations occur in natural dunes areas, while uninfected populations occur on agricultural fields (chapter 2, Reumer *et al.* 2010). Population genetics of *T. coeruleus* shows that there is occasional gene flow from uninfected to infected populations, but not vice versa (chapter 3, Reumer *et al.* in prep). This might explain why the *Wolbachia* infection, until now, never has arrived in the Dutch agricultural fields.

Alternatively, differences between two habitats may select for different *Wolbachia* infection frequencies or reproductive modes. By comparing two populations of *T. coeruleus* that differ in habitat, *Wolbachia* infection and mode of reproduction, I found several differences in life history traits (chapter 4, Reumer *et al.* in prep). An uninfected arrhenotokous population from an agricultural field and a *Wolbachia*-infected thelytokous population from a natural Dutch dune area differed in clutch size, female life span, female weight and female nutrient concentrations. However, in order to determine if and how these life history differences are related to differences in ecology, *Wolbachia* infection or mode of reproduction, life history traits of more populations of *T. coeruleus* should be investigated. This might shed more light on the co-existence of *Wolbachia*-infected and uninfected population of *T. coeruleus* in

geographically close proximity.

In most cases of *Wolbachia*-induced parthenogenesis, the infection is fixed and the entire host population consists of females (Huigens & Stouthamer 2003). However, both in *T. coeruleus* and *A. japonica* a small number of male offspring is regularly produced in the otherwise thelytokously reproducing populations (*T. coeruleus*: chapter 2, Reumer *et al.* 2010; *A. japonica*: Mitsui *et al.* 2007, chapter 6, Reumer *et al.* 2012). In *A. japonica*, this male production seems to be a maladaptive side-effect of incomplete co-adaptation between *Wolbachia* and host because of a relatively young *Wolbachia* infection (chapter 6, Reumer *et al.* 2012). In *T. coeruleus*, the *Wolbachia* infection seems to be older than in *A. japonica* (chapter 3, Reumer *et al.* in prep). Therefore, co-adaptation between *Wolbachia* and *T. coeruleus* could evolve over a longer period of time and male production in thelytokous populations of *T. coeruleus* probably has to be explained in a different way.

In *A. japonica*, more than half of the males produced by *Wolbachia*-infected females were also infected with *Wolbachia* (chapter 6, Reumer *et al.* 2012). This was the exact same strain of *Wolbachia* that causes parthenogenesis in females (chapter 7, Kraaijeveld *et al.* 2011b). This PI-*Wolbachia* strain seems to induce vestigial cytoplasmic incompatibility in infected males (chapter 7, Kraaijeveld *et al.* 2011b). In *T. coeruleus*, none of the males from thelytokous populations were infected with *Wolbachia* (chapter 2, Reumer *et al.* 2010).

Apart from the effect a PI-*Wolbachia* strain may have on infected males, it can also have severe consequences for females. In most cases of *Wolbachia*-induced parthenogenesis, the infection is fixed and the entire host population consists of females (Huigens & Stouthamer 2003). In the absence of males and sexual reproduction, genes involved in sexual reproduction are not actively maintained by selection. Accumulation of neutral mutations or selection against the maintenance of costly sexual traits may lead to their loss or deterioration (Carson *et al.* 1982, Pijls *et al.* 1996, Pannebakker *et al.* 2005, Kraaijeveld *et al.* 2009). In addition, females may loose the ability to reproduce sexually due to 'functional virginity mutations' that may spread concomitantly with the *Wolbachia* infection through a population (Stouthamer *et al.* 2010, King & Hurst 2010). Virginity mutations arise during the early stages of PI-*Wolbachia* infection and affect traits in females involved in sexual reproduction, e.g. mating or egg fertilization. Accumulation of neutral mutations or selection against the maintenance of costly traits arise after a longer period of parthenogenetic reproduction and can potentially affect all traits involved in sexual reproduction, e.g. courtship behaviour or pheromone production. Many insect species with female-only populations exhibit deterioration or loss of female sexual traits, such as attractiveness, receptiveness and morphology of sexual organs (Pijls *et al.* 1996, Gottlieb & Zchori-Fein 2001, Kraaijeveld *et al.* 2009). In *T. coeruleus*, I found a longevity cost of mating or sperm storage. Selection against the maintenance of costly sexual traits appears to have resulted in the degradation of receptivity and

spermathecal morphology of thelytokous females (chapter 4, Reumer *et al.* in prep). However, arrhenotokous and thelytokous females were equally attractive to males. Although thelytokous *T. coeruleus* females never mated in the lab, I found clues for occasional matings between males and thelytokous females in the field (chapter 3, Reumer *et al.* in prep). Because of these sporadic matings, females may still be exposed to costs of mating due to male manipulations and adaptations of females against these manipulations keep evolving. However, I cannot exclude that these traits (also) have degraded due to functional virginity mutations or accumulation of neutral mutations. In *A. japonica*, females from thelytokous populations that were cured from their *Wolbachia* infection with antibiotics were not capable of sexual reproduction (Kremer *et al.* 2009). Moreover, *A. japonica* males never exhibited courtship towards thelytokous females; thelytokous females were not attractive to males. Because the *Wolbachia* infection in *A. japonica* seems to be relatively young, these traits may have degraded due to functional virginity mutations or strong selection against the maintenance of costly traits, rather than accumulation of neutral mutations.

The comparison of populations that are infected with parthenogenesis-inducing *Wolbachia* and thus reproduce asexually and populations that are not infected and thus reproduce sexually, increases the understanding of the evolution of sexual reproduction. The consequences of asexual reproduction at a genetical and phenotypical level can be studied by comparing individuals from PI-*Wolbachia*-infected and uninfected populations. In addition, the traits that are involved in sexual reproduction and the rates at which these traits deteriorate and are lost can be studied by comparing different species that differ in the age of their PI-*Wolbachia* infection.

