Phylogeny and host-symbiont interactions of thelytoky inducing *Wolbachia* in Hymenoptera

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-Stellingen-

(1) In tegenstelling tot de gegevens over het *ftsZ*-gen blijkt fylogenetisch uit de *wsp*-gen- en de 'spacer 2 region'- data dat *Wolbachia* geassocieerd met de pissebed *Armadillidium vulgare* in grote mate afwijkt van de andere typen *Wolbachia*.
Dit proefschrift.

(2) Aangezien thelytokie-inducerende *Wolbachia* van gefixeerde gastheer-lijnen relatief goed aangepast zijn aan de gastheer, zijn deze typen *Wolbachia* waarschijnlijk het minst geschikt om naar een niet-verwante gastheer over te brengen.
Dit proefschrift.

(3) Omdat *Wolbachia* maternaal overerft, is het het meest efficiënt om micro-injecties uit te voeren in een *Nasonia*-lijn die de 'maternal sex ratio-distorter' bevat aangezien deze lijn het hoogste percentage vrouwelijke nakomelingen heeft.
Parker, E.D. and Orzack, S.H. (1985) Genetic variation for the sex ratio in *Nasonia vitripennis*. *Genetics* **110**: 93-105.

(4) De vergelijking van arrhenotoke en thelytoke lijnen van *Trichogramma minutum* door Wang & Smith (1996) heeft geen informatieve waarde aangezien de auteurs de nucleaire- en *Wolbachia*-component niet afzonderlijk hebben bekeken.
Wang, Z. and Smith, S.M. (1996) Phenotypic differences between thelytokous and arrhenotokous *Trichogramma minutum* from *Zeiraphera canadensis*. *Entomologia Experimentalis et Applicata* **78**: 315-323.

(5) Aangezien vitamine C naast antioxidatieve ook oxidatieve kenmerken heeft, zou de supplement-industrie onderzoek moeten financieren naar de relevantie van deze bevindingen voor de groeiende groep consumenten.
Levine, M., Daruwala, R.C., Park, J.B., Rumsey, S.C., Wang, Y.H. (1998) Does vitamin C have a pro-oxidant effect? *Nature* **395**: 231.

(6) De slogan 'the only solution for pollution is dilution' is niet alleen voor homeopaten onacceptabel.

(7) In het kader van de Europese integratie zou de noodkreet "HELP!", die in veel Europese landen erg verschillend is, naar één eurotaal omgezet moeten worden.

(8) Spellingscontrole-programma's verslechteren de parate taalkennis.

Stellingen behorende bij het proefschrift:

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Wageningen, 7 mei 1999. Marnix M. M. van Meer

Je stabiliteit is even groot als je bereidheid om haar op het spel te zetten, in het vertrouwen dat je opnieuw stabiliteit zult bereiken in een nieuwe situatie (R. Alon).

Voorwoord

Hier voor u ziet u het resultaat van 5 jaar zwoegen en ik wil mij graag richten tot iedereen die, direct en indirect, een bijdrage heeft geleverd tot het stand komen van dit proefschrift. Richard Stouthamer, ik ben je dankbaar voor je hulp, interessante wetenschappelijke gesprekken en het feit dat je meestal klaarstond. Joop van Lenteren, bedankt voor je ondersteuning bij mijn laatste hoofstukken van het proefschrift. Jeroen Witteveldt, student die het aandurfde een 6-maands afstudeervak bij mij te doen om de gecompliceerde micro-injectie experimenten uit te voeren en tevens een moleculair onderwerp heeft gedaan. Ik vond onze samenwerking zeer prettig en een perfecte symbiose. Isabel Silva, jouw openheid, nauwkeurigheid inzake experimenten, en gezelligheid kan ik zeer waarderen en onze gezamenlijke projecten waren mede in de laatste jaar daardoor stimulerend en plezierig. In dat laatste jaar ben ik tevens Michel Roskam dankbaar, vanwege zijn hulp met de enorme hoeveelheid practische werk dat uitgevoerd moest worden, zonder jou zou ik nu nog een jaar bezig zijn! Bertha Koopmanschap, jouw ondersteuning bij de vele aspecten behorende het moleculair lab. heeft mijn leven ook minder gecompliceerd gemaakt. Menno Schilthuizen, bedankt voor corrigeren van mijn manuscripten, het heeft zeker geholpen tot de lees- en publiceerbaarheid ervan. Tevens was door jouw aanwezigheid en die van Eddy Dijkstra mede gezellig op het moleculair lab. Astrid Schoenmaker, jouw assistentie met de laatste loodjes van het practisch werk hebben me een paar minder slapeloze nachten bezorgd. Hanneke van Heest, ook bedankt voor het geven van de *Trichogramma* kweekcursus in 3 lessen. Tenslotte, Frenk van Kan, bedankt voor het practisch werk wat je tijdens mijn eerste jaren van mijn onderzoek hebt uitgevoerd.

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Marnix
5 Januari 1999, Wageningen

Contents

General introduction

History

Wolbachia are endocellular bacteria that belong to the family of Rickettsia (α-proteobacteria) and infect numerous species of arthropods (Werren, 1995ab). *Wolbachia* was first described in the gonads of the mosquito *Culex pipiens* by Hertig and Wolbach (1924) and was officially named *Wolbachia pipientis* in an additional microscopical study of the same species (Hertig, 1936). The effects of this bacterial infection on its host was not clear till Yen and Barr (1971) established that cytoplasmic incompatibility (CI) was associated with the presence of *Wolbachia*. CI is a significantly reduced egg hatch in crosses between infected males and uninfected females whereas all other crosses are fully compatible. Since then CI-inducing *Wolbachia* have been detected in numerous other arthropod species, including flour beetles, weevils, planthoppers, fruit flies and *Aedes* mosquitoes (Wade and Stevens, 1995; Hsiao and Hsiao, 1995; Noda, 1984; Binnington and Hoffmann, 1989; Sinkins *et al.*, 1995a). In related research, several maternally-inherited sex ratio distorters were discovered (reviewed in Hurst *et al.*, 1993) covering a wide array of different microorganisms including spiroplasmas in fruit flies, male-killing Ricketssia in ladybird beetles, male killing microsporidia in mosquitoes, feminizing (genetic males are converted into functional females) bacteria in isopods and thelytoky-inducing (unfertilized eggs develop into daughters) bacteria in hymenopteran parasitoids.

Phylogeny of *Wolbachia*

During the 1990's the establishment of modern molecular tools made it possible to characterize the CI-*Wolbachia* and the different sex ratio distorters by sequencing the 16S ribosomal DNA. According to these sequences, close relatives of CI inducing *Wolbachia* are *Ehrlichia*, *Cowdria* and *Anaplasma* which are pathogens of mammals transmitted through arthropod vectors (O'Neill *et al.*, 1992). Two other phylogenetic studies based on 16S rDNA (Rousset *et al.*, 1992b; Stouthamer *et al.*, 1993) showed that thelytoky inducing (T-) bacteria in parasitoids and feminizing (F-) bacteria in isopods were closely related to CI-*Wolbachia* and formed a monophyletic group. Sequence divergence among different *Wolbachia* strains was up to only 3% and the group was divided into two subgroups (type I and II; Stouthamer *et al.*, 1993).

An unexpected finding was that *Wolbachia* strains inducing CI, T or F did not form distinct groups and CI- and T- *Wolbachia* were found in both subgroups. Because 16S rDNA variation was relatively limited, other genes like 23S, the cell cycle gene *ftsZ* (Holden *et al.*, 1993), the groE-homologous operon (Masui *et al.*, 1997) and a gene coding for an outer membrane protein (*wsp*) (Braig *et al.*, 1998) were sequenced to improve phylogenetic resolution between different *Wolbachia* strains. The 23S rDNA data did not improve phylogenetic resolution (Rousset *et al.*, 1992b).

An extensive phylogenetic study of different *Wolbachia* strains based on the *ftsZ* gene showed a maximum sequence difference of 15% between the different strains. The two divisions were confirmed and were named groups A and B, corresponding to type II and I respectively (Werren *et al.*, 1995a). Based upon synoymous substitution rates of *ftsZ*, group A and B *Wolbachia* diverged from each other 58-67 million years ago (Werren *et al.*, 1995a) and both groups were found in a wide variety of insect species. The maximum sequence difference of the *ftsZ* gene was relatively low within group A (3%) and suggested that extensive horizontal transmission has occurred recently in this group (Werren *et al.*, 1995a).

Finally, a phylogenetic study was done based on the *wsp* gene, which exhibited higher variation between *Wolbachia* strains than the *ftsZ* gene (14% variation within the least variable group A *Wolbachia*) (Zhou *et al.*, 1998). This study showed that the *Wolbachia* clade was subdivided into supergroups A and B (synonymous to group A and B) in which twelve distinct groups were recognised based on 2.5% sequence difference (Zhou *et al.*, 1998). In addition, the *wsp* gene seemed better at associating the *Wolbachia* (CI or T) phenotypic modification of the host with their phylogenetic position (Zhou *et al.*, 1998; Bourtzis *et al.*, 1998) compared to the *ftsZ* gene.

Distribution and horizontal transfer of *Wolbachia*

A recent survey in Panama have shown *Wolbachia* to be present in at least 16.9% of sampled insect species belonging to many major insect orders, such as Coleoptera, Diptera, Hemiptera, Hymenoptera, Lepidoptera and Orthoptera (Werren *et al.*, 1995b). *Wolbachia* was not detected in Odonata but only two species were tested from this order. Other studies showed that *Wolbachia* also infects several isopod species (Crustacea) (Rousset *et al.*, 1992b), mite species (Chelicerata) (Breeuwer, 1997) and even a filarial worm (Nematoda) (Sironi *et al.*, 1995).

Phylogenetic studies based on 16S rDNA and *ftsZ* showed that the phylogenies of host and symbiont were not congruent and suggested that horizontal transfer occurred frequently

between different insect orders. Indeed, several microinjection studies showed that *Wolbachia* can adapt to different cellular environments and could even be transferred from the mosquito species *Aedes albopictus* to *Drosophila simulans* (Braig *et al.*, 1994). How horizontal transmission occurs in the field is unknown, but there are a few indirect indications. It was found that *Wolbachia* of the parasitoid *Nasonia* spp. and its host *Protocalliphora* sp. clustered together based on the *ftsZ* gene suggesting *Wolbachia* exchange between these species (Werren *et al.*, 1995a). In another study Schilthuizen *et al.* (1997) found that in the distinct *Wolbachia* group of the parasitoid *Trichogramma*, the phylogenies of the host and *Wolbachia* were not congruent either and excluded host symbiont cospeciation as the explanation for the pattern. It was therefore hypothesised, but not yet shown, that horizontal transmission occurred in the host of *Trichogramma* namely, Lepidopteran eggs. *Trichogramma* can superparasitize these eggs so that infected and uninfected larvae can reside in the same eggs.

Types of reproductive alterations in hosts due to *Wolbachia* infection

1. Cytoplasmic incompatibility

There are two forms of CI: unidirectional and bidirectional. Unidirectional CI is expressed when sperm from an infected male fuses with an uninfected egg, resulting in zygotic death in diploid species and in parahaploid mites, or in male production in hymenopteran haplodiploid species. All other crosses are compatible (Table 1a). Bidirectional incompatibility typically occurs when a male and a female harbour a different strain of *Wolbachia* that are mutually incompatible (Table 1b).

The mechanism of CI is unknown, but it involves a two component system of modification of the sperm and neutralization of this modification by the egg (= rescue). Sperm does not contain *Wolbachia* and yet CI is expressed. Probably, the bacteria in the testes modify the proteins of the developing sperm. When the egg harbours the same strain of *Wolbachia* it can rescue this specific modification, otherwise incompatibility is expressed. This model is confirmed by individuals which are infected with two different strains of *Wolbachia* and such superinfections create new compatibility types (Merçot *et al.*, 1995; Sinkins *et al.*, 1995a). Superinfected females can rescue sperm from single-infected males and superinfected males, while single-infected females can only rescue sperm from males harbouring the same *Wolbachia* strain (Table 1b).

Table 1. Crossing schemes of the CI phenotype which is associated with *Wolbachia* infection in arthropod hosts of diploid-diploid arthropods, parahaploid mites and haploid-diploid Hymenoptera. Shadowed boxes represent the putative CI crossings.

a. unidirectional incompatibility

Crossing / Offspring	♀ x ♂	♀$^{w(a)}$ x ♂	♀ x ♂$^{w(a)}$	♀$^{w(a)}$ x ♂$^{w(a)}$
diploid-diploid	♀ ♂	♀$^{w(a)}$ ♂$^{w(a)}$	-	♀$^{w(a)}$ ♂$^{w(a)}$
parahaploid	♀ ♂	♀$^{w(a)}$ ♂$^{w(a)}$	-	♀$^{w(a)}$ ♂$^{w(a)}$
haploid-diploid	♀ ♂	♀$^{w(a)}$ ♂$^{w(a)}$	♂ ♂	♀$^{w(a)}$ ♂$^{w(a)}$

w(a): infected with CI inducing *Wolbachia* strain 'a'; -: no offspring

b. bidirectional incompatibility

Crossing / Offspring	♀$^{w(a)}$ x ♂	♀$^{w(a)}$ x ♂$^{w(b)}$	♀ x ♂$^{w(ab)}$	♀$^{w(ab)}$ x ♂$^{w(ab)}$
diploid-diploid	♀$^{w(ab)}$ ♂$^{w(ab)}$	-	-	♀$^{w(ab)}$ ♂$^{w(ab)}$
haplo-diploid	♀$^{w(ab)}$ ♂$^{w(ab)}$	♂$^{w(b)}$ ♂$^{w(a)}$	♂ ♂	♀$^{w(ab)}$ ♂$^{w(ab)}$

w(a): infected with CI inducing *Wolbachia* strain 'a' ; w(b): infected with CI inducing *Wolbachia* strain 'b'; w(ab): infected with both *Wolbachia* strains 'a' and 'b'; -: no offspring.

c. CI 'rescuing' *Wolbachia*

Crossing / Offspring	♀$^{w(a-)}$ x ♂	♀$^{w(a+)}$ x ♂$^{w(a-)}$	♀ x ♂$^{w(a-)}$	♀$^{w(a-)}$ x ♂$^{w(a+)}$
diploid-diploid	♀$^{w(a-)}$ ♂$^{w(a-)}$	♀$^{w(a+)}$ ♂$^{w(a+)}$	♀ ♂	♀$^{w(a-)}$ ♂$^{w(a-)}$

w(a+): infected with CI inducing *Wolbachia* strain 'a' ; w(a-): infected with CI 'rescuing inducing *Wolbachia* strain 'a'; -: no offspring.