Absence of migration and gene flow between *Wolbachia*-infected and uninfected populations because of geographical or ecological boundaries, together with the ongoing co-evolution between *Wolbachia* and host, and deterioration and/or loss of genes that are no longer used, may lead to larger differences between populations. In theory, these differences may eventually lead to speciation between *Wolbachia*-infected and uninfected populations.

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Nederlandse samenvatting

Dit hoofdstuk is gebaseerd op de algemene introductie (hoofdstuk 1) en algemene discussie (hoofdstuk 8) van dit proefschrift. Om de leesbaarheid te verhogen zijn alle literatuurverwijzingen weggelaten. Aan het einde van dit hoofdstuk is een verklarende woordenlijst toegevoegd.

Seksuele en aseksuele voortplanting

Binnen de eukaryoten[1] is seksuele voortplanting[2] de meest voorkomende manier van voortplanting. Er zijn veel theorieën die het succes van seksuele voortplanting proberen te verklaren. Deze theorieën zijn onder te verdelen in twee grote groepen: de mutatietheorieën en de ecologietheorieën. In de mutatietheorieën wordt uitgelegd dat seksuele recombinatie[3] kan zorgen voor de afvoer van schadelijke mutaties[4] uit het DNA, terwijl bij aseksuele voortplanting[5] mutaties, inclusief schadelijke, zich kunnen ophopen in het DNA. In de ecologietheorieën wordt uitgelegd dat organismen die zich seksueel voortplanten zich sneller kunnen aanpassen dan aseksuele organismen wanneer ze te maken krijgen met andere organismen, zoals parasieten, predatoren en concurrenten. Al deze theorieën hebben de overkoepelende gedachte dat seksuele voortplanting kan zorgen voor snellere aanpassing aan een complexe omgeving dan aseksuele voortplanting. Bovendien zal seksuele voortplanting op de lange termijn voordeliger zijn dan aseksuele voortplanting.

Seksuele voortplanting brengt echter ook een aantal kosten met zich mee. Aangezien de fitnessnadelen[6] van seksuele voortplanting zo groot zijn, is het eigenlijk opmerkelijk dat de meeste eukaryoten zich seksueel voortplanten. Deze fitnessnadelen van seksuele voortplanting (en dus fitnessvoordelen van aseksuele voortplanting) worden beschreven in een theorie die 'de tweevoudige kosten van seksuele voortplanting' wordt genoemd. Op populatieniveau gezien: omdat een aseksueel vrouwtje alleen maar dochters produceert (door zichzelf te klonen), zal al haar nageslacht ook weer bijdragen aan de generatie daarna (in theorie zal ieder individu ook weer minimaal één individu in de volgende generatie produceren). Daarentegen zal slechts de helft van het nageslacht (bij een 50/50 sekse ratio) van een seksueel vrouwtje bijdragen aan de generatie daarna (er zijn telkens twee individuen nodig, een mannetje en een vrouwtje, om één individu in de volgende generatie te kunnen produceren). Omdat er dus minder individuen nodig zijn om net zoveel nageslacht te produceren in de volgende generatie, kan een aseksuele populatie sneller groeien dan een seksuele

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populatie. Wanneer deze twee populaties in competitie zouden zijn, zal de aseksuele populatie de seksuele populatie wegconcurreren omdat hij sneller kan groeien. Op genniveau[7] gezien: een aseksueel vrouwtje geeft al haar DNA door aan de volgende generatie, terwijl een seksueel vrouwtje slechts de helft van haar DNA doorgeeft aan haar nageslacht. Een aseksueel vrouwtje heeft daarom een hogere fitness dan een seksueel vrouwtje en een grotere verwantschap tussen moeder en nageslacht zal zorgen voor selectie[8] ten gunste van aseksuele voortplanting.

Aan de hand van de voor- en nadelen van seksuele en aseksuele voortplanting, kan worden voorspeld dat in simpele omgevingen zonder veel interacties met andere organismen en de omgeving, en/of op de korte termijn, aseksuele voortplanting voordeliger zal zijn. In complexe omgevingen met veel interacties, en/of op de lange termijn, zal echter seksuele voortplanting voordeliger zijn. Ondanks dat seksuele voortplanting de meest voorkomende manier van voortplanting is, zal aseksuele voortplanting waarschijnlijk relatief vaak voorkomen in simpele omgevingen. Dit idee wordt ondersteund door een aantal recente studies aan invertebraten[9]. Deze studies laten zien dat aseksuele soorten en populaties relatief vaker voorkomen in landbouwgebieden en andere door mensen verstoorde gebieden en minder vaak in natuurlijke gebieden. Omdat er vaak weinig soorten voorkomen in door mensen verstoorde gebieden worden deze gebieden als simpel beschouwd. In natuurlijke gebieden zijn vaak veel ingewikkelde interacties tussen organismen en tussen organismen en de omgeving, waardoor deze gebieden als complex worden beschouwd.

In invertebraten wordt aseksuele voortplanting vaak veroorzaakt door een infectie met micro-organismen, zoals de bacteriën *Wolbachia*, *Cardinium* en *Rickettsia*. In dit proefschrift beperk ik me tot het micro-organisme *Wolbachia*.

Wolbachia

Wolbachia is een intracellulaire, symbiotische[10] bacterie, behorend tot de orde Rickettsiales binnen de klasse α-Proteobacteria. *Wolbachia* infecteert een groot aantal verschillende arthropoden, zoals insecten, spinnen, mijten, schorpioenen en pissebedden, maar *Wolbachia* is ook gevonden in rondwormen. De typesoort voor het geslacht *Wolbachia* is *Wolbachia pipientis*, die voor het eerst beschreven is in de mug *Culex pipiens* in 1936 door Hertig. Het geslacht *Wolbachia* kan worden onderverdeeld in zes, en waarschijnlijk acht, grote supergroepen (A-H). *Wolbachia* van supergroep C en D zijn alleen bekend in rondwormen. De andere supergroepen worden voornamelijk gevonden in arthropoden, waarin supergroep A en B het meeste voorkomen. Het wordt geschat dat 66% van alle insectensoorten (gedeeltelijk) is geïnfecteerd met *Wolbachia*. Verschillende insectensoorten zijn met meerdere *Wolbachia* soorten geïnfecteerd, die zelfs uit verschillende supergroepen afkomstig kunnen zijn.

Wolbachia wordt via de moeder overgedragen, omdat een spermacel te klein is om de bacterie te kunnen huisvesten. Daarom is verticale overdracht (van moeder

op dochter) de belangrijkste overdrachtsmethode van *Wolbachia* binnen bestaande gastheren[11]. Maar omdat de stambomen van de verschillende soorten *Wolbachia* en hun gastheren niet overeenkomen, komt horizontale overdracht van *Wolbachia* tussen gastheren waarschijnlijk ook vaak voor. In verschillende experimenten is het gelukt om *Wolbachia* horizontaal over te brengen, zowel tussen individuen binnen één gastheersoort als tussen verschillende gastheersoorten. Bovendien laat een recent veldonderzoek aan een sluipwespsoort zien dat horizontale overdracht ook voorkomt binnen een gastheersoort tijdens de eerste fase na de initiële besmetting met *Wolbachia*. Het lijkt er dus op dat horizontale overdracht van *Wolbachia* niet alleen een rol speelt in de verspreiding van *Wolbachia* tussen gastheersoorten, maar ook tussen individuen binnen nieuw geïnfecteerde soorten.

Omdat *Wolbachia* wordt overgedragen van moeder op dochter, heeft *Wolbachia* er voordeel bij wanneer zijn gastheer meer vrouwen dan mannen produceert. Om zijn eigen overdracht naar de volgende generatie te verbeteren, is *Wolbachia* in staat om verschillende veranderingen te veroorzaken in het voortplantingsmechanisme van zijn gastheer, zoals cytoplasmatische incompatibiliteit, feminisatie, mannendoding en parthenogenese[12].

Cytoplasmatische incompatibiliteit (CI) is het meest voorkomende effect van een *Wolbachia*-infectie. CI door *Wolbachia* is beschreven in mijten, pissebedden en veel insectenordes, zoals de Coleoptera (kevers), Diptera (vliegen, muggen), Hemiptera (wantsen, bladluizen, cicaden), Hymenoptera (mieren, hommels, bijen, wespen), Lepidoptera (vlinders) en Orthoptera (krekels, sprinkhanen). CI is een verandering in het voortplantingsmechanisme dat zorgt voor incompatibiliteit tussen sperma- en eicellen. CI-*Wolbachia* past de spermacellen van geïnfecteerde mannetjes tijdens de aanmaak van sperma op zo'n manier aan dat er uit eitjes die met deze spermacellen worden bevrucht geen embryo kan ontwikkelen. Eitjes van vrouwtjes die geïnfecteerd zijn met CI-*Wolbachia* hebben een reddingsmechanisme dat die verandering van het sperma ongedaan kan maken, waardoor er wel een embryo kan ontwikkelen. Wanneer het juiste reddingsmechanimse niet aanwezig is in het eitje, bijvoorbeeld omdat het vrouwtje niet geïnfecteerd is met *Wolbachia* of omdat ze met een andere soort *Wolbachia* is geïnfecteerd dan het mannetje, dan zijn sperma en eitje incompatibel. Als de verandering van het sperma niet ongedaan wordt gemaakt, dan loopt de vorming van de mannelijke en vrouwelijke pronuclei niet in fase met elkaar tijdens de eerste mitotische[13] deling in het embryo. In diploïde[14] soorten zal dit leiden tot de dood van het embryo, terwijl in haplodiploïde[15] soorten zich alleen mannelijk nageslacht zal ontwikkelen.

Feminisatie door *Wolbachia* is gevonden in pissebedden en de insectenordes Hemiptera en Lepidoptera. Bij door *Wolbachia* geïnduceerde feminisatie ontwikkelen genetische mannetjes zich tot functionele vrouwtjes, bijvoorbeeld door de onderdrukking van mannelijke hormonen.

Mannendoding door *Wolbachia* is beschreven in pseudoschorpioenen en de in-

sectenordes Coleoptera, Diptera en Lepidoptera. Met *Wolbachia* geïnfecteerde mannetjes worden gedood tijdens hun ontwikkeling als embryo's, zodat de vrouwelijke embryo's meer nutriënten ter beschikking hebben.

Parthenogenese-inducerende (PI) *Wolbachia* is tot nu toe alleen beschreven in haplodiploïde soorten, zoals tripsen, mijten en Hymenoptera. In ongeïnfecteerde haplodiploïde organismen ontwikkelen bevruchte eitjes zich tot diploïde dochters en onbevruchte eitjes tot haploïde zonen. Deze vorm van seksuele voortplanting wordt ook wel arrhenotokie genoemd. PI-*Wolbachia* zorgt ervoor dat haploïde eitjes diploïd worden door de verdeling van de homologe chromosomen te verstoren tijdens de eerste mitotische deling na de meiose[16], zodat diploïde dochters zich kunnen ontwikkelen uit onbevruchte eitjes. Deze vorm van aseksuele voortplanting wordt ook wel thelytokie genoemd.

In dit proefschrift beperk ik me tot parthenogenese-inducerende *Wolbachia* in Hymenoptera. Ik heb de dynamica, oorzaken en gevolgen van door *Wolbachia* geïnduceerde parthenogenese bestudeerd in twee soorten sluipwespen, *Tetrastichus coeruleus* en *Asobara japonica*.