The cytogenetic mechanism of CI has been described for several species (Lassy and Karr, 1996; Reed and Werren, 1995; Callaini, 1994) and was related to early mitotic defects in fertilized eggs of incompatible crosses leading to zygotic death. The severity of CI expression is probably dependent on different factors, such as *Wolbachia* density, host- and symbiont genotype.

Wolbachia density was positively correlated with the expression of CI (Breeuwer and Werren, 1993; Sinkins *et al.*, 1995b). In addition, antibiotic partially-cured individuals showed a decreased CI compared to the untreated counterparts (Breeuwer and Werren, 1993; Clancy and Hoffmann, 1998). Finally, decreasing *Wolbachia* density and CI expression correlates with the age of the host (Clancy and Hoffmann, 1998). This rule of

density and severity of expression is only valid within species because a study in different *Drosophila* species showed that there are differences in CI expression which were not correlated with the density of *Wolbachia* (Bourtzis *et al.*, 1996).

Secondly, host genotype can influence CI expression as shown in microinjection studies. For example, *Wolbachia* expressing high CI (*w*Ri) in *D. simulans* has been transferred to the sibling species *D. melanogaster* which showed relative low CI (Boyle *et al.*, 1993). This suggests that nuclear genes of *D. melanogaster* influence the CI expression of *w*Ri. In a follow-up study, *Wolbachia* from *D. melanogaster* (*w*Mel) was transferred to *D. simulans*. *W*Mel also induces slight CI in *D. melanogaster* but relatively high CI in transinfected *D. simulans*, confirming the hypothesis that the host genome influences the CI expression of *Wolbachia* (Poinsot *et al.*, 1998). Microsocopical studies showed that this low CI in *D. melanogaster* was probably caused by a tenfold lower number of infected cysts in the testes of males compared to the number of infected cysts in *D. simulans* (Poinsot *et al.*, 1998). For males it is advantageous to lower the number of infected cysts because non-modified sperm can fertilize all eggs (infected and non infected) in the population.

Finally, symbiont genotype can also play a role in the expression of CI. *Drosophila mauritiana* harbours a *Wolbachia* strain (*w*Ma) which does not induce CI (Giordano *et al.*, 1995). When this strain was transferred to *D. simulans*, no CI was expressed either. When *w*Ri was transferred to *D. mauritiana*, CI was expressed. These results suggest that the *Wolbachia* strain genotype was responsible for the expressed host phenotype. In a follow up of this study, eggs infected with *w*Ma were able to rescue sperm from males carrying a closely related CI inducing *Wolbachia* strain (Table 1c; Bourtzis *et al.*, 1998).

2. Feminization

To date, *Wolbachia* inducing feminization (F) has only been found in isopods (Crustacea). Laboratory studies with *Armadillidium vulgare* (Oniscidae) showed that some mothers produced highly female-biased offspring and that this trait was maternally inherited. Inoculation studies showed that this trait was infectious and that it could be transferred to other individuals that do not have this trait (Legrand and Juchault, 1970). The feminizing bacterium acts by suppressing an androgenic gland which results in the transformation of genetic males into neofemales. The neofemales have to mate with a male to produce offspring (Table 2). Genetic analysis of this symbiont based on 16S rDNA and *ftsZ* showed that it belonged to the monophyletic group of *Wolbachia* (Rousset *et al.*, 1992b; Werren *et al.*, 1995a). Population studies of *A. vulgare* showed the prevalence of suppressor genes against F-*Wolbachia* (Rigaud and Juchault, 1992).

Table 2. Crossing schemes of the F phenotype which is associated with *Wolbachia* infection in isopods.

Crossing	♀ x ♂	♀$^{w(f)}$	♂$^{w(f)}$ → ♀$^{w(f)}$ x ♂
Offspring	♀ ♂	-	♀$^{w(f)}$ ♀$^{w(f)}$

w(f): infected with feminizing inducing *Wolbachia* strain 'f'; -: no offspring

3. Fecundity increase

An increase of fecundity of infected hosts has been found for the egg parasitoid *Trichogramma bourarachae* (Girin and Boulétreau, 1995). Infected females produced at least two times more offspring than the cured counterparts. Back-cross experiments showed that this trait was maternally inherited. PCR on *Trichogramma* strains producing high offspring numbers showed they were infected with *Wolbachia* from group A (Vavre *et al.*, 1999). In this *Wolbachia* infected species, no other reproductive alterations have been found.

4. Thelytoky

In Hymenoptera, the most common mode of reproduction is arrhenotoky, in which males are produced from haploid, unfertilized eggs and females from diploid, fertilized eggs. Thelytoky is a less common mode of reproduction, in which both fertilized and unfertilized eggs produce diploid females (Table 3). In several wasp species thelytoky is caused by bacteria of the genus *Wolbachia* (Rousset *et al.*, 1992b; Stouthamer *et al.*, 1993; Van Meer *et al.*, 1995; Zchori-fein *et al.*, 1995). These bacteria alter the segregation patterns of chromosomes in unfertilized eggs. Diploidy is restored by preventing chromosome segregation in the first mitotic division (anaphase) which leads to the development of a homozygous, thelytokous female (Stouthamer and Kazmer, 1994). The infected thelytokous wasps can be reverted to arrhenotoky by treatment with several specific antibiotics (e.g. rifampicin, tetracycline) or high temperatures (>30°C) (Stouthamer *et al.*, 1990a). *Wolbachia*-induced thelytoky is present in different families of Hymenoptera but have not yet been detected in other orders (Stouthamer, 1997; Table 4). The infection can be fully established in a population so that only thelytokous females are present (= fixed population). But there are also cases, especially in the genus *Trichogramma*, where thelytokous females co-exist with arrhenotokous ones (= mixed population). In mixed populations, thelytokous females are still able to mate and produce daughter offspring sexually (Stouthamer and Kazmer, 1994).

A previously published model, which describes the dynamics of *Wolbachia*-infected *Trichogramma* wasps in mixed populations (Stouthamer, 1997), indicates that when the *Wolbachia* transmission efficiency is high and when *Wolbachia* impact on host fitness is within a certain range, the infection will go to fixation. However, for several years, field collections did not show large shifts in population infection percentages (Stouthamer, pers. comm.). Therefore countermeasures against the infection are likely be present in these mixed populations. Model studies suggest that nuclear factors like the heterosis effect (Schoenmaker *et al.*, 1998), supressor genes (Stouthamer, 1997) or a non Mendelian paternal-sex-ratio (*psr*) factor (Van Tilborg and Stouthamer, 1998) could stabilize this infection polymorphism in a population. *PSR* is a supernumerary 'B chromosome' and when a male, carrying *psr*, mates with an infected female, her offspring will be male biased (Table 3). Only recently, *Trichogramma* spp individuals were collected from the field and crossing studies including microscopal studies showed they carry such a *psr* factor (Stouthamer, unpublished).

Table 3. Crossing schemes of the T-phenotype which are associated with *Wolbachia* infection in haploid-diploid Hymenoptera.

Crossing	♀ x ♂	♀	♀$^{w(t)}$	♀$^{w(t)}$ x ♂	♀$^{w(t)}$ x ♂^{psr}
Offspring	♀ ♂	♂ ♂	♀$^{w(t)}$ ♂$^{w(t)}$	♀$^{w(t)}$ ♀$^{w(t)}$	♂^{psr} ♂^{psr} ♀$^{w(t)}$

w(t): infected with thelytokoy-inducing *Wolbachia* strain 't'; *psr*: males harbouring the paternal sex ratio factor; -: no offspring

Implications of the use of thelytokous wasps in biological control

Thelytokous reproduction can result in a higher production of daughters per female than arrhenotokous reproduction (Stouthamer and Luck, 1993). This can be advantageous in biological control because only female wasps kill the pest species. The following potential advantages of using thelytokous females v.s. arrhenotokous females in biological control were postulated by Stouthamer (1993):

(1) Faster population growth of the thelytokous population

(2) Reduction of production costs because no resources are 'wasted' for the production of males

(3) Easier establishment in the field because arrhenotokous forms must mate to produce female offspring. When wasp population density is low, males and females may have difficulties in finding each other. Thelytokous females do not have this problem and can still reproduce effectively at low host densities.

Disadvantages of thelytokous reproduction may be:

(1) Thelytokous females are homozygous and because sexual reproduction is absent, genetic variation in populations is relatively low (Plantard *et al.*, 1998). Therefore, sexual forms will likely be able to adapt faster to changes in the environment. In addition, hosts may evolve countermeasures against parasitization, which could be circumvented only by the coevolution of the parasitoid. Evidence for the above is limited and a preliminary study with fixed thelytokous populations of *Encarsia formosa* against whitefly hosts did not support evidence for this hypothesis (Stouthamer, pers. comm.).

(2) If sexual forms are made thelytokous artificially by the injection of *Wolbachia*, it is unclear what the ecological implications are when these novel infected species are released for biological control. There is concern that inundative release of parasitoids may have an impact on non-target species or interfere with naturally occuring parasitoids (for a review see Simberloff and Stiling, 1996). Because thelytokous wasps can have faster population growth and they can still reproduce effectively at low host densities, it should be taken into consideration that releasing thelytokous species may pose an extra threat in the ecology of non target species compared to releasing arrhenotokous wasps.

Overview of thesis and research objectives

1. Phylogeny

Objective: To identify genes that improve phylogenetic resolution between *Wolbachia* strains and to determine whether *Wolbachia* strains inducing different host phenotypic modifications (CI ore T) are monophyletic.

Initially, *Wolbachia* was taxonomically identified by the sequencing of their 16S rDNA. A disadvantage of the 16S rDNA is that it is relatively conserved and could not be used to distinguish closely related *Wolbachia* strains. In addition, *Wolbachia* inducing CI or T did not form monophyletic groups. Sequencing of a more variable gene of *Wolbachia* may adequately resolve the relationships between distinct *Wolbachia* strains that display different reproductive phenotypes. The cell cycle gene *ftsZ* (Holden *et al.*, 1993) showed higher variation but it was still limited especially in group A (Werren *et al.*, 1995a). Therefore, the spacer 2 region and 5S rDNA was sequenced from different strains to determine whether this region would improve the phylogenetic resolution (Chapter 2). In addition, the membrane protein of *Wolbachia* (Braig *et al.*, 1998) was sequenced of

several T-*Wolbachia* strains because an earlier study showed that this gene had the largest sequence difference among many CI-*Wolbachia* strains (Zhou *et al.*, 1998) (Chapter 3).

2. Host-symbiont interactions

Objective: Thelytoky inducing *Wolbachia* is transferred to *Drosophila simulans* to determine if T-*Wolbachia* can induce CI in a novel host.

Phylogenetic studies showed that the *Wolbachia* clade can be subdivided into groups A and B (Werren *et al.*, 1995a). In addition, this study showed *Wolbachia* that induce similar effects on hosts are not monophyletic and are present in both groups. Very closely related *Wolbachia* can have quite different effects on their host and this finding led to several hypotheses. One of them is that a non-specific *Wolbachia* strain induces a distinct host response, e.g. a *Wolbachia* strain inducing thelytoky in a hymenopteran host would induce CI in a diploid-diploid host. To test this hypothesis, we transferred T-*Wolbachia* from the fly pupal parasitoid *Muscidifurax uniraptor* to the fruitfly *Drosophila simulans* and investigated whether this infection had any effect on its new host (Chapter 4).

3. Microinjection protocol

Objective: To develop a microinjection protocol for the hymenopteran *Nasonia vitripennis* for prospective T-*Wolbachia* transfer studies in this species.

Wolbachia transfer between species has been successfully performed by microinjection of CI-*Wolbachia* in the egg of the host species. The newly transfected species exhibited similar CI as the donor species, although the intensity of the CI expression could be different (Boyle *et al.*, 1993; Poinsot *et al.*, 1998). Because no microinjection protocol was available for parasitoids, such a protocol had to be developed. Initially, it was tried with the fly pupal parasitoid *Muscidifurax raptor* but several practical problems in the injection procedure could not be solved. We shifted to the gregarious fly pupal parasitoid host *Nasonia vitripennis* because large quantities of eggs can be collected relatively easy compared to *M. raptor* and many other hymenopteran species. This facililitated the testing of the different steps of the micro-injection procedure. In addition, *N. vitripennis* is naturally infected with *Wolbachia* so it is capable of harbouring this bacterium. A *Drosophila* injection protocol was not suitable for this species. Therefore different injection protocols (Santamaria, 1986; Chang and Wade, 1994) were combined and optimal conditions were determined (Chapter 5).

Table 4. Known cases of thelytokous reproduction in which evidence exists of *Wolbachia* involvement.