Gevolgen van door *Wolbachia* geïnduceerde parthenogenese

In de meeste gevallen van door *Wolbachia* geïnduceerde parthenogenese is de infectie gefixeerd en bestaat de hele populatie uit vrouwtjes die zich parthenogenetisch voortplanten. In de afwezigheid van mannetjes en seksuele voortplanting, worden genen die betrokken zijn bij seksuele voortplanting niet meer actief in stand gehouden door selectie. Ophoping van mutaties of selectie tegen het in stand houden van kostbare seksuele eigenschappen kan leiden tot de beschadiging of het verlies van deze genen. Omdat mannelijke eigenschappen niet tot uiting komen in aseksuele populaties, zullen deze eigenschappen waarschijnlijk door mutaties beschadigen en verdwijnen. Vrouwelijke seksuele eigenschappen, die wel tot uiting komen maar niet worden gebruikt, kunnen daarnaast ook worden weggeselecteerd als ze kostbaar zijn om in stand te houden. Bovendien kunnen vrouwtjes hun vermogen om zich seksueel voort te planten ook verliezen door zogenaamde 'functionele maagdelijkheidmutaties', die zich tegelijk met de *Wolbachia*-infectie door een populatie kunnen verspreiden. Mutaties die er voor zorgen dat vrouwtjes geen eitjes meer bevruchten, zullen een selectief voordeel hebben in de aanwezigheid van vrouwtjes die geïnfecteerd zijn met PI-*Wolbachia*. Vrouwtjes met zulke maagdelijkheidmutaties zullen meer zonen produceren die vervolgens meer paringsmogelijkheden hebben door de verhoogde productie van dochters door met PI-*Wolbachia* geïnfecteerde vrouwtjes. Maagdelijkheidmutaties kunnen ontstaan tijdens de eerste fase na de initiële besmetting met PI-*Wolbachia* en ze kunnen invloed hebben op vrouwelijke eigenschappen die betrokken zijn bij seksuele voortplanting, zoals bijvoorbeeld de paring en de bevruchting van haar eitjes. Ophoping van mutaties en selectie tegen het in stand houden van kostbare eigenschappen ontstaan na een langere periode van parthenogenetische

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voortplanting en kunnen van invloed zijn op alle mogelijke eigenschappen, zowel mannelijke als vrouwelijke, die betrokken zijn bij seksuele voortplanting, zoals bijvoorbeeld baltsgedrag en feromoonaanmaak.

Wanneer zulke beschadigingen of verlies van genen die betrokken zijn bij seksuele voortplanting gedurende een lange tijd kunnen ontstaan en evolueren, kunnen aseksuele soorten of populaties na verloop van tijd niet meer in staat zijn tot seksuele voortplanting. Veel soorten insecten die populaties hebben met alleen vrouwtjes vertonen beschadiging of verlies van vrouwelijke seksuele eigenschappen.

Tetrastichus coeruleus

Tetrastichus coeruleus (Hymenoptera: Eulophidae) parasiteert de kever *Crioceris asparagi* (het aspergehaantje). *T. coeruleus* vrouwtjes leggen hun eitjes, meerdere tegelijk, in de eitjes van *C. asparagi*. Uit zo'n eitje wordt vervolgens gewoon een larve van *C. asparagi* geboren. Wanneer deze larve zich verpopt, komen de eitjes van *T. coeruleus* uit en eten de keverlarve van binnen uit op. Uit de pop van *C. asparagi* komt vervolgens geen kever, maar een aantal sluipwespen. *T. coeruleus* voedt zich als adult ook met de inhoud van de eitjes van *C. asparagi*. *T. coeruleus* is synovigeen, wat betekent dat vrouwtjes gedurende (bijna) heel hun leven nieuwe eitjes aanmaken.

De kever *C. asparagi* leeft op de asperge (*Asparagus officinalis*). Aspergeplanten groeien op zandgronden, zoals duingebieden langs de kust, maar ook als monocultuur op landbouwgronden. *A. officinalis* is inheems in West-Azië, Europa en Noord-Afrika en wordt al duizenden jaren gebruikt als consumptiegewas. *C. asparagi* staat bekend als plaagsoort in de aspergeteelt en de sluipwesp *T. coeruleus* kan mogelijk gebruikt worden als biologisch bestrijdingsmiddel tegen deze kevers. *A. officinalis* is geïntroduceerd in de Verenigde Staten voor de teelt van asperges en in 1859 werd *C. asparagi* voor het eerst waargenomen in het noordoosten van de Verenigde Staten. Later, waarschijnlijk al in 1863, maar zeker in 1909, werd ook *T. coeruleus* waargenomen. Bovendien zag men dat deze sluipwesp de populatie aspergehaantjes onder controle hield door zich met eitjes van deze kevers te voeden en er haar eigen eitjes in te leggen. Er werden echter alleen vrouwtjes van *T. coeruleus* gevonden, zowel wanneer ze in het veld werden gevangen als wanneer ze voor meerdere generaties in het lab werden gekweekt. Dit wijst erop dat de populaties van *T. coeruleus* in het noordoosten van de Verenigde Staten zich parthenogenetisch voortplanten. Eerder onderzoek in ons lab vond ook aanwijzingen dat een aantal Nederlandse populaties van *T. coeruleus* geïnfecteerd zijn met *Wolbachia* die parthenogenese veroorzaakt.

Asobara japonica

Asobara japonica (Hymenoptera: Braconidae) parasiteert verschillende soorten fruitvliegen. *A. japonica* vrouwtjes leggen hun eitjes, één per keer, in de larven van fruitvliegen. *A. japonica* komt voor in Japan. Populaties van *A. japonica* op de grote hoofdeilanden van Japan hebben scheve geslachtsverdelingen met voornamelijk vrouwtjes (92.7% - 99.2% vrouwtjes). De populaties van *A. japonica* op de kleine subtropische zuidelijke eilanden van Japan hebben gelijke verhoudingen mannetjes en vrouwtjes. De populaties op de hoofdeilanden zijn geïnfecteerd met parthenogenese-inducerende *Wolbachia*, terwijl de populaties op de kleinere zuidelijke eilanden niet zijn geïnfecteerd.

Wolbachia in *T. coeruleus* en *A. japonica*

Het interessante is dat zowel *T. coeruleus* als *A. japonica* populaties hebben die geïnfecteerd zijn met *Wolbachia* en populaties die niet geïnfecteerd zijn. In beide soorten planten de populaties die met *Wolbachia* zijn geïnfecteerd zich voort via parthenogenese, terwijl de ongeïnfecteerde populaties zich seksueel voortplanten. Ook wordt in beide soorten regelmatig een kleine hoeveelheid mannen geproduceerd in de zich normaal gesproken parthenogenetisch voortplantende populaties.

Er zijn slechts een paar andere sluipwespsoorten bekend die zowel seksuele als parthenogenetische populaties hebben. In de sluipwespen *Apoanagyrus diversicornis*, *Telenomus nawai* en *Leptopilina clavipes* komen met PI-*Wolbachia* geïnfecteerde en ongeïnfecteerde populaties allopatrisch[17] voor. Gemengde populaties van met PI-*Wolbachia* geïnfecteerde en ongeïnfecteerde individuen zijn slechts bekend van een aantal soorten *Trichogramma* sluipwespen. In de meeste gevallen van door *Wolbachia* geïnduceerde parthenogenese is de infectie gefixeerd en bestaat de hele populatie uit vrouwtjes die zich parthenogenetisch voortplanten.

In dit proefschrift bestudeer ik de dynamica van met *Wolbachia* geïnfecteerde en ongeïnfecteerde populaties van *T. coeruleus* en *A. japonica*. Daarnaast heb ik de productie van mannetjes in zich normaal gesproken parthenogenetisch voortplantende populaties onderzocht.

Overzicht proefschrift

In dit proefschrift bestudeer ik door *Wolbachia* geïnduceerde parthenogenese in twee soorten sluipwespen, *Tetrastichus coeruleus* (hoofdstuk 2, 3, 4 & 5) en *Asobara japonica* (hoofdstuk 6 & 7). Ik heb de populatiegenetica van geïnfecteerde en ongeïnfecteerde populaties van beide soorten bestudeerd aan de hand van nucleair[18] en mitochondriaal[19] DNA, om meer te weten te komen over de populatiedynamica en de verspreiding van de *Wolbachia*-infectie door de populaties na de initiële besmetting (hoofdstuk 3 & 6). Ik heb de verschillen tussen geïnfecteerde en ongeïnfecteerde

populaties onderzocht, met betrekking tot de frequentie van de *Wolbachia*-infectie, het voortplantingsmechanisme, de ecologie en de levensloopeigenschappen[20] (hoofdstuk 2 & 4). Verder heb ik de gevolgen van een PI-*Wolbachia*-infectie onderzocht, door te kijken naar de seksuele functionaliteit van *T. coeruleus* vrouwtjes en *A. japonica* mannetjes (hoofdstuk 5 & 7).

Resultaten en discussie

De populatiegenetica laat zien dat er tussen gastheersoorten verschillende scenario's kunnen zijn waarmee een parthenogenese-inducerende *Wolbachia*-infectie zich kan verspreiden. Een recent onderzoek aan de met PI-*Wolbachia* geïnfecteerde sluipwesp *Leptopilina clavipes* was het eerste dat liet zien dat horizontale overdracht van *Wolbachia* een belangrijke rol speelt in de verspreiding van *Wolbachia* tussen individuen en populaties binnen een nieuwe gastheersoort. Seksuele voortplanting tijdens de eerste fase na de initiële besmetting met *Wolbachia* zou de variatie in het nucleair DNA van *L. clavipes* kunnen verklaren, maar niet de variatie in het mitochondriaal DNA. Net als in *L. clavipes* laat de variatie in het mitochondriaal DNA van *A. japonica* zien dat, na de initiële besmetting, de *Wolbachia*-infectie zich via horizontale overdracht (naast de normale verticale overdracht) heeft verspreid door de populaties van *A. japonica*. Ik heb helaas geen informatie over het nucleair DNA van *A. japonica*. Er is echter geen variatie te zien in het mitochondriaal DNA van *T. coeruleus*. Dat wijst erop dat de *Wolbachia*-infectie zich, na de initiële besmetting, enkel via verticale overdracht heeft verspreid door de populaties van *T. coeruleus*, en niet via horizontale overdracht. De variatie in het nucleair DNA van *T. coeruleus* doet vermoeden dat vrouwtjes hebben gepaard met mannetjes tijdens de eerste fase na de initiële besmetting met *Wolbachia* toen er nog mannetjes aanwezig waren. Mogelijk doen ze dat nog steeds af en toe, alhoewel mannetjes slechts zelden voorkomen in de parthenogenetische populaties. In geen van de drie genoemde sluipwespen is variatie gevonden in het DNA van *Wolbachia*. Daarom kunnen we concluderen dat alle drie de soorten geïnfecteerd zijn geraakt met een enkele soort *Wolbachia* die zich vervolgens op verschillende manieren door de populaties heeft verspreid.