Taxon	heat[a]	antib.[a]	wolb.[a]	i.s.[b]	cop.[c]	References
Tenthredinoidea						
Pristiphora erichsonii	+	?	?	?	?	Smith, 1955
Chalcidoidea						
Pteromalidae						
Muscidifurax uniraptor	+	+	+	f	-	Legner, 1985a, b; Stouthamer *et al.*, 1993, 1994
Spalangia fuscipes	?	?	+	?	?	Werren *et al.*, 1995a; Van Meer *et al.*, 1999
Aphelinidae						
Aphelinus asynchus	+	?	?	?	?	Schlinger & Hall, 1959
Aphytis chilensis	?	?	+	?	?	Gottlieb *et al.*, 1998
A. chrysomphali	?	?	+	?	?	Gottlieb *et al.*, 1998
A. diaspidis	?	?	+	?	?	Zchori-Fein *et al.*, 1994, 1995
A. lignanensis	?	+	+	?	+	Zchori-Fein *et al.*, 1994, 1995
A. mytilaspidis	?	?	?	m	+	Rössler & DeBach, 1973
A. yanonensis	?	+	+	?	?	H. Nadel pers com; Werren *et al.*, 1995a
Encarsia formosa	?	+	+	f	-	Zchori-Fein *et al.*, 1992; Van Meer *et al.*, 1995, 1999 Werren *et al.*, 1995a
Eretmocerus staufferi	?	?	+	?	?	Van Meer *et al.*, 1999
Signiforidae						
Signiphora borinquensis	+	?	?	?	+	Quezada *et al.*, 1973
Encyrtidae						
Aponanagyrus diversicornis	+	+	+	f	+	Pijls *et al.*, 1996; Van Meer *et al.*, 1999
Coccidoxenoides peregrinus	+	?	+	f	+	Flanders, 1965; Van Meer *et al.*, 1999
Ooencyrtus submetallicus	+	?	?	?	-	Wilson & Woolcock 1960a,b Wilson, 1962
O. fecundus	+	?	?	?	?	Laraichi, 1978
Plagiomerus diaspidis	+	?	?	?	-	Gordh & Lacey, 1976
Trechnites psyllae	?	+	?	?	?	Unruh pers. com.
Habrolepis rouxi	+	?	?	?	?	Flanders, 1965
Platygasteridae						
Amitus fuscipennis	?	?	+	?	?	Van Meer *et al.*, 1999
Trichogrammatidae						
Trichogramma brevicapillum	+	+	+	m	+	Stouthamer *et al.*, 1990a, b, Werren *et al.*, 1995a

T. chilonis	+	+	+	m	+	Stouthamer *et al.*, 1990a, b, Chen *et al.*, 1992, Schilthuizen *et al.*, 1997
T. cordubensis	+	+	+	f	+	Cabello & Vargas 1985, Stouthamer *et al.*, 1990b, 1993
T. deion	+	+	+	m	+	Stouthamer *et al.*, 1990ab, 1993; Schilthuizen *et al.*, 1997; Van Meer *et al.*, 1999
T. embryophagum	+	+	?	?	+	Birova, 1970; Stouthamer *et al.*, 1990b
T. evanescens (rhenana)	+	+	?	?	+	Stouthamer *et al.*, 1990b
T. kaykai	?	+	+	m	+	Stouthamer & Kazmer, 1994; Schilthuizen *et al.*, 1997, 1998; Van Meer *et al.*, 1999
T. minutum	?	?	?	m	?	Wang & Smith, 1996
T. nubilale	?	?	+	?	?	Schilthuizen *et al.*, 1997; Van Meer *et al.*, 1999
T. oleae	+	+	+	?	+	Louis *et al.*, 1993
T. platneri	+	+	?	m	+	Stouthamer *et al.*, 1990a
T. pretiosum	+	+	+	m	+	Orphanides & Gonzalez, 1970; Stouthamer *et al.*, 1990a, b Schilthuizen & Stouthamer, 1997
T. sibericum	+	?	+	?	?	Schilthuizen & Stouthamer, 1997 Van Meer *et al.*, 1999
T. sp	+	?	?	?	?	Bowen & Stern, 1966
T. telengai	+	?	?	?	?	Sorakina, 1987
Cynipoidea						
Hexicola sp. *near websteri*	+	?	?	?	?	Eskafi & Legner, 1974
Leptopilina australis	?	?	+	?	?	Werren *et al.*, 1995a; Van Meer *et al.*, 1999
L. clavipes	?	?	+	?	?	Werren *et al.*, 1995a
Diplolepis rosae	?	?	+	f	-	Stille & Dävrig, 1980; Van Meer *et al.*, 1995, 1999
Diplolepis spinosissima	?	?	+	?	?	Plantard *et al.*, 1998

The evidence is classified as males following heat treatment (heat), males following antibiotic treatment (antib.), molecular evidence for *Wolbachia* presence (wolb.). In addition, information is given about the infection status of the population i.e. if the thelytokous forms are found in populations where thelytoky is fixed in the population or if it occurs mixed with arrhenotoky (i.s.), and if the males and females are capable of successfull copulations (cop.).

[a] +, Evidence exists; ? information not available.

[b] f, Thelytoky fixed in population; m, thelytoky and arrhenotoky occur in populations; ?, information not available.

[c] Copulations are successfull (+) or not (-).

[d] Mating and sperm transfer take place, but no successful fertilization of eggs.

[e] Mating of males of the thelytokous line is succesfull with closely related arrhenotokous females, but not with thelytokous females.

4. Female fitness studies of different T-*Wolbachia* infected *Trichogramma* populations

Objective: To determine whether *Wolbachia* strains from fixed populations have less impact on their host fitness compared to *Wolbachia* strains from mixed populations.

In previous research, fitness studies of T-*Wolbachia* were carried out with the fly pupal parasitoid *Muscididuax uniraptor* from a fixed population (Horjus and Stouthamer, 1995) and three different *Trichogramma* lines from one fixed and two mixed populations (Stouthamer and Luck, 1993). While no negative effect of *Wolbachia* was found for *M. uniraptor*, all *Trichogramma* lines showed a negative impact of the infection on total offspring production while infected or uninfected females of the fixed population produced an equal number of daughter offspring. We extended these previous studies by determining *Wolbachia* impact on various life history parameters of six additional *Trichogramma* lines from mixed and fixed populations (Chapter 6).

5. Quality assessments of *Wolbachia* infected versus non infected lines in greenhouse conditions

Objective: To study whether *Wolbachia* infected thelytokous wasps do equally well as arrhenotokous wasps in biological control.

Theoretically, in biological control, thelytokous females may be better control agents than sexual forms (Stouthamer, 1993). However, since *Wolbachia* can have a negative impact on female fecundity (Stouthamer and Luck, 1993), other fitness parameters like host search behaviour or dispersion could also be affected. This hypothesis was indirectly tested by comparing the parasitization efficacy of two species of infected and conspecific 'cured' *Trichogramma* lines in greenhouse compartments (Chapter 7).

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Spacer 2 region and 5S rDNA variation of *Wolbachia* strains involved in cytoplasmic incompatibility or sex-ratio distortion in arthropods

Abstract – Bacteria in the genus *Wolbachia* are widespread in arthropods and can induce sex-ratio distortion or cytoplasmic incompatibility in their host. The phylogeny of *Wolbachia* has been studied using 16S rDNA and the cell cycle gene *ftsZ*, but sequence variation of those genes is limited. The spacer 2 region (SR2) was amplified to determine whether this region would improve phylogenetic resolution. The SR2 of *Wolbachia* is 66 bp long, shows higher variation than *ftsZ* and has very low homology with closely related bacteria. Due to the small length of SR2 of *Wolbachia*, little phylogenetic information could be retrieved.

Introduction

Bacteria of the genus *Wolbachia* (α Proteobacteria, Rickettsiales) are widespread in arthropods and are inherited cytoplasmically. The infection can result in different alterations in the mode of reproduction of the host such as cytoplasmic incompatibility (CI), thelytoky (T) or feminization (F) (reviewed in Werren and O'Neill, 1997).

The 16S rDNA sequence data from different *Wolbachia* strains suggest they are closely related (< 3% difference) and can be subdivided into two distinct groups (type I and II) (Stouthamer *et al.*, 1993). Other genes analyzed from *Wolbachia* are the 23S rDNA (Rousset *et al.*, 1992b) and the bacterial cell-cycle *ftsZ* gene (Holden *et al.*, 1993; Werren *et al.*, 1995a). An extensive phylogenetic study of different *Wolbachia* strains based on the *ftsZ* gene has been carried out and revealed a maximum sequence difference of 15% between the strains. The *Wolbachia* clade was subdivided into group A and B which diverged from each other 58-67 million years ago (Werren *et al.*, 1995a).

Although *ftsZ* variation is much higher than 16S rDNA, it is still limited, especially among *Wolbachia* in group A (2.7 % maximum) (Werren *et al.*, 1995a). In addition, no congruence in host effect (CI, T or F) and phylogenetic relation of the different *Wolbachia* strains is found.

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Sequencing of a more variable gene of *Wolbachia* may adequately resolve the relationships between distinct *Wolbachia* strains displaying different reproductive phenotypes.

Non-coding regions are expected to have higher mutation rates than relative conserved coding genes such as *ftsZ*. Non-coding DNA regions, which are relatively easy to amplify, are intergenic transcribed spacers (IGS) and are located between 16S and 23S rRNA (spacer 1 region) or between 23S and 5S rRNA coding genes (spacer 2 region). *Wolbachia* lacks a spacer 1 region (SR1) but has a spacer 2 region (Bensaadi-Merchemek *et al.*, 1995). Previous studies showed that the spacer 2 region (SR2) of the several *Rickettsia sp.* was highly variable in comparison with 16S rDNA data (Stothard *et al.*, 1994). This high variation suggested that it could be a powerful tool for detailed phylogenetic study of closely related Rickettsial species. In this paper, the SR2 and 5S rDNA variation of several *Wolbachia* strains is analysed to determine its potential for fine-scale phylogenetic resolution. The SR2 and 5S rDNA were also sequenced for *Cowdria ruminantium*, a bacterium closely related to *Wolbachia* (Stouthamer *et al.*, 1993) and for the male killing bacterium of *Adalia bipunctata*, which is more distantly related (Werren *et al.*, 1994).

Materials and methods

Host and origin of the α *Proteobacteria* – The SR2 of eight different *Wolbachia* strains, *Cowdria ruminantium* and the male killing bacterium of *Adalia bipunctata* was sequenced (Table 1).

DNA extraction – Total insects (from isofemale lines) were thoroughly ground in 100µL STE buffer (O'Neill *et al.*, 1992) with a sterile polypropylene pestle and a phenol extraction was performed (Sambrook *et al.*, 1989).

Primer design and PCR – The 23S and 5S rDNA genes of *Rickettsia prowazekii*, *R. bellii* and *R. rickettsii* (Genbank Database accession numbers U11018, U11015 and U11022 respectively) were aligned to search for conserved regions in those genes. The primers Ri-23S (5' GTTTGAGAGGATCTGCC 3') and Ri-5S (5' CTCCCATGCCTTATGAC 3') amplified 237 bp of 23S rDNA, the SR2 and 81 bp of 5S rDNA of *Adalia bipunctata* male killing bacterium, but PCR with *Wolbachia* and *Cowdria ruminantium* resulted in no product. By aligning the published 23S-SR2-5S sequence of *Wolbachia* from *Culex pipiens* (U50394) (Bensaadi-Merchemek *et al.*, 1995) with the previously mentioned *Rickettsia* species, the following primers were designed: Wol-23S (5' CCAGTTGATAGGCTA 3'), Wol-5S1 (5' CTTGGCAACGAC CTAC 3') and Wol-5S2 (5' GTTTCATCGCTCTACT 3').

The primers Wol-23S and Wol-5S1 amplify 61 bp of 23S rDNA, the SR2 and 92 bp of 5S rDNA (Wol-5S1). The primers Wol-23S and Wol-5S2 amplify the same region including the complete 5S rDNA and 29 bp of the 3' flanking sequence of 5S rDNA.

For PCR, the temperature profile was 94°C for 3 min (1 cycle); 94°C for 1 min, 44°C - 42°C (ramp -1.5°C/ min), 72°C for 30s (10 cycles) and 30 cycles of 94°C for 1 min, 43°C for 45 sec, 72°C for 30 sec. All PCR products were cloned into a T-tailed vector (TA- cloning kit; Invitrogen, San Diego, USA). Plasmids were isolated using the QIA-prep Spin plasmid kit (Qiagen GmbH, Hilden, Germany) and sequenced on an ABI automated sequencer.

Table 1. Host and origin of the α Proteobacteria used in this study: (T: thelytoky; F: feminization; CI: cytoplasmic incompatibility).

Host species	α proteobacterium	Geographic origin	Reference
Trichogramma deion TX	*Wolbachia* group B (T)	Sanderson, Texas, USA	Stouthamer *et al.*, 1993
Trichogramma deion SW	*Wolbachia* group B (T)	Mojave desert, California, USA	Schilthuizen and Stouthamer, 1997
Leptopilina australis	*Wolbachia* group B (T)	Emmeloord, the Netherlands	Werren *et al.*, 1995a
Encarsia formosa	*Wolbachia* group B (T)	[Koppert BV] Netherlands	Van Meer *et al.*, 1995
Armadillidium vulgare	*Wolbachia* group B (F)	Wageningen, the Netherlands	te Beest *et al.*, 1998
Drosophila simulans	*Wolbachia* group A/Adm (CI)	Riverside, California, USA	Rousset *et al.*, 1992a
Muscidifurax uniraptor	*Wolbachia* group A/Adm (T)	Puerto Rico	Stouthamer *et al.*, 1993
Trichopria drosophilae	*Wolbachia* group A/Atc (T)	Benin, Africa	Werren *et al.*, 1995a
Tissue culture	*Cowdria ruminantium*	Mozambique	-
Adalia bipunctata	male killing bacterium	Cambridge, England	Werren *et al.*, 1994

Sequence alignment and phylogenetic analysis – Sequences were manually aligned using the sequence editor program ESEE 3.0 (Cabot, 1995). Phylogenetic trees were constructed by the neighbor-joining method (Saitou and Nei, 1987) with the MEGA program (v1.01) (Kumar *et al.*, 1993) and were midpoint rooted. Deletions and insertions in *ftsZ* and the SR2 were treated as single gaps and as one additional state. Kimura's 2-parameter distances (K) and nucleotide frequencies were determined by the MEGA program.

The SR2 sequences of this study have been deposited in GenBank under accession numbers AF051549-051558. For SR2 analysis, the dataset was supplemented with SR2 of *Culex pipiens* (U50394) (Bensaadi-Merchemek *et al.*, 1995) and *Tribolium confusum* (U97353) (Fialho and Stevens, 1997). In addition, six 16S rDNA sequences and ten *ftsZ* sequences were retrieved from Genbank: accession numbers (16S rDNA) M21789, X62432, X61768, L02884, AF045189 and L02822; (*ftsZ*) U28210, U28209, U28201, U28196, U28208, U28914, U28190, U28178, U28186 and U74474.

Results and discussion

PCR was performed with the different primer sets on the AB bacterium of *A. bipunctata*, *C. ruminantium* and on the *Wolbachia* strains. The Ri-23S and Ri-5S primers failed to amplify the SR2 from *C. ruminantium* and from several of the *Wolbachia* strains. These primers were therefore not suitable to use. Sequence analysis of the SR2 of *Wolbachia* of *Culex pipiens* (Bensaadi-Merchemek *et al.*, 1995) revealed that the Ri-5S primer hybridizes within a region of 5S rDNA that contains a 8 basepair deletion, accounting for the lack of amplification. Primer combination Wol-23S and Wol-5S1, amplified the SR2 of all *Wolbachia* strains in contrast to the combination Wol-23S and Wol-5S2 which only amplified the SR2 of the *Wolbachia* of *L. australis*, *E. formosa* and *T. deion* SW (group B *Wolbachia*; Table 1). The primer Wol-5S2 hybridizes with the flanking region of 5S rDNA which is probably more variable among the different *Wolbachia* strains than 5S rDNA itself.

No substantial length variation was found for the *Wolbachia* SR2 with the exception of the *Wolbachia* strain from *A. vulgare* which has a 10 bp deletion in comparison with the other group B *Wolbachia* SR2 sequences. The SR2 is 65 bp long for group A and 66 bp for group B *Wolbachia*. The SR2 sequences of group A contained a gap in the same position (Table 2).

The SR2 of *C. ruminantium* is 67 bp in length and showed low homology with other SR2 sequences from Genbank or with the SR2 of *Wolbachia*. Finally, the SR2 of the AB bacterium is 215 bp in length; it could not be aligned with those of *C. ruminantium* or *Wolbachia*, but showed similarity with the SR2 sequence of *R. rickettsii* (data not shown). According to data on the citrate synthase gene, *R. rickettsii* belongs to a separate clade to that of the AB bacterium (Roux *et al.*, 1997), but the lack of SR2 sequences of *Rickettsia* species more closely related to the AB bacterium explains this fact.

Table 2. Aligned SR2 sequences of *Wolbachia*.

L. australis	CTTTCTATATGTGCATATGCAGTGTTAAATATTAAATTAAAATTTATTAGGTAAGAAATTTTTGTT
C. pipiens	·····G·····A··C·····
E. formosa	·····A·····
T. confusum	·····G···A··T·····
T. deion TX	·····T·····G··T···GC·····T·····
T. deion SW	·····T·····G···A··T···G·····
A. vulgare	·····AT·····----------TG·····A··
T. drosophilae	·····G····TG·····G··G·····-G···AA·T·····A··
D. simulans Ri	·····TG·····G·····-G···A··T·····A··
M. uniraptor	·····TG·G·····G·····-G···A··T·····A··

· ·: alignment; - : gap.

Kimura's 2-parameter distances (K) of the SR2 sequences among the different *Wolbachia* strains was maximally 0.07 within group A, 0.14 within group B and 0.18 between groups A and B. These values are higher than for *ftsZ* (0.02, 0.04 and 0.14, respectively), 5S rDNA (0.01, 0.07 and 0.11, respectively) or 16S rDNA (0.04 between group A and B). These results indicate that 5S rDNA variation within the *Wolbachia* clade is considerably higher than for 16S rDNA. The same phenomenon is found when sequences of 16S rDNA and 5S rDNA of *C. ruminantium*, *R. prowazekii* and *Wolbachia* are compared (Table 3). These data are in contrast to previous studies of three human α proteobacterian pathogens in which 5S rDNA variation is comparable to the variation found in 16S rDNA (Minnick and Stiegler, 1993). An explanation for this finding could be that in these species 16S and 23S rDNA units are linked with SR1 (Roux and Raoult, 1995) and interaction can occur between the different spacer regions, as shown for enterococci (Naimi *et al.*, 1997). This interaction may lead to a more comparable variation between the ribosomal subunits.

A discrepancy in variation was found between *ftsZ*, and the SR2 data because high K values for SR2 can concur with low and high values for *ftsZ*. Such an example can be found when comparing *C. pipiens* with *T. deion* TX or *T. drosophilae* (Table 3). The maximum level of SR2 sequence variation has probably been reached within a shorter time period than for *ftsZ* and is then restricted. In order to estimate maximum variation between different *Wolbachia* strains, K was calculated for the third codon position of the *ftsZ* gene. This K value is considerably higher (0.30 maximum) between groups A and B *Wolbachia*, compared with the maximum K of 0.18 for the SR2 (all positions). Statistical analysis revealed that the maximum K values of *ftsZ* (3rd position) and the SR2 (all positions) are significant different ($p=0.039$, 2x2 contingency table with Yates correction) and confirms that the mutation rate in the SR2 is restricted.

This restriction may be induced by interaction of the SR2 with the 5' flanking sequence and a part of 23S rDNA (Bensaadi-Merchemek *et al.*, 1995).

Table 3. Kimura 2-parameter distance*‡ between the different *Wolbachia* strains, *C. ruminantium*§ and *R. prowazekii*.

		Trichogramma deion TX	*Encarsia formosa*	*Trichopria drosophilae*	*Muscidifurax uniraptor*	*Cowdria ruminantium*	*Rickettsia prowazekii*
Culex pipiens	16S	1.58	1.81	-	3.13	14.02	18.9
	5S	4.53	3.38	9.41	10.71	43.25	41.31
	ftsZ	3.26	3.38	12.02	12.28	-	-
	SR2	13.66	3.10	14.13	10.20	n.d.	n.d.
Trichogramma deion TX	16S		2.68	-	3.89	15.2	20.63
	5S		1.10	4.55	5.75	36.94	35.45
	ftsZ		3.83	12.07	12.07	-	-
	SR2		11.60	13.90	13.90	n.d.	n.d.
Encarsia formosa	16S			-	3.55	15.11	20.41
	5S			5.75	6.99	39.03	37.49
	ftsZ			11.39	12.18	-	-
	SR2			15.94	11.94	n.d.	n.d.
Trichopria drosophilae	16S				-	-	-
	5S				1.10	33.02	28.03
	ftsZ				2.24	-	-
	SR2				6.57	n.d.	n.d.
Muscidifurax uniraptor	16S					13.76	19.24
	5S					34.94	26.34
	ftsZ					-	-
	SR2					n.d.	n.d.
Cowdria ruminantium	16S						19.42
	5S						34.40
	ftsZ						-
	SR2						n.d.

*Values were multiplied by 100; ‡Gap sites and missing information data were removed in the pairwise comparisons only. §*C. ruminantium* 16S rDNA data was taken from a different strain than from the SR2 data; - data not available; n.d. not determined.

Slightly different trees were obtained for the *ftsZ* and the extended SR2 sequences (± 219 bp) which also includes the 3' flanking sequences of 23S rDNA (61 bp) and the major part of 5S rDNA (92 bp). The phylogenetic tree based on the extended SR2 region resulted in an A and B division and a subdivision of group A into Atc and Adm *Wolbachia* (Fig. 1) as similarly found for *ftsZ* (Werren *et al.*, 1995a). *Wolbachia* of *Armadillidium vulgare* and *Encarsia formosa* clustered differently in the two trees, but bootstrap values for *A. vulgare* were less than 50% in both trees, indicating its phylogenetic position is not clear.

In spite of the high degree of variation, the SR2 had relative restricted phylogenetic information due to its small length and is therefore not suitable for resolving the relationships between distinct *Wolbachia* strains displaying different reproductive phenotypes (CI, T or F).

However, recent molecular research on *Wolbachia* has provided two new promising candidates. The groE homologous operon (Masui *et al.*, 1997) has been sequenced and part of this operon (800 bp) showed much higher degree of variation within group A *Wolbachia* than for *ftsZ*. A second candidate is the *wsp* gene (±550 bp), coding for an outer membrane of *Wolbachia* (Braig *et al.*, 1998). Sequencing results of this gene showed at least three times higher variation within *Wolbachia* group A and B than *ftsZ* (Zhou *et al.*, 1998).

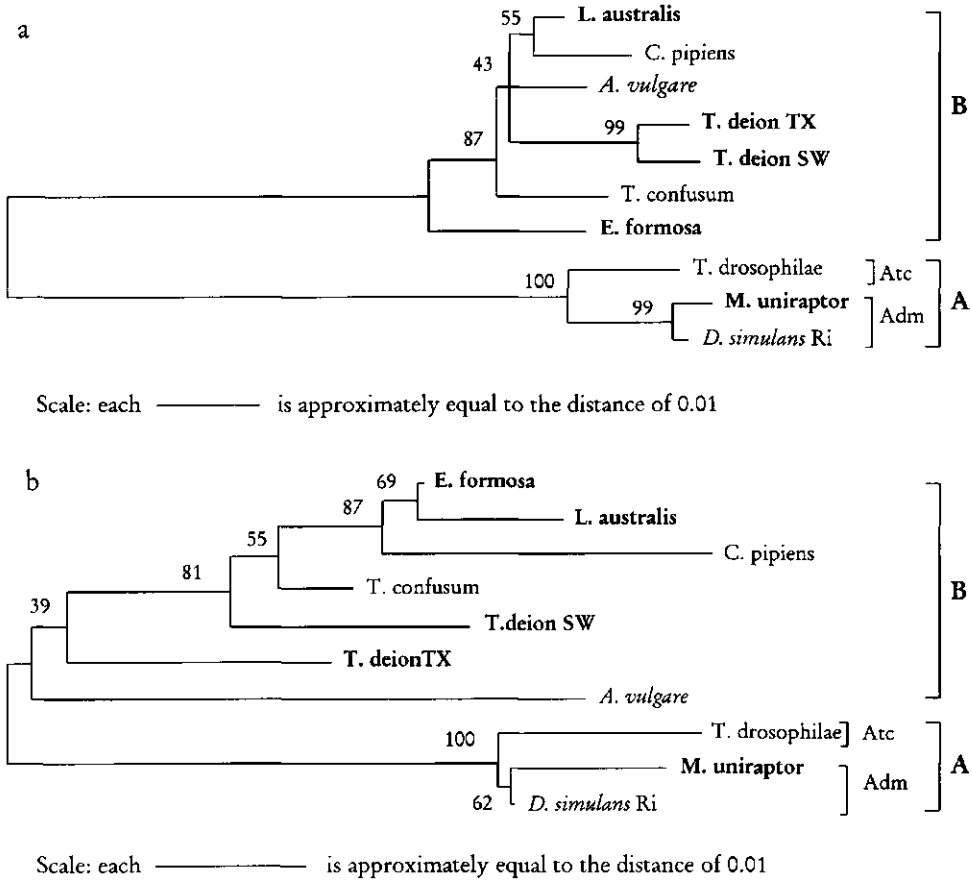

Figure 1. Phylogenetic relationships among *Wolbachia* strains from different insect hosts based on (a) *ftsZ* and (b): SR2 plus portion (61 bp) of the flanking sequence of 23S and 92 bp of 5S. Thelytoky and feminization associated *Wolbachia* are shown in bold and italic respectively. The trees were constructed using the neighbor-joining implementation. The numbers on the nodes indicate percentages of 500 bootstrap replicates.

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Phylogeny of the arthropod endosymbiont *Wolbachia* based on the *wsp* gene

Abstract – Bacteria of the genus *Wolbachia* (Rickettsiae) are widespread in arthropods and can induce cytoplasmic incompatibility (CI), thelytoky (T) or feminization (F) in their host. Recent research on the *wsp* gene of mainly CI inducing (CI-) *Wolbachia* has shown that this gene evolves at a much faster rate than previously sequenced genes such as 16S or *ftsZ*. As a result this gene appears to be very useful in subdividing the *Wolbachia* and twelve groups have been distinguished to date. Here we extend the *Wolbachia wsp* data set with fifteen T-*Wolbachia*, one F-*Wolbachia* and three other CI-*Wolbachia* strains. The results showed: (i) the addition of seven groups; (ii) no relation between host phenotype and *Wolbachia* phylogenetic position and (iii) possible horizontal *Wolbachia* transfer between the moth *Ephestia kuehniella* and its parasitoid *Trichogramma* spp.

Introduction

Many species of different insect orders carry obligatory symbionts such as bacteria, yeasts and flagellates (Buchner, 1965). Without the symbionts these insects show either stunted growth or do not survive at all (e.g. Nogge, 1976; Prosser and Douglas, 1991; Costa *et al.*, 1993). Besides these obligatory host-symbiont relationships, insects can also carry facultative microorganisms which interfere with their host's mode of reproduction. A well-known example is *Wolbachia* which was first described by Hertig (1936) in *Culex pipiens*. Surveys have shown *Wolbachia* to be present in at least 16% of sampled insect species (Werren *et al.*, 1995b). *Wolbachia* can cause a number of different reproductive alterations in their hosts, which facilitate the spread of the infection through a population: (1) Cytoplasmic incompatibility (CI) in which we can distinguish uni- and bidirectional incompatibility. Unidirectional incompatibility occurs when sperm from infected males cannot successfully fertilize eggs from uninfected females, while all other crosses are fertile. Bidirectional incompatibility occurs when a male and a female harbor a different strain of *Wolbachia* that are mutually incompatible. An incompatible cross leads to the loss of the paternal genome, resulting in only male offspring in haplodiploid hymenoptera and embryonic death in diploid species or in parahaploid mites.

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These phenomena have been found in several insect orders and also in isopods and mites (Legrand *et al.*, 1980; Noda, 1984; Wade & Stevens, 1985; Hoffmann, 1988; Merçot *et al.*, 1995; Perrot-Minnot *et al.*, 1996; Breeuwer, 1997); (2) Thelytoky (T), a form of parthenogenesis, where unfertilized eggs from *Wolbachia*-infected females develop into females, whereas they normally would develop into males. This effect is found in several hymenopteran species (Rousset *et al.*, 1992b; Van Meer *et al.*, 1995; Stouthamer *et al.*, 1993; Stouthamer, 1997); (3) Feminization (F), is found in isopods where genetic males are converted into functional females (Rousset *et al.*, 1992b; Juchault *et al.*, 1994); (4) Fecundity increase of the host and has been found for the egg parasitoid *Trichogramma bourarachae* (Girin and Boulétreau, 1995). In this *Wolbachia* infected species, no other reproductive alteration has been found.

Despite the wide variety of effects that different *Wolbachia* strains have on their hosts and their presence in a broad range of arthropods, there is less than 3% difference in the 16S rDNA of several *Wolbachia* strains (O'Neill *et al.*, 1992; Stouthamer *et al.*, 1993). To improve phylogenetic resolution, other *Wolbachia* genes have been analyzed such as 23S rDNA (Rousset *et al.*, 1992b), the bacterial cell-cycle *ftsZ* gene (Holden *et al.*, 1993; Werren *et al.* 1995a) and spacer-2 region (SR2) including 5S rDNA (Fialho *et al.*, 1997; Van Meer *et al.*, 1999).

Based on the *ftsZ* gene, the *Wolbachia* clade has been subdivided into group A and B which diverged from each other 58-67 million years ago (Werren *et al.*, 1995a). *Wolbachia* from both groups are found in a wide variety of insect species. A maximum sequence variation of 15% was reported between group A and B but was relatively low within group A (3%). This suggests that extensive horizontal transmission has occurred recently in this group (Werren *et al.*, 1995a). The 23S rDNA data yielded similar phylogenies as the 16S data and did not improve phylogenetic resolution (Rousset *et al.*, 1992b). SR2 variation was larger than *ftsZ* variation (6% variation in group A) but did not improve resolution due to its small size (van Meer *et al.*, 1999). All of the phylogenies published to date showed that the various effects (CI, T, F) induced by the *Wolbachia* were polyphyletic.

Recent research on *Wolbachia* genes resulted in two new candidates for detailed phylogenetic studies: (1) the groE-homologous operon showed more variation than *ftsZ* (Masui *et al.*, 1997) but no large sequence data set exists of this gene; (2) the *wsp* gene, that codes for an outer membrane protein of *Wolbachia* (Braig *et al.*, 1998), also exhibited higher variation than the *ftsZ* gene (14% variation within the least variable group A *Wolbachia*) and an extensive sequence database was available (Zhou *et al.*, 1998).