Zowel *T. coeruleus* als *A. japonica* hebben populaties die geïnfecteerd zijn met *Wolbachia* en populaties die niet geïnfecteerd zijn. De reden voor dit verschil in infectiefrequentie tussen populaties lijkt te verschillen tussen de twee soorten. Nieuwe bacteriële infecties kunnen zich razendsnel verspreiden in gastheerpopulaties. Er zijn invasies gerapporteerd van de bacteriën *Wolbachia* in de fruitvlieg *Drosophila simulans* in California en *Rickettsia* in de wittevlieg *Bemisia tabaci* in Arizona waarbij de infectiefrequentie van 0% naar bijna complete fixatie ging in minder dan 100 generaties. Wanneer echter de gastheerpopulaties allopatrisch voorkomen kan de verspreiding van een bacterie minder makkelijk gaan. Net als *T. coeruleus* en *A. japonica* heeft de sluipwesp *L. clavipes* ook met *Wolbachia* geïnfecteerde en ongeïnfecteerde populaties. Geïnfecteerde populaties komen voor in Noord-Europa,

terwijl in Spanje ongeïnfecteerde populaties voorkomen. In het gebied tussen de Pyreneeën en het Centraal-Massief in Frankrijk worden echter geen populaties van *L. clavipes* gevonden. Dit gebied lijkt op te treden als geografische barrière tussen de geïnfecteerde en ongeïnfecteerde populaties, waardoor er geen migratie tussen de populaties kan plaatsvinden. Er lijkt ook een geografische barrière aanwezig te zijn tussen de geïnfecteerde en ongeïnfecteerde populaties van *A. japonica*. Met *Wolbachia* geïnfecteerde populaties van *A. japonica* komen voor op de twee grote Japanse hoofdeilanden, terwijl ongeïnfecteerde populaties op de kleine zuidelijke eilanden van Japan voorkomen. De geografische afstand tussen de eilanden is waarschijnlijk te groot voor *Wolbachia* om de zuidelijke eilanden te invaderen. Daarentegen komen de geïnfecteerde en ongeïnfecteerde populaties van *T. coeruleus* in Nederland relatief dicht bij elkaar voor. Ze komen echter in verschillende habitats voor: met *Wolbachia* geïnfecteerde populaties van *T. coeruleus* komen voor in de Nederlandse duingebieden, terwijl ongeïnfecteerde populaties op aspergevelden voorkomen. De populatiegenetica van *T. coeruleus* laat zien dat er wel af en toe migratie is van de ongeïnfecteerde naar de geïnfecteerde populaties, maar niet andersom. Dit zou kunnen verklaren waarom de *Wolbachia*-infectie tot nu toe (nog) niet voorkomt op de Nederlandse aspergevelden.

Verschillende habitats kunnen echter ook selecteren voor verschillende infectiefrequenties van *Wolbachia* of voor verschillende voortplantingsmechanismen. Door twee populaties van *T. coeruleus* met elkaar te vergelijken die verschillen in habitat, *Wolbachia*-infectie en voortplantingsmechanisme, heb ik een aantal verschillen gevonden in levensloopeigenschappen. Een ongeïnfecteerde seksuele populatie van een aspergeveld in Brabant en een met *Wolbachia* geïnfecteerde parthenogenetische populatie van het duingebied Meijendel verschilden in legselgrootte (aantal wespen per aspergehaantje), vrouwelijke levensduur, gewicht van een vrouwtje en samenstelling van de nutriëntreserves van een vrouwtje. De twee populaties lijken verschillende levensloopstrategieën te hebben. De vrouwtjes uit Brabant lijken meer te investeren in overleving, lichaamsgrootte en legselgrootte, terwijl de vrouwtjes uit Meijendel actiever lijken te zijn. Om echter te bepalen of en hoe deze verschillen in levensloopeigenschappen te maken hebben met de verschillen in habitat, *Wolbachia*-infectie en voortplantingsmechanisme, moeten er meer populaties van *T. coeruleus* onderzocht worden op deze levensloopeigenschappen. Hierdoor zouden we uiteindelijk het relatief dicht bij elkaar voorkomen van met *Wolbachia* geïnfecteerde en ongeïnfecteerde populaties van *T. coeruleus* beter kunnen begrijpen.

In de meeste gevallen van door *Wolbachia* geïnduceerde parthenogenese is de infectie gefixeerd en bestaat de hele populatie uit vrouwtjes die zich parthenogenetisch voortplanten. Echter zowel in *T. coeruleus* als in *A. japonica* worden regelmatig enkele mannetjes geproduceerd in de parthenogenetische populaties. In *A. japonica* lijkt deze productie van mannetjes een bijproduct te zijn van de nog niet complete aanpassing van *Wolbachia* aan zijn gastheer, omdat de infectie in *A. japonica* nog

relatief jong is. Aan de hand van mitochondriaal DNA heb ik vastgesteld dat de *Wolbachia*-infectie in *T. coeruleus* ouder is dan die in *A. japonica*. Daarom hebben *Wolbachia* en *T. coeruleus* langer de tijd gehad om zich aan elkaar aan te passen. De productie van mannetjes in parthenogenetische populaties van *T. coeruleus* moet dus waarschijnlijk op een andere manier worden verklaard.

Meer dan de helft van de mannetjes die door geïnfecteerde *A. japonica* vrouwtjes worden geproduceerd zijn ook geïnfecteerd met *Wolbachia*. De *Wolbachia* in deze mannetjes blijkt van precies dezelfde soort *Wolbachia* te zijn als die in *A. japonica* vrouwtjes parthenogenese veroorzaakt. Deze parthenogenese-inducerende *Wolbachia* lijkt cytoplasmatische incompatibiliteit te veroorzaken wanneer deze zich in mannetjes bevindt. Geen van de mannetjes die door geïnfecteerde *T. coeruleus* vrouwtjes worden geproduceerd zijn geïnfecteerd met *Wolbachia*.