This study showed that the *Wolbachia* clade was subdivided into supergroup A and B (synonymous to group A and B) in which twelve distinct groups were recognized based on the grouping criterion of 2.5% sequence difference (Zhou *et al.*, 1998). In addition, the *wsp* gene seemed better at associating the *Wolbachia* effect with their phylogenetic position since it was shown that all *Wolbachia* strains inducing no CI effect in *Drosophila* were able to rescue closely related strains that do induce CI (Bourtzis *et al.*, 1998).

The study of Zhou *et al.* (1998) focused on CI-*Wolbachia* and from each supergroup, only one T-*Wolbachia* strain was sequenced including the fly parasitoid *Muscidifurax uniraptor* (supergroup A) and the egg parasitoid *Trichogramma deion* (supergroup B). Those T-*Wolbachia* strains did not cluster with CI-*Wolbachia* strains (Zhou *et al.*, 1998).

To determine whether the T-*Wolbachia* strains represent distinct groups, fifteen T-*Wolbachia* strains were sequenced and combined with the *wsp* sequences of Zhou *et al.* (1998). In addition, this data set was supplemented with one CI-*Wolbachia* strain from the parasitoid *Trichopria drosophilae* because in the *ftsZ* tree this strain formed a separate clade (Atc) within the relatively conserved group A (Werren *et al.*, 1995a).

Secondly, a *Wolbachia* strain from the *Trichogramma* host *Ephestia kuehniella* was sequenced to look for evidence of horizontal transmission of *Wolbachia* between host and parasitoid. Such a phenomenon has been suggested for the parasitoids of the genus *Nasonia* and their fly host *Protocalliphora* sp. because their *Wolbachia* strains, based on the *ftsZ* sequences, clustered together (Werren *et al.*, 1995a). Horizontal transmission has also been suggested for *Wolbachia* within the *Trichogramma* genus. All *Trichogramma Wolbachia* were monophyletic according to the *ftsZ* gene and cospeciation of host and symbiont was excluded as an explanation because the phylogenetic trees of *Trichogramma* and their *Wolbachia* were not congruent (Schilthuizen and Stouthamer, 1997). These results suggested that horizontal transmission sometimes occurs inside a common host (Lepidopteran eggs) of *Trichogramma* parasitoids (Schilthuizen and Stouthamer, 1997). To look for different example of parasitoids using a common host, three *Wolbachia* strains from the thelytokous parasitoids of the greenhouse whitefly *Trialeurodes vaporariorum* were included to determine if their T-*Wolbachia* strains were monophyletic too. Finally, one F-*Wolbachia* strain of the isopod *Armadillidium vulgare* was included to determine whether this strain would form a separate group.

A phylogenetic reconstruction was done based on all *wsp* sequences using the neighbor joining and the maximum parsimony implementation. Furthermore, Kimura distance values of *wsp*, *ftsZ* and SR2 between the *Wolbachia* strains were calculated and compared.

Materials and methods

Host species – The different host species used for *Wolbachia* DNA isolation are shown in Table 1.

Table 1. Host and origin of the *Wolbachia* strains used in this study.

Host	Origin	References
Amitus fuscipennis	CIAT, Cali, Colombia	Viggiani, 1991
Apoanagyrus diversicornis	CIAT, Cali, Colombia	Pijls *et al.*, 1996
Armadillidium vulgare	Wageningen, the Netherlands	Te Beest *et al.*, 1998
Coccidoxenoides peregrinus	South-Africa	Flanders, 1965; this study
Diplolepis rosae	Wageningen, The Netherlands	Van Meer *et al.*, 1995
Encarsia formosa	Koppert, The Netherlands	Van Meer *et al.*, 1995
Ephestia kuehniella	Koppert, The Netherlands	Rousset *et al.*, 1992b
Eretmocerus staufferi	Texas, USA	Rose and Zolnerowich, 1997
Leptopilina australis	Emmeloord, The Netherlands	Werren *et al.*, 1995a
Spalangia fuscipes	Leiden university, lab strain	Werren *et al.*, 1995a
Torymus bedeguaris	Wageningen, The Netherlands	Schilthuizen and Stouthamer, 1998
Trichogramma kaykai	LC110, El Paso Mountains, CA, USA	Schilthuizen *et al.*, 1998
Trichogramma nubilale	234, Nova Scotia, Canada	Schilthuizen and Stouthamer, 1997
Trichogramma kaykai	JT6-3, San Bernardino, CA, USA.	this study
Trichogramma deion	SW436, Mojave desert, CA, USA	Schilthuizen and Stouthamer, 1997
Trichogramma sibericum	SIB, Canada	Schilthuizen and Stouthamer, 1997
Trichogramma kaykai	AW7-5, Sky valley, Riverside CA, USA	Schilthuizen *et al.*, 1998
Trichogramma bourarachae	Divor, Portugal	Silva and Stouthamer, 1997
Trichopria drosophilae	Benin, Africa	Werren *et al.*, 1995a

DNA extraction – Two different protocols were used for isolating *Wolbachia* DNA. STE extraction was performed as described by O'Neill *et al.* (1992): approximately 100 entire *Trichogramma* individuals were homogenized in 49 µL STE-buffer with 1 µL proteinase K (20mg/ml). The mixture was incubated for one hour at 37°C followed by 5 min at 95°C to inactivate the proteinase K. For the other species, the amount of STE-buffer was adjusted according to the insect size. For phenol extraction the following protocol was used: 100 *Trichogramma* wasps were homogenized in 150 µL STE-buffer and 150 µL phenol was added followed by 15 min shaking. After centrifuging for 10 min, the upper phase was transferred to a new vial and 150 µL chloroform/isoamyl alcohol (24:1) was added. The mixture was vigorously shaken for 10 min followed by 10 min centrifugation.

The upper phase of the solution was transferred to a new vial, mixed with 15 µL Sodium acetate (3M, pH 5,5) and 375 µL 96% ethanol was added. This mixture was incubated at -80°C degrees for one hour and then centrifuged for 20 min at 4°C. The pellet was washed with 70% ethanol and dried and 50 µL of distilled water was added.

PCR amplification – The *wsp* region of the *Wolbachia* DNA was amplified with the primers *wsp*-81F (forward) and *wsp*- 691R (reverse) (Braig *et al.*, 1998). Infected strains of *Nasonia vitripennis* E13, *Trichogramma deion* and *T. kaykai* were used as positive controls. Uninfected *Drosophila simulans*, *Trichogramma spp.* and *N. vitripennis* were used as negative control. For assessing primer specificity, *Cowdria ruminantium*, a bacterium closely related to *Wolbachia*, was also tested. For PCR, a temperature profile of 94°C for 3 min (1 cycle); 94°C for 1 min, 55°C for 1 min and 72°C for 1 min (40 cycles) was utilized.

Cloning and sequencing – The PCR product was isolated using the freeze-squeeze method (Sambrook *et al.*, 1989) and directly cloned into a T-tailed vector (TA- cloning kit; Invitrogen, San Diego). Plasmids were isolated using the QIA-prep Spin plasmid kit (Qiagen) and sequenced on an ABI automated sequencer. Clones (same strand) were sequenced twice when amino-acid translation showed frameshifts. The *wsp* sequences of this study have been deposited in GenBank under accession numbers AF071909-071927.

Phylogenetic analysis – The data set consisted of 19 *wsp* sequences from this study combined with the *wsp* sequences of Zhou *et al.* (1998). From the 28 *wsp* sequences of Zhou et al. (1998), five sequences from group A and two from group B were removed since they were identical or almost identical to *wsp* sequences in the same group. All *wsp*-sequences were aligned using the ClustalX program (Thompson *et al.*, 1994) followed by manual modifications based on amino acid translation of the different genes. The third hypervariable region of the gene (Braig *et al.*, 1998) was excluded because no accurate alignment could be made. The data set was analyzed by using the Neighbor-Joining implementation (Saitou and Nei, 1987) from the PHYLIP software package version 3.5c (Felsenstein, 1995). Deletions and insertions in *wsp* were treated as an additional state. Bootstrap analysis was done with 1000 replications. The data set was also analyzed by maximum parsimony using PAUP 3.1 (Swofford, 1993). Heuristic searches were performed, gaps were treated as fifth base and bootstrap analysis was done with 500 replications. All phylogenetic trees were midpoint rooted because no suitable outgroup was available. Kimura's 2-parameter distances (K) were determined by the MEGA program (v 1.01, Kumar *et al.* 1993). Sequence data of *wsp*, *ftsZ* and SR2 of several *Wolbachia* strains were retrieved from Genbank.

Results

Primer specificity

PCR amplification with *wsp* primers (81F, 691R) was successful for all hosts that were known to be infected using the *ftsZ* primers of Werren *et al.* (1995a) and Holden *et al.* (1993). None of the negative controls did result in PCR amplification. In addition, no reaction occurred with *Cowdria ruminantium* DNA, a bacterium closely related to *Wolbachia*.

Wolbachia *phylogeny*

We combined our data set (19 *Wolbachia* sequences including 18 insects and 1 isopod host species) with 21 *Wolbachia* sequences from the data set of Zhou *et al.* (1998) and examined the nucleotide sequences of the *wsp* gene. The sequences showed a maximum divergence of 16% within supergroup A, 23% within supergroup B and 23% between supergroup A and B. These values are much higher than those for the *ftsZ* sequences (3%, 6% and 15% respectively) (Werren *et al.*, 1995a).

Figure 1 shows the tree based on the general data set using the neighbor-joining implementation after bootstrapping 1000 times. The topology shows the division of *Wolbachia* into two supergroups, A and B which corresponds with 16S rDNA, *ftsZ* , SR2 and the previous *wsp* data (O'Neill *et al.*, 1992; Rousset *et al.*, 1992b; Stouthamer *et al.*, 1993; Werren *et al.*, 1995a; Van Meer *et al.*, 1999; Zhou *et al.*, 1998).

Using the grouping criterion of 2.5% sequence difference as proposed by Zhou *et al.* (1998), seven new groups (two in supergroup A and five in B) were added to the twelve already found. The CI-*Wolbachia* strain from *T. drosophilae* which was placed in a distinct clade (Atc, group A) in the *ftsZ* tree (Werren *et al.*, 1995a) was most closely related to *G. austeni* but differed sufficiently (≥9%) to represent a new group (Dro). By adding the Kue and Dro group to supergroup A, bootstrap values in the phylogenetic tree were greatly improved (≥80) for *M. uniraptor* and *D. simulans* (Ri) compared to Zhou *et al.*, (1998) where these values were less than 50 (Fig. 1). In supergroup B, *A. diversicornis* and *E. formosa* (Hymenoptera) were not sufficiently related to the existing groups to be included and therefore represented two additional groups (Div and For respectively). The *Trichogramma Wolbachia* were subdivided into three adjacent groups Dei, Sib and Kay. The isopod *A. vulgare* represented a new group (Vul) and was most closely related to the Pip group. The *wsp* sequence of *A. vulgare* differed by at least 13% from all other strains which suggests that it is a distant group from all others.

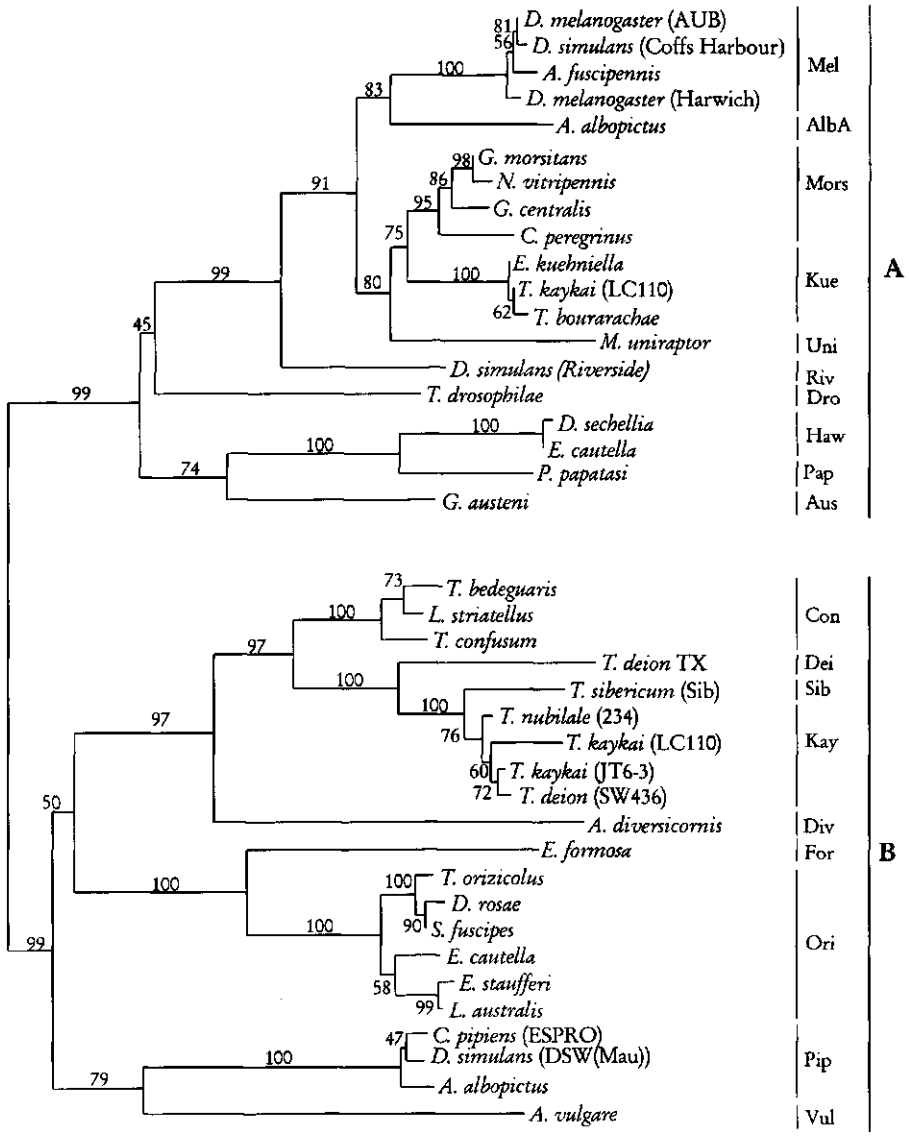

Figure 1. Phylogenetic tree of *Wolbachia* based on the *wsp* sequences. Name of the host arthropod species is followed by the group designation. The tree has been constructed by the Neighbor Joining algorithm (Saitou and Nei, 1987) using the Kimura distance and is midpoint rooted. Numbers on the nodes indicate percentages of 1000 bootstrap replicates.

The phylogenetic tree with maximum parsimony implementation was identical in topology to the Neighbor Joining tree and showed only slight differences in subdivisions within groups (tree not shown).

Wolbachia *phylogeny and phenotype*

An overview of *Wolbachia* strains and the expressed phenotype per clade is given in Table 2. These results show no correlation between phenotype and phylogenetic position, both CI- and T-*Wolbachia* are found within the same group. F-*Wolbachia* from *A. vulgare* represents a distinct group (Vul).

Horizontal transmission

The occurrence of similar *Wolbachia* retrieved from unrelated hosts confirms the notion that *Wolbachia* sometimes undergo horizontal transmission and is in agreement with results of *ftsZ* data. Because the *wsp* gene evolves at a faster rate than *ftsZ*, these sequences can be used more accurately to determine the potential horizontal transmission between different hosts. However, in many cases it remains unclear how such transfers could have taken place because the connection between the species is obscure. One example is found in the Ori group where *S. fuscipes* (a *Drosophila* parasitoid) and *D. rosae* (a gall former on wild species of *Rosa*) have a similar *wsp* sequences that differ only by 0.2%.

Other host species with very similar *wsp* sequences are found within the three adjacent *Trichogramma Wolbachia* groups (Dei, Kay and Sib) and in the Kue group (supergroup A) where *E. kuehniella*, *T. bourarachae* and *T. kaykai* (LC110) cluster together. The *wsp* sequence of *E. kuehniella* was identical to the *wsp* sequence of *T. kaykai* (LC110) and differed in 1 base pair only from *T. bourarachae*. LC110 is also infected with a *Wolbachia* strain from the Kay clade (supergroup B). PCR on LC110 wasps with the *ftsZ* primers of Werren *et. al* (1995a) resulted in a faint band for supergroup A *Wolbachia* and a clear band for supergroup B *Wolbachia*.

In addition, restriction analysis was done on the *wsp* sequences. The *wsp* sequences from the Kay group have a *Bgl* II restriction site while this site is absent in the Kue group sequences. Restriction analysis showed that the concentration of the distinct bands was much higher for the Kay *Wolbachia* (data not shown) and confirms the results with the *ftsZ* primers. To avoid the possibility that the Kue *Wolbachia* DNA found in LC110 and *T. bourarachae* was the remains of *E. kuehniella* egg material the wasps consumed during their larval stage, we reared both lines for an extra generation on uninfected *Mamestra brassica* eggs.

Table 2. *Wolbachia* group nomenclature and Genbank accession numbers (GB #)

Super-group	Group	*Wolbachia* host species	Associated *Wolbachia* strain (reference strain in bold)	Phenotype	GB #
A	Mel	*D. melanogaster* (Aub)	***w*Mel**	CI	AF 020063
		D. simulans (Coffs harbour)	*w*Cof	NE	AF 020067
		A. fuscipennis	*w*Fus	T	AF 071909
		D. melanogaster (Harwich)	*w*MelH	NE	AF 020066
	Alba	*A. albopictus* (Houston)	***w*AlbA**	CI	AF 020058
	Mors	*G. morsitans*	***w*Mors**	?	AF 020079
		N. vitripennis	*w*VitA	CI	AF 020081
		G. centralis	*w*Cen	?	AF 020078
		C. peregrinus	*w*Per	T	AF 071914
	Kue	*E. kuehniella*	***w*Kue**	?	AF 071911
		T. kaykai (LC110)	*w*KayA	T	AF 071912
		T. bourarachae	*w*Bou	Fec	AF 071913
	Riv	*D. simulans* (Riverside)	***w*Ri**	CI	AF 020070
	Uni	*M. uniraptor*	***w*Uni**	T	AF 020071
	Ha	*D. sechellia*	***w*Ha**	CI	AF 020073
		E. cautella	*w*CauA	CI	AF 020075
	Pap	*P. papatasi*	***w*Pap**	?	AF 020082
	Aus	*G. austeni*	***w*Aus**	?	AF 020077
	Dro	*T. drosophilae*	***w*Dro**	CI	AF 071910
B	Con	*T. confusum*	***w*Con**	CI	AF 020083
		L. striatellus	*w*Stri	CI	AF 020080
		T. bedeguaris	*w*Bed	T/?	AF 071915
	Dei	*T. deion* (TX)	***w*Dei**	T	AF 020084
	Sib	*T. sibericum* (SIB)	***w*Sib**	T	AF 071923
	Kay	*T. kaykai* (JT6-3)	***w*KayB**	T	AF 071924
		T. kaykai (LC110)	*w*KayLC	T	AF 071927
		T. deion (SW 436)	*w*DeiSW	T	AF 071925
		T. nubilale (234)	wNub	T	AF 071926
	Div	*A. diversicornis*	***w*Div**	T	AF 071916
	For	*E. formosa*	***w*For**	T	AF 071918
	Ori	*T. orizicolus*	***w*Ori**	CI	AF 020085
		D. rosae	*w*Ros	T/?	AF 071922
		S. fuscipes	*w*Fu	T/?	AF 071921
		E. cautella	*w*CauB	CI	AF 020076
		E. staufferi	*w*Sta	T/?	AF 071919
		L. australis	*w*Aus	T	AF 071920
	Pip	*C. pipiens* (ESPRO)	***w*Pip**	CI	AF 020061
		D. simulans (DSW/MAU)	*w*Ma	-	AF 020069
		A. albopictus (Houston)	*w*AlbB	CI	AF 020059
	Vul	*A. vulgare*	***w*Vul**	F	AF 071917

CI = Cytoplasmatic incompatibility, F = feminization, Fec = Fecundity increase, NE = no effect or rescue effect of CI (Bourtzis *et al.*, 1998), T = Thelytoky, T/? = *Wolbachia* and thelytoky are both observed, but no curing experiments have been performed, ? = unknown.

PCR and restriction analysis experiments were repeated and again the wasps were found to be infected with the same Kue group *Wolbachia*.

Another parasitoid group which shares a common host are the whitefly parasitoids *E. staufferi*, *E. formosa* and *A. fuscipennis*. However, their corresponding *Wolbachia* strains are not closely related and they are present in both supergroups (in Ori, For and Mel groups respectively).

Kimura-2 parameter distance comparisons among the different genes of Wolbachia strains

The Kimura-2 parameter distance (K) of *ftsZ*, SR2 and *wsp* between the different *Wolbachia* strains was calculated to test if mutation rates of different *Wolbachia* genes were congruent with each other (Table 2). These results show relative low K(*ftsZ*) values corresponding with widely varying K(SR2) or K(*wsp*) values. It can also be seen that relatively low K(SR2) values correspond with relatively high K(*wsp*) values and high K(SR2) values with relative low K(*wsp*) values.

Discussion

The *wsp* gene sequence variation is much higher (Zhou *et al.*, 1998; this study) compared to the *ftsZ* gene (Werren *et al.*, 1995a) or the spacer 2 region (Van Meer *et al.*, 1999). Comparison of Kimura distances of SR2 and *wsp* suggests that evolution in the *wsp* gene is initially fast but slows down in time due to saturation of the third codon position. This finding is supported by the equal variation (23%) within supergroup B and between supergroup A and B and contrasts with *ftsZ* and SR2 data where variation between supergroup A and B is at least a factor two higher than within supergroup B (Werren *et al.*, 1995a; van Meer *et al.*, 1999).

The grouping threshold of 2.5% variation as suggested by Zhou *et al.* (1998) resulted in the addition of seven new groups to the existing twelve. Although the resolution between the different *Wolbachia* strains has been improved by using the *wsp* gene, CI- and T-*Wolbachia* do not constitute monophyletic groups. This result is similar to the phylogenies based on 16S and *ftsZ* sequences (Stouthamer *et al.*, 1993; Werren *et al.*, 1995a). F-*Wolbachia* from *A. vulgare* represents a new group but additional F-*Wolbachia* strains have to be sequenced to determine if feminization is monophyletic based on this gene.

Several mechanisms have been proposed for this lack of concordance between *Wolbachia* phylogeny and the reproductive phenotype that they generate in their host (Van Meer *et al.*, 1995): (1) the existence of plasmids or phages associated with *Wolbachia* that carry the

genes responsible for the phenotype; (2) the host determines the effect on an aspecific *Wolbachia* infection; (3) genes inducing CI or T are highly similar and can be derived from each other by a simple mutation. The first mechanism could be possible but so far plasmids in the α Proteobacteria have only been found in the root symbiont *Rhizobium sp.* (Pardo *et al.*, 1994) and not in bacteria more closely related to *Wolbachia*. Phages have been found in the male-killing spiroplasma of *Drosophila*, where each spiroplasma type seemed to be associated with their specific phages (Cohen *et al.*, 1987) and electron-microscope studies revealed the existence of virus-like particles in *Wolbachia* (Wright *et al.*, 1978). But in various other studies, they have not been found in *Wolbachia* (Martin *et al.*, 1973; Wright and Wang, 1980; Louis and Nigro, 1989). The second mechanism is not supported because various micro-injection studies show that similar effects were found in the new host-*Wolbachia* combination as in the original one (Braig *et al.*, 1994; Giordano *et al.*, 1995; Juchault *et al.*, 1994) although evidence exists that nuclear genes can mediate the severity of CI expression (Boyle *et al.*, 1993; Bordenstein *et al.*, 1998). Finally, the last mechanism is difficult to test but analysis of *in vivo* synthesized proteins of *Wolbachia* revealed that the size of some proteins correlated with the phenotype they generate in their host. Analysis and sequencing of these proteins might resolve this issue (Sasaki *et al.*, 1998).

Table 3. Kimura 2-parameter distance[1,2] between the different *Wolbachia* strains based on *ftsZ*, the spacer-2 region (SR2) and *wsp*.

		Encarsia formosa	*Trichogramma deion* TX	*Armadillidium vulgare* (Wag)[3]	*Trichopria drosophilae*	*Muscidifurax uniraptor*	*Drosophila simulans* Ri
Leptopilina australis	*ftsZ*	2.60	2.27	1.29	11.36	11.94	11.78
	SR2	1.54	9.78	9.67	18.07	13.90	11.94
	wsp	9.86	18.65	18.24	15.56	19.32	22.31
Encarsia formosa	*ftsZ*		3.83	2.82	11.50	12.29	12.17
	SR2		11.60	9.67	15.94	11.94	10.05
	wsp		20.87	19.67	17.98	25.10	24.03
Trichogramma deion TX	*ftsZ*			2.27	12.31	12.31	12.19
	SR2			7.71	13.90	13.90	11.94
	wsp			27.17	17.66	30.06	23.77
Armadillidium vulgare (Wag)	*ftsZ*				11.90	12.16	12.04
	SR2				12.06	9.84	7.71
	wsp				26.28	23.06	20.29
Trichopria drosophilae	*ftsZ*					2.24	2.02
	SR2					6.57	4.84
	wsp					17.06	14.00
Muscidifurax uniraptor	*ftsZ*						0.42
	SR2						1.56
	wsp						11.40

[1]Values were multiplied by 100. [2]Gap sites and missing information data were removed in the pairwise comparisons only. [3]*ftsZ* sequence of this strain (Te Beest *et al.*, 1998) is identical to the strain used in the study of Werren *et al.* (1995a). Genbank accession numbers of *ftsZ*: U28178, U28186, U28190, U28196, U28208, U28210; SR2: AF051549, AF051551-051554, AF051556, AF051557; *wsp*: see Table 2.

Several species of *Trichogramma* have been tested for their *Wolbachia* and they all appear to harbour closely related *Wolbachia* (Kay, Dei and Sib groups). These groups do not contain any *Wolbachia* strains isolated from hosts other than *Trichogramma*. Three possible explanations may be given for this pattern: (1) T-*Wolbachia* have cospeciated with their wasp host; (2) there is an occasional horizontal transmission between *Trichogramma* spp. when individuals of two species share a host; (3) the different *Trichogramma* species will occasionally take up the *Wolbachia* of their Lepidopteran host which happen to be a *Wolbachia* of the Kay, Sib or Dei groups. The first possibility has been excluded by Schilthuizen and Stouthamer (1997) since the host and the *Wolbachia* phylogenies were not congruent. For the second explanation, it is unclear how *Wolbachia* exchange can occur. The mechanism could be explained by predation of an infected parasitoid by an uninfected one, or by "blood-blood" contact between infected and uninfected individuals as found in isopods (Rigaud and Juchault, 1995). So far, infection by oral ingestion has not been established for the isopod *A. vulgare* (Juchault *et al.*, 1974) or for *Trichogramma brassicae* (Pintureau *et al.*, 1993). The third possibility is supported because *wsp* sequences of the Lepidopteran host *E. kuehniella* are identical to the *wsp* sequence of *T. kaykai* (LC110) and differ in 1 basepair only with the *wsp* sequence of *T. bourarachae*. Both *Trichogramma* species were reared on *E. kuehniella* eggs for at least 3 years in our laboratory. It is not known whether they were already infected with Kue *Wolbachia* in the field but we know that not all *Trichogramma* species or lines take up Kue *Wolbachia* when reared on infected host eggs (unpublished data). Both *Trichogramma* species are not sympatric as *T. kaykai* (LC110) was collected from California and *T. bourarachae* from Portugal (Silva and Stouthamer, 1997). Experiments showed that *Wolbachia* infection in *T. bourarachae* resulted in a fitness increase of the host (Girin and Boulétreau, 1995). In *T. kaykai* (LC110) this Kue infection coexisted with *Wolbachia* (Kay) of group B. This Kay *Wolbachia* is probably involved in the induction of thelytoky and it is not yet known if double infection has an additional effect on the host. Additional experiments have to be performed to assess the influence of the Kue *Wolbachia* in LC110.

Although we found evidence of *Wolbachia* exchange between host and parasitoid, it remains unclear if this is an exceptional or more general mode of horizontal *Wolbachia* transmission within the *Trichogramma* genus. The number of identified *Wolbachia* of Lepidoptera is limited (O'Neill *et al.*, 1992; Rousset *et al.*, 1992b; Werren *et al.*, 1995b) but so far no Lepidopteran species have yet been found with a Kay, Dei or Sib *Wolbachia*. Therefore, we can not discriminate between the second or third proposed explanation and in fact, they are not mutually exclusive.

In our analysis, we included three thelytokous parasitoids of the whitefly *Trialeurodes vaporariorum* to determine if sharing of this common host would also lead to a monophyletic

group of *Wolbachia*. In contrast to *Trichogramma* spp., these parasitoids belong to different families of Hymenoptera. *E. formosa* and *E. staufferi* both belong to the Aphelinidae whereas *A. fuscipennis* belong to the Platygasteridae. Their T-*Wolbachia* belong to distantly related groups and are present in both supergroups. These results suggest that for parasitoids, both sharing of hosts and belonging to the same genus may be a critical factor in the interspecies exchange of *Wolbachia*.