Behalve het effect dat een PI-*Wolbachia*-infectie kan hebben op geïnfecteerde mannetjes, kan de infectie ook verstrekkende gevolgen hebben voor vrouwtjes. In de meeste gevallen van door *Wolbachia* geïnduceerde parthenogenese is de infectie gefixeerd en bestaat de hele populatie uit vrouwtjes. In de afwezigheid van mannetjes en seksuele voortplanting, worden genen die betrokken zijn bij seksuele voortplanting niet meer actief in stand gehouden door selectie. Ophoping van neutrale mutaties of selectie tegen het in stand houden van kostbare seksuele eigenschappen kan leiden tot de beschadiging of het verlies van deze genen. Daarnaast kunnen vrouwtjes het vermogen om zich seksueel voort te planten verliezen door zogenaamde 'functionele maagdelijkheidmutaties'. Veel soorten insecten die populaties hebben met alleen vrouwtjes vertonen beschadiging of verlies van seksuele eigenschappen, zoals de aantrekkelijkheid van vrouwtjes, het openstaan van vrouwtjes voor paring en de morfologie van geslachtsorganen. In *T. coeruleus* heb ik gevonden dat paring of spermaopslag ten koste gaat van de levensduur van een vrouwtje. Selectie tegen het in stand houden van kostbare seksuele eigenschappen lijkt ervoor gezorgd te hebben dat parthenogenetische vrouwtjes minder graag willen paren. Bovendien zien hun spermathecae[21] er anders uit dan die van seksuele vrouwtjes. Spermathecae van seksuele vrouwtjes zijn mooi rond, terwijl die van parthenogenetische vrouwtjes uitgerekt zijn en allerlei uitstulpingen kunnen vertonen. Seksuele en parthenogenetische vrouwtjes zijn echter wel even aantrekkelijk voor mannetjes. Hoewel parthenogenetische vrouwtjes van *T. coeruleus* nooit met mannetjes paarden tijdens paringsexperimenten in het lab, heb ik wel aanwijzingen gevonden dat er af en toe paringen in het veld plaatsvinden tussen mannetjes en vrouwtjes van parthenogenetische populaties. Door dit sporadische contact tussen mannetjes en vrouwtjes, zijn vrouwtjes nog steeds blootgesteld aan de kosten die een paring met zich meebrengt door de manipulatie van mannetjes. De aanpassingen van vrouwtjes tegen deze manipulaties blijven dus evolueren. Ik kan echter niet uitsluiten dat de bereidwilligheid van parthenogenetische vrouwtjes om te paren en haar spermathecae ook beschadigd kunnen zijn geraakt door functionele maagdelijkheidmutaties of

ophoping van neutrale mutaties. *A. japonica* vrouwtjes van parthenogenetische populaties die van hun *Wolbachia*-infectie zijn genezen door middel van antibiotica, zijn niet in staat tot seksuele voortplanting. *A. japonica* mannetjes vertonen ook nooit baltsgedrag naar vrouwtjes van parthenogenetische populaties; parthenogenetische vrouwtjes zijn dus niet aantrekkelijk voor mannetjes. Omdat de *Wolbachia*-infectie in *A. japonica* nog relatief jong is lijken deze eigenschappen beschadigd te zijn geraakt door functionele maagdelijkheidmutaties of sterke selectie tegen het in stand houden van kostbare eigenschappen en niet door ophoping van neutrale mutaties.

Door een vergelijking te maken tussen populaties die zijn geïnfecteerd met parthenogenese-inducerende *Wolbachia* en zich dus aseksueel voortplanten en populaties die niet zijn geïnfecteerd en zich dus seksueel voortplanten, kunnen we meer te weten komen over de evolutie van seksuele voortplanting. De gevolgen van aseksuele voortplanting op zowel genetisch als fenotypisch niveau kunnen bestudeerd worden door individuen van met PI-*Wolbachia* geïnfecteerde en ongeïnfecteerde populaties met elkaar te vergelijken. Verder kunnen de eigenschappen die betrokken zijn bij seksuele voortplanting en de snelheden van beschadiging of verlies van deze eigenschappen worden bestudeerd door gastheersoorten die verschillen in leeftijd van de PI-*Wolbachia*-infectie met elkaar te vergelijken.

De afwezigheid van migratie tussen met *Wolbachia* geïnfecteerde en ongeïnfecteerde populaties door geografische of ecologische barrières, samen met de voortdurende co-evolutie tussen *Wolbachia* en zijn gastheer, en de beschadiging of het verlies van genen die niet langer worden gebruikt, kunnen leiden tot steeds grotere verschillen tussen populaties. In theorie zouden deze verschillen uiteindelijk kunnen leiden tot soortsvorming tussen met *Wolbachia* geïnfecteerde en ongeïnfecteerde populaties.

[1] De eukaryoten vormen één van de drie domeinen van het leven. In tegenstelling tot prokaryoten, bevatten de cellen van eukaryoten een celkern. In deze celkern ligt het DNA opgeslagen, dat van het cytoplasma wordt gescheiden door een membraan. De andere twee domeinen, Archaea en Bacteria, behoren tot de prokaryoten en hebben cellen zonder celkern. Het DNA zweeft dan los door de cel.

[2] Bij seksuele voortplanting wordt het DNA van twee individuen van een soort gecombineerd in een nieuw individu in de volgende generatie. Vaak wordt het DNA van twee verschillende seksen of geslachten gecombineerd door de versmelting van een eicel en een spermacel.

[3] Bij recombinatie wordt het erfelijk materiaal (de genen[7]) van de ouders herschikt, zodat het nieuwe individu een andere combinatie van genen heeft dan zijn ouders.

[4] Een mutatie is een verandering in het DNA van een organisme. Mutaties kunnen gunstig, neutraal of schadelijk zijn.

[5] Bij aseksuele voortplanting is slechts één ouder betrokken. Het DNA van deze ouder wordt in zijn geheel overgebracht naar een nieuw individu in de volgende generatie.

[6] Met de fitness van een gen[7] of individu wordt de relatieve toename van het aantal kopieën van dat gen of het aantal nakomelingen van dat individu in de volgende generatie ten opzichte van andere genen of individuen bedoeld. Als een individu relatief veel nakomelingen krijgt ten opzichte van andere individuen, dan is zijn fitness hoog.