An additional case of double infection in a thelytokous species beside LC110 was found in the parasitoid *S. fuscipes* by Werren *et al.* (1995a). However we could not confirm these results because PCR with *ftsZ* primers specific for group A and B (Werren et al, 1995a) amplified only group B *Wolbachia*. Double infection has been described for several CI-*Wolbachia* strains (Rousset et al, 1992b; Sinkins *et al.*, 1995a; Werren *et al.*, 1995a) and create a novel compatibility type because they can rescue single infected sperm, but singly infected eggs do not rescue double infected sperm (Merçot *et al.*, 1995; Rousset and Solignac, 1995; Perrot-Minnot *et al.*, 1996). This explains why double infections can exist in populations. For thelytoky, double infections are rare (Werren *et al.*, 1995a) and in arthropods carrying feminizing *Wolbachia* they have not yet been reported. For thelytoky or feminization, double infection could be stable if this leads to a fitness increase as found in *T. bourarachae* for example (Girin and Boulétreau, 1995).

We expanded the *Wolbachia* clade with seven new groups. When more related *Wolbachia* strains are found, some groups may be subdivided. Because the choice of a reference strain is arbitrary, it may happen that when closely related strains are added, one may shift to another reference strain in order to subdivide the group. For example in the *Trichogramma* Kay group, we chose to cluster all *T. kaykai* strains and to make a distinct group for *T. sibericum* even though some strains of *T. kaykai* differed less than 2.5% from this *T. sibericum*. A primer based system for the groups (Zhou *et al.*, 1998) will facilitate identification of infections or superinfections in various hosts but the design of specific primers will become more difficult with the increase in the number of closely related groups as found for the *Trichogramma Wolbachia*.

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Cross-order transfer of *Wolbachia* from *Muscidifurax uniraptor* (Hymenoptera: Pteromalidae) to *Drosophila simulans* (Diptera: Drosophilidae)

Abstract – Bacteria of the genus *Wolbachia* are widespread in arthropods and are cytoplasmically inherited. They can induce different effects on the host such as cytoplasmic incompatibility (CI), thelytoky (T) or feminization (F). In some *Wolbachia*-infected hosts, no effect (N) has been found. Successful transfer of *Wolbachia* by microinjection from one host to an uninfected one has been established with CI, F, N-*Wolbachia* but not with T-*Wolbachia*. Here, we describe a transfer experiment of T-*Wolbachia* from the parasitoid *Muscidifurax uniraptor* to *Drosophila simulans*. The infection could be detected in the new host for several generations by polymerase chain reaction (PCR). However, no specific effects on the host were detected, and the bacteria were not stably maintained.

Introduction

Intracellular bacteria of the genus *Wolbachia* (Rickettsiales) are found in many different arthropod species. Although infection with these bacteria was known from mosquitoes at the beginning of this century (Hertig, 1936), it was only with the availability of polymerase chain reaction (PCR) technology at the end of the 1980s that the widespread occurrence of these bacteria was revealed. Screening of insect species from different orders from Panama revealed an infection rate of 16% (Werren *et al.*, 1995b).

Wolbachia are cytoplasmically inherited and can have a number of different effects on their hosts: (1) cytoplasmic incompatibility (CI), which is a crossing incompatibility in which sperm from *Wolbachia*-infected males cannot successfully fertilize eggs from uninfected females, whereas all other crosses are fertile (Yen and Barr, 1971; Trpis *et al.*, 1981; Noda, 1984; Wade and Stevens, 1985; Hoffmann *et al.*, 1986; Breeuwer and Werren, 1990); (2) thelytoky (T), which is found in Hymenoptera - unfertilized eggs from *Wolbachia*-infected females develop into females, whereas they normally would develop into males (Stouthamer *et al.*, 1990a, 1993; Zchori-Fein *et al.*, 1995); (3) feminization (F), which has only been found in isopods (Crustacea) in which the infection causes genetic males to

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reproduce as females (Juchault *et al.*, 1994); (4) no apparent effect (N) on the reproduction of infected individuals at all, and this has been found in several *Drosophila* species (Giordano *et al.*, 1995; Hoffmann *et al.*, 1996).

The phylogeny of *Wolbachia* has been studied extensively based on several *Wolbachia* genes: 16S rDNA (O'Neill *et al.*, 1992; Rousset *et al.*, 1992b; Stouthamer *et al.*, 1993) and the cell cycle gene *ftsZ* (Holden *et al.*, 1993; Werren *et al.*, 1995a). These studies show that two main groups of sequences can be distinguished which have been named group A and group B (Werren *et al.*, 1995a). However, it appears that *Wolbachia* inducing similar effects on hosts are not monophyletic and are present in both groups. Very closely related *Wolbachia* can have quite different effects on their host. This absence of an association between monophyly and the different effects on the host has led to several hypotheses. One hypothesis is that a non-specific *Wolbachia* strain induces a distinct host response, e.g. a *Wolbachia* strain inducing (CI) in one host would induce thelytoky in a hymenopteran host, but so far this has not been found. Alternative hypotheses for the lack of congruence between effect on host and phylogeny are: (1) *Wolbachia* genes coding for a certain effect (T, CI or F) are located on a plasmid which can jump relatively easily from one *Wolbachia* strain to another; (2) *Wolbachia* genes inducing T, CI or F can cross over between different strains in double-infected hosts; (3) *Wolbachia* genes causing T, CI or F are highly congruent and can evolve readily from each other.

One method to determine whether (1) a specific *Wolbachia* strain causes a distinct effect on the host or (2) different hosts experience different effects with the same *Wolbachia* strain, is to create new combinations of host and symbiont. Such transfer experiments have been carried out successfully with CI-*Wolbachia* among several *Drosophila* spp. both intraspecifically (Rousset and De Stordeur, 1994) and interspecifically (Boyle *et al.*, 1993; Clancy and Hoffmann, 1997) and interspecifically in the coleopteran *Tribolium confusum* (Chang and Wade, 1994). In addition, interfamily transfer of CI-*Wolbachia* has been realized. *Wolbachia* from the mosquito *Aedes albopictus* were transferred to the fruitfly *D. simulans* and this revealed that the transinfected *Drosophila* strain became bidirectionally incompatible with all other naturally infected strains (Braig *et al.*, 1994). F-*Wolbachia* causing feminization have also been transferred inter- and intraspecifically in several species of isopods (Juchault *et al.*, 1994) and induced similar effects in their hosts. Finally, N-*Wolbachia* causing no obvious effect on host were transferred from *D. mauritiana* and to *D. simulans* in which they also failed to cause any obvious effect (Giordano *et al.*, 1995). From these studies it became clear that similar effects were found in the new host-*Wolbachia* combination as in the original one.

No such transfer experiments have been performed with T-*Wolbachia*. Here, we report on experiments we have conducted to determine whether T-*Wolbachia* found in the hymenopteran *Muscidifurax uniraptor* would cause a specific effect in the dipteran host *Drosophila simulans*. It was determined whether injection of T-*Wolbachia* induced mortality in *D. simulans* eggs, and if the infection would become established in the new host. Secondly, when the infection succeeded, effects on the host were studied by: (1) measuring egg mortality of infected eggs; and (2) performing crosses to investigate whether T-*Wolbachia* were able to induce CI in its new host. CI-*Wolbachia* were also injected in uninfected *D. simulans* to verify the microinjection protocol.

Materials and Methods

Insect strains – The insect and *Wolbachia* strains used in the injection experiment are shown in Table 1.

Table 1. Insect species and *Wolbachia* strains used in the microinjection experiment. Ooplasm was isolated from the donor species (D) and injected into the acceptor (A).

Species		*Wolbachia* strain abbreviation	Effect in host	Abbreviation for new trans-infected species
Drosophila simulans Riverside	D	*w*Ri	CI	-
Muscidifurax uniraptor	D	*w*Uni	T	-
Drosophila simulans Watsonville	A	injected with *w*Ri	CI	DSW(CI)
Drosophila simulans Watsonville	A	injected with *w*Uni	?	DSW(T)

Flies were maintained in bottles containing *Drosophila* medium [20 g agar, 135g sugar, 38g yeast, 0.12g nipagine (10g/L nipagine in 70% ethanol) made up in 1 L water] and incubated at 20°C. The fly pupa parasitoid *M. uniraptor* originated from Puerto Rico (Legner, 1985a) and was reared on *Musca domestica* pupae and incubated at 25°C.

Microinjection – Microinjection was performed according to the method of Santamaria (1986) and Boyle et al. (1993) with some modifications. Embryos were collected over a 30 min period, transferred to double-sided sticky tape on a slide, dechorionated manually and transferred to a new slide. The embryos were partially desiccated by exposure to the air for up to 4 min (depending on the relative humidity) and subsequently covered with heavy mineral oil (Sigma, no. 400-5). Using a microcapillary needle (Boehringer femtotips) ooplasm containing *Wolbachia* (wUni or wRi) was drawn from infected eggs and then injected into the posterior poles of uninfected *D. simulans* Watsonville embryos before pole

cell formation. Injected embryos were incubated overnight at 25°C (18h). The following day, hatching larvae were isolated at two-hour intervals and transferred to vials with *Drosophila* medium. After pupation and eclosion, isofemale lines were started. Adult virgin females were crossed with uninfected males, because some males from the injected eggs failed to mate or were sterile. A sample of the injected eggs was used to measure the mortality of eggs, larvae and pupae.

Rearing of isofemale lines – In the first generation after injection, all females were tested for *Wolbachia* infection. From the offspring of each of these isofemale lines, five new isofemale lines were started. After 1 week these five females were tested for *Wolbachia* infection. Offspring from infected mothers were used to start new isofemale lines. In the 3rd-7th generations, new DSW(T) lines were established from females that had the highest proportion of infected offspring. Adult transinfected females were tested for *Wolbachia* infection by PCR amplification of the *fts*Z gene (Holden *et al.*, 1993).

Egg mortality, fecundity and incompatibility testing – To investigate whether *w*Uni infection in DSW(T) caused egg mortality, the number of hatched eggs of infected DSW(T) females was counted. Females were taken from an infected DSW(T) line and the uninfected DSW line (control). They were placed on agar dishes covered with fresh yeast. After 48h, these females were placed on a new agar dish with yeast. When the agar dishes had been incubated at 20°C for 48h, the numbers of eggs and larvae were counted. At the end of the experiment, females were prepared for PCR to check for *Wolbachia* infection. Data from uninfected females from the DSW(T) line, according to PCR, were removed before analysis of the results. Experiments were performed in duplicate. The numbers of eggs from infected and non-infected mothers were also compared.

Egg mortality was used to indicate reproductive incompatibility. To test whether *w*Uni induced CI in DSW(T), matings between transinfected males and uninfected females were set up. For each cross, twenty 1- to 2-day-old DSW virgin females were each allowed to mate with one 5-day-old DSW(T) male in the second generation or 1-day-old male in the fourth generation. As control, ten virgin DSW females (from the same batch as above) were crossed with uninfected DSW males. To confirm the unmated status of these females, a control vial of 10 virgin females was taken. All DSW females were checked for *Wolbachia* infection to confirm their uninfected status. After 24h, males were removed and tested for *Wolbachia* infection. Females were allowed to lay eggs for two days on agar covered with fresh yeast. After removing the females, the dishes were incubated for 48h, and the numbers of eggs and larvae were counted. Experiments were performed in duplicate and the mortality rates were compared with a Kruskal-Wallis test.

DNA extraction and PCR – *Drosophila* flies were homogenized with a sterile pestle in 100 µL STE (100mM NaCl, 10mM Tris, 1mM EDTA, pH 8.0) (O'Neill *et al.*, 1992) and incubated with 2 µL Proteinase K (20mgmL^{-1}) for 1 h at 37°C, followed by 5 min at 95°C. Samples were spun for 2 min in a centrifuge and 1 µL of the supernatant was used as template in subsequent PCR reactions. The *Wolbachia ftsZ* gene was amplified using specific *ftsZ* primers (Holden *et al.*, 1993): 5'- GGACCGGATCCGTATGCCGATTGCAGAGC TTG-3' and 5'-GGACCGAATTCGCCATGAGTATTCACTTGGCT-3'. For PCR, a temperature profile of 94°C for 2 min (1 cycle); 94°C for 1 min; 55°C for 1 min; 72°C for 1 min (40 cycles) was utilized.

Confirmation of the identity of the Wolbachia *strain in the infected* Drosophila *host* – *FtsZ* sequences of *Wolbachia* from *D. simulans* Riverside *w*Ri and *M. uniraptor w*Uni were retrieved from Genbank (accession numbers U28178 and U28186) and analysed for differences (Werren *et al.*, 1995a). The *ftsZ* primers (Holden *et al.*, 1993) amplify the region 494bp-1221bp of the *ftsZ* gene, which includes a part of the 3'flanking sequence. The 792 bp sequences (including the primers) differ in four nucleotides, and *Pvu* II cuts at position 1120 of the *w*Ri *ftsZ* sequence. *Pvu* II restriction digestion results in two fragments of 659 and 133 bp in the case of the *ftsZ* of *w*Ri *Wolbachia*.

Results

Mortality rates of injected eggs

Mortality rate differences were found between eggs injected with *Drosophila* cytoplasm containing *w*Ri or *Muscidifurax* cytoplasm containing *w*Uni (Table 2). Eggs injected with *w*Uni showed higher hatching rates than eggs injected with *w*Ri. However, this difference is not seen when mortality rates of larvae or pupae are compared. Of the resulting adult females, approximately 50% produced offspring.

Wolbachia *infection rates*

One positive DSW(CI) line was found from 11 lines injected with *w*Ri. From this infected mother, all offspring were infected (five individuals tested per generation) in the third and consecutive generations. The infection remained stable for at least 10 generations. Of 41 isofemale lines injected with *w*Uni, ten lines had infected offspring rates ranging from 20-80%. The percentage infected offspring of infected mothers decreased to 60% at maximum in successive generations, and no infected offspring were found in the seventh and consecutive generations (Table 3). PCR with the *ftsZ* primers was performed on *D. simulans* Watsonville

(DSW) and Riverside, *M. uniraptor* and the transinfected lines DSW(CI) and DSW(T). No product was amplified from DSW while all others samples resulted in a PCR product of the expected size (792 bp). Restriction analysis on these *ftsZ* products confirmed the identity of the *w*Uni strain in *D. simulans* (data not shown).

Table 2. Mortality rates of different life stages of *Drosophila simulans* Watsonville injected with different cytoplasm types and *Wolbachia* strains.