[7] Genen zijn de eenheden van erfelijk materiaal in het DNA. Genetica is de wetenschap die erfelijkheid beschrijft, waarbij de genen worden bestudeerd.

[8] Natuurlijke selectie zorgt ervoor dat individuen die het beste aan hun omgeving zijn aangepast de meeste kans hebben om te overleven en meer nakomelingen zullen krijgen dan minder goed aangepaste individuen. Natuurlijke selectie is het mechanisme achter evolutie.

[9] Invertebraten zijn alle dieren zonder wervelkolom.

[10] Symbiose is het samenleven van twee verschillende organismen. Symbiose kan gunstig (mutualisme) zijn voor beide partners (de symbionten), maar het kan ook voordelig zijn voor slechts één van beide partners. In het laatste geval kan de symbiose voor de tweede partner neutraal (commensalisme) of schadelijk (parasitisme) zijn. Ook kan symbiose noodzakelijk zijn voor de overleving van één of beide partners (obligaat mutualisme).

[11] Een gastheer is een organisme waarin een ander organisme leeft. Bij symbiose is de gastheer de grootste van de partners en huisvest hij de tweede partner.

[12] Parthenogenese of maagdelijke voortplanting is een vorm van aseksuele voortplanting. Vrouwtjes die zich parthenogenetisch voortplanten kunnen nakomelingen krijgen zonder daarbij een mannetje nodig te hebben. De nakomelingen komen dan uit onbevruchte eieren.

[13] Mitose of kerndeling is onderdeel van de celcyclus. Voor de mitose wordt het DNA in een cel eerst verdubbeld. Tijdens de mitose worden de twee identieke DNA-kopieën vervolgens uit elkaar gehaald en verdeeld over twee nieuwe cellen die ontstaan door deling van de oude cel.

[14] In eukaryoten is het DNA onderverdeeld in kleinere stukjes die chromosomen worden genoemd. Bij diploïde organismen hebben alle individuen, zowel mannetjes als vrouwtjes, twee sets chromosomen. Eén set is afkomstig van de vader en één set is afkomstig van de moeder. Een eicel en een zaadcel moeten versmelten om nageslacht te produceren.

[15] Bij haplodiploïde organismen hebben alleen de vrouwtjes twee sets chromosomen; één set van de vader en één set van de moeder. Mannetjes hebben slechts één set chromosomen, afkomstig van hun moeder. Een vrouwtje hoeft niet te paren om nageslacht te produceren. Bevruchte eitjes hebben twee sets chromosomen, zijn dus diploïd en ontwikkelen zich tot dochters. Onbevruchte eitjes hebben slechts één set chromosomen, zijn dus haploïd en ontwikkelen zich tot zonen.

[16] Bij de meiose of reductiedeling worden de geslachtcellen, eicellen en zaadcellen, gevormd. In tegenstelling tot bij de mitose, worden bij de meiose de twee homologe chromosoomsets die iedere diploïde cel bevat uit elkaar gehaald en verdeeld over twee nieuwe cellen die ontstaan door deling van de oude cel. De nieuwe cellen bevatten ieder slechts één set chromosomen en zijn dus haploïd.

[17] Allopatrische populaties zijn geografisch van elkaar gescheiden, waardoor er geen contact is tussen de populaties. Sympatrische populaties komen in hetzelfde gebied voor en staan dus met elkaar in contact.

[18] Nucleair DNA is het DNA van een organisme dat zich in de celkern (de nucleus) bevindt. Nucleair DNA wordt zowel via de vader als via de moeder overgeërfd.

[19] Mitochondriaal DNA is het DNA dat zich in de mitochondriën van een cel bevindt. Mitochondriën zijn celorganellen die voor de energiehuishouding van een cel zorgen. Mitochondriaal DNA wordt alleen via de moeder overgeërfd.

[20] Levensloopeigenschappen zijn betrokken bij de belangrijke gebeurtenissen in het leven van een organisme, zoals voortplanting en overleving. Belangrijke levensloopeigenschappen zijn bijvoorbeeld levensduur, aantal en grootte van de nakomelingen, lichaamsgrootte, ontwikkelingsduur, leeftijd waarop een organisme volwassen is en verder alle eigenschappen die bij de levensloop betrokken zijn.

[21] Spermathecae zijn de organen van een vrouwtje waarin ze het sperma van een mannetje opslaat na een paring.

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Curriculum Vitae

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Courses: Dutch, English, French, Greek, Mathematics B, Physics, Chemistry and Biology

2001-2004	Bachelor Biology, Utrecht University

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Bachelor Thesis 'Gargano, a Miocene insular fauna', supervised by Dr. Wilma Wessels and Dr. Albert van der Meulen (group Stratigraphy and Paleontology, faculty Geosciences, Utrecht University) about the vertebrate fauna and insular evolution on Gargano, a Mediterranean island during the Miocene, 8 million years ago.

2004-2006	Master Biogeology, Utrecht University

Master Thesis '*Democricetodon* (Rodentia, Cricetidae) from Sandelzhausen (Upper Freshwater Molasse, Germany)', supervised by Dr. Wilma Wessels (group Stratigraphy and Paleontology, faculty Geosciences, Utrecht University) about the taxonomic description of two cricetid species from the genus *Democricetodon* and the reconstruction of the biostratigraphical position of their sampling location Sandelzhausen, Germany.

Master Thesis 'Sexual conflict in the parasitoid wasp *Leptopilina clavipes* (Hymenoptera: Figitidae)', supervised by Dr. Ken Kraaijeveld (section Animal Ecology, Institute of Biology Leiden, Leiden University) about the different interests of males and females in sexual reproduction in the parasitoid wasp *Leptopilina clavipes*.

2007-2012   PhD Biology, Leiden University

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I reviewed manuscripts of others for the peer-reviewed journals Heredity, Molecular Ecology, Oikos and Phytoparasitica.

Conference presentations

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