	Egg to Larva			Larva to pupa		
Injected	Alive	Dead	Total	Alive	Dead	Total
*w*Ri	98	142	240	58	40	98
*w*Uni	140	75	215	66	74	140
	Larva to adult			**Pupa to adult**		
Injected	Alive	Dead	Total	Alive	Dead	Total
*w*Ri	48	50	98	48	10	58
*w*Uni	52	88	140	52	14	66

	Adults		
Injected	Total females	Total females with offspring	Total males
*w*Ri	22	11	26
*w*Uni	25	11	27

Effects on host resulting from w*Uni infection in* D. simulans

In the second generation after injection, effects on the host caused by the *w*Uni infection in DSW(T) were studied by determining female fecundity and egg mortality rates from infected mothers. No differences were found between infected and DSW mothers (Table 4).

Secondly, expression of CI by infected *w*Uni males was studied. If *w*Uni induces CI in its host, reduced egg hatching rates should be found in the crossing of DSW ♀ x DSW(T) ♂. But no reduced egg hatching rates were found in the putative incompatible crossing in comparison with the control crossing (Table 4).

In the fourth generation, similar egg mortality results were obtained from the putative CI and control crossing (Table 4). Egg mortality from DSW(T) mothers was determined in the fourth generation as well but, because of the low transmission rate (26%; Table 3) insufficient positive samples were obtained.

Table 3. Number of isofemale lines of *D. simulans* tested, number of individuals tested per line and offspring infection rates from 2nd to 10th generations of DSW(T) injected females.

Generation after injection	No. of isofemale lines tested	No. of individuals per isofemale line	% infected offspring
2	31	5	0
	3	10	20
	3	5	40
	1*	40	48
	1	5	60
	2	5	80
3	1	5	0
	1	5	20
	3	5	60
4	1*	38	26
	3	10	40
5	2	5	0
	1	5	20
	3	5	60
6	2	10	0
	1	10	50
7	2	20	0
8, 9, 10	2	5	0

* Individuals from these isofemale lines were used in egg mortality and CI experiments.

Discussion

It was not known whether the injection of foreign cytoplasm into *D. simulans* eggs would result in higher mortality. Because of the different microinjection conditions between the actual experiments, our experimental set-up is not suitable for addressing this issue. However, comparing the mortality in both experiments it becomes clear that *Drosophila* eggs can survive the introduction of foreign cytoplasm (Table 2). In addition, *w*Uni injection does not result in excessive mortality in the different developmental stages of *Drosophila*.

Wolbachia could be detected by PCR in the DSW(CI) and DSW(T) lines in the second generation after injection. The transmission was 100% in the second generation for DSW(CI) and was stable for at least 10 generations. For DSW(T), however, the highest transmission rate was found in the second generation (80%) and decreased to 0% in the seventh generation.

Effects on the host were studied for DSW(T) in the second and fourth generations. Because *D. simulans* is not adapted to thelytoky, egg mortality was expected to occur. But no significantly higher mortality was found in eggs from infected mothers in the second generation, which corresponded with the decreasing transmission rates. In addition, no fecundity differences could be found between infected and non-infected females. Because *Wolbachia* can induce CI in *Drosophila* (Hoffman *et al.*, 1986), this phenomenon was tested for with the *w*Uni transinfected *Drosophila* strains. No CI could be detected in the second and fourth generations after injection in the incompatible crosses. In other cytoplasm injection experiments, *Wolbachia* effects in the host could be detected as early as two generations after injection (Boyle *et al.*, 1993). In the injection experiment of Braig *et al.* (1994), *Wolbachia* could only be detected after six generations by PCR. But the set-up of this experiment was different because purified *Wolbachia* were injected which could have an effect on *Wolbachia* fitness or result in a difference in injected *Wolbachia* dose.

Table 4. Number of eggs with standard error (SE) and mean egg mortality with standard deviation (SD) from different mating combinations between uninfected *Drosophila simulans* (DSW) and *w*Uni infected DSW(T), two and four generations after injection (crosses b and f represent the putative CI crossing).

Generation tested (after infection)	Cross (female x male)	Eggs/ female ± SE (number of females tested)	Mean egg mortality ± SD	Egg mortality comparison
2	a: DSW x DSW	79 ± 6 (10)	12.7 ± 7	a vs. b P=0.42
	b: DSW x DSW(T)	86 ± 8 (12)	10.3 ± 10	
	c: DSW x DSW	64 ± 7 (9)	4.8 ± 5	c vs. d P=0.64
	d: DSW(T) x DSW(T)	78 ± 9 (9)	3.6 ± 5	
4	e: DSW x DSW	59 ± 5 (10)	8.4 ± 5	e vs. f P=0.09
	f: DSW x DSW(T)	56 ± 8 (7)	5.3 ± 5	

Although the *ftsZ* sequences of *w*Ri and *w*Uni are closely related (0.4% difference), no permanent establishment of *Wolbachia* from *M. uniraptor* infection occurred in *D. simulans*. Because *w*Uni infection could be detected for several generations, *Wolbachia* had to be present in the eggs. However, no effects on the host could be detected and transmission rates decreased after each generation. Because no correlation was found between the decreased transmission rates and egg mortality, it remains unclear why the infection is not stable in its new host. One explanation might be that, when no CI mechanism is present, this being the driving force for spread of *Wolbachia* through the population (Turelli, 1994), *Wolbachia* could disappear from the population.

However, transfer experiments with N-*Wolbachia* revealed that the new hosts had 100% infected offspring as well, indicating that no CI mechanism was necessary for their persistence (Giordano *et al.*, 1995). A second explanation for the infection not being stable is that *w*Uni is not physiologically adapted to its new host. Consequently the *Wolbachia* density would not rise sufficiently, explaining the fact that no effects on the host were observed (Boyle *et al.*, 1993; Breeuwer and Werren, 1993). As found in *D. serrata*, a novel host for *Wolbachia*, *Wolbachia* from *D. simulans* Riverside were successfully transferred to this species but maternal transmission was relatively low (Clancy and Hoffmann, 1997) indicating that host symbiont interactions are important in the success in the establishment of the infection. An example of *Wolbachia* transmission failure was found in isopods. Injection experiments revealed that *Wolbachia* could be transferred between closely related species of isopods (*Armadillidium nasatum* to *A. vulgare*). However, transmission of *Wolbachia* from *Chaetophiloscia elongata* to *A. vulgare*, which are more distant related species, failed (Juchault *et al.*, 1994). This might be a similar case where *Wolbachia* were not adapted to their new host. On the other hand, phylogenetic work on *Wolbachia* (Werren *et al.*, 1995a) provides evidence that in some cases this bacterium has successfully bridged large phylogenetic distances in its interspecific movement and it remains unknown what permits particular inter-order transfers to occur successfully. When practical microinjection problems are overcome, future experiments could reveal whether *w*Uni can be transferred to other hymenopteran species and if similar phenotypes are induced as in the original host.

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Development of a microinjection protocol for the parasitoid *Nasonia vitripennis*

Introduction

Microinjection of eggs is increasingly used in genetic research on arthropods and can have several practical applications (for a review see Ashburner *et al.*, 1998). This procedure has been used to study transposon-host interaction in several dipteran species such as *Drosophila melanogaster* and *Ceratitis capitata* (Spradlin and Rubin, 1982; Loukeris *et al.*, 1995). DNA injections have been carried out for genetic modification of some mosquito vectors such as *Aedes aegypti*, *Aedes triseriatus* (McGrane *et al.*, 1988; Morris *et al.*, 1989) and *Anopheles gambiae* (Miller *et al.*, 1987). Genetic transformation of vector species may be used to prevent the transmission of pathogens from mammal host to arthropod host (Marshall, 1998). In Lepidoptera, DNA has been injected in the silkworm *Bombyx mori* (Nikolaev *et al.*, 1989) and the pink bollworm *Pectinophora gossypiella* (Peloquin *et al.*, 1997).

Microinjection can also be used to study host-symbiont interactions. A major focus in this field has been on the rickettsial endosymbiont *Wolbachia*. Infection of an arthropod host with *Wolbachia* can result in cytoplasmic incompatibility (CI) that leads to a significantly reduced egg hatch in crosses between infected males and uninfected females whereas all other crosses are fully compatible. *Wolbachia* infection can also induce thelytoky, in which unfertilised eggs develop into females, or feminization (males are converted into functional females) in their host (for a review see Werren and O'Neill, 1997). *Wolbachia* has been transferred by microinjection into several *Drosophila* species (Boyle *et al.*, 1993; Rousset and De Stordeur, 1994; Giordano *et al.*, 1995; Clancy and Hoffmann, 1997) and in the Coleopteran *Tribolium confusum* (Chang and Wade, 1994). *Wolbachia* transfer may have several useful applications. For example, CI inducing *Wolbachia* could be transferred to a new host in order to spread a genetic modified type into a natural population (Beard *et al.*, 1998; Sinkins *et al.*, 1997). Infection of sexual reproducing parasitoids with thelytoky inducing *Wolbachia* can be potentially advantageous in biological control situations, because thelytokous parasitoids produce more daughters compared to normal reproducing parasitoids (Stouthamer, 1993).

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Finally, for prospective studies on fertilisation and gamete maturation, a microinjection protocol, to introduce sperm into eggs, has been developed for the sawfly *Athalia rosae* (Hymenoptera; Tentredinidae) (Sawa and Oishi, 1989).

Microinjection experiments are mainly performed in dipterans where one specific developed injection protocol could be used for several *Drosophila* or mosquito species. For other insect species, microinjection protocols are limited, because they require their own specific protocols. These protocols consist of distinct steps requiring different conditions that have to be adjusted to reduce mortality. Furthermore, after injection the surviving larvae of some species need to be reared on artificial media, that are not always available.

We developed a microinjection protocol for the gregarious fly pupa parasitoid *Nasonia vitripennis* (Hymenoptera, Pteromalidae). This species was chosen because, similarly to *Drosophila*, extensive genetic research has been done on this species. This includes studies on gene linkage maps (Saul *et al.*, 1965), extrachromosomal factors such as a paternal sex ratio distorter (Nur *et al.*, 1988), a maternal sex ratio distorter (Skinner, 1982), the son killer trait (Skinner, 1985) and studies on the endosymbiont *Wolbachia* (Breeuwer and Werren, 1990, 1993). In addition, large quantities of eggs can be collected relatively easily from this species and facilitates the testing of the different stages of the protocol. Initially, a *Drosophila* injection protocol was used but none of the injected *N. vitripennis* eggs survived. Therefore, different microinjection protocols (Santamaria, 1986; Chang and Wade, 1994) were combined and optimal conditions for each step were determined. In addition, an *in vitro* incubation step for the larvae was developed. In this paper we describe a protocol that makes microinjection an available tool for this species.

Protocol

Cultures

For the experiments, the *N. vitripennis* lines Ti277c and AMf6 were used. The line AMf6 was developed in our laboratory, for other experimental purposes, by introgression of the MI genotype (Parker and Orzack, 1985) in the Asym cytotype (Breeuwer and Werren, 1990) for six generations. Line Ti277c was derived from Line Ti277 (Saul *et al.*, 1965) that was infected with a group A *Wolbachia* (Perrot-Minot *et al.*, 1996). Ti277 wasps were cured from infection by feeding them honey containing the antibiotic tetracycline (0,25 mg/ml) for three consecutive generations. Line AMf6 has red eyes while line Ti277c has 'tinged' eyes. This characteristic can be used to distinguish non-treated wasps from wasps derived from the injection procedure. *Nasonia vitripennis* wasps were given honey and *Calliphora*

sp. pupa (Lazebo BV, the Netherlands) as food source. The wasps were reared at 25°C with a light-dark regime of L16:D8. Under these conditions, the lifecycle from egg to adult took two weeks.

Egg collection

To collect large quantities of eggs of similar age, three-day-old *N. vitripennis* wasps (AMf6) were deprived of fly pupae 24 h before the experiment. Two *N. vitripennis* females were placed together with one *Calliphora* pupa per vial for one hour (20 replicates). After ovipositon, the pupa was opened with a scalpel and the pupal shell was removed. Using a binocular microscope, the eggs were collected with a fine needle and placed in a Petri dish containing an agarlayer (2 % agar w/v in water).

Fixation of the eggs

Before microinjection, *N. vitripennis* eggs need to be attached to slides. Rubber cement and double sided tape 3M have been successfully used for *Drosophila* and *Tribolium* respectively (Chang and Wade, 1994; Giordano *et al.*, 1995). For *N. vitripennis*, 3M tape resulted in the lowest mortality and rubber cement proved to be highly toxic (Table 1).

Table 1. Hatching rates of *N. vitripennis* eggs fixed on different brands of double sided tape or rubber cement.

Brand	n total eggs	n survival (%)
Sellotape	120	97 (80.8%)
3M Co.	42	40 (95.2%)
TESA	80	65 (81.3%)
Rubber cement*	50	0 (0.0%)

*Ta Fong Che Ind.Co. LTD.

Dehydration of the eggs

Before injection, eggs needed to be dehydrated because the turgor of freshly laid eggs was too high to perform injections without rupturing them. *Drosophila* eggs are dehydrated by dechorionation of the egg followed by exposure to air. But, in contrast to *Drosophila* eggs, the chorion layer of *N. vitripennis* eggs is relatively flexible and penetrable so that dechorionation was not necessary for microinjection. The intact eggs were dehydrated by exposing them in different concentrations of ethanol or they were dried by incubating them in a desiccator containing $CaCl_2$. The optimum drying time was determined (Table 2). The ethanol treatment dehydrated the eggs, but the turgor varied considerably.

A more consistent result was obtained when the eggs were dried in the desiccator. Subsequent experiments showed that the optimal drying time was around 55-65 min.

Table 2. Hatching rates of *Nasonia* eggs after different dehydration methods followed by microinjection† and incubation in Ringer solution.

Treatment		time	n total eggs	n survival (%)
Ethanol[1]	40%	10'	30	0 (0.0%)
	45%	10'	59	3 (5.1%)
	50%	10'	60	0 (0.0%)
	60%	5'	53	10 (18.9%)
	70%	5'	55	5 (9.1%)
	70%	7'	31	1 (3.2%)
	85%	5'	30	0 (0.0%)
Desiccator		20'	30	0 (0.0%)
		60'	26	6 (23.1%)
		90'	20	7 (35.0%)

† Microinjection consisted of only puncturing the eggs.
[1] Ethanol 99% (w/v)

Injection of the eggs

After initial dehydration, the eggs were covered with oil until hatching to prevent further dehydration and to restrict cytoplasm loss after injection. Three different oils were tested to identify the least toxic one (Table 3). All three oils gave good larval survival rates and experiments were continued with paraffin.

Table 3. Hatching rates of *N. vitripennis* eggs after being covered with paraffin or different kinds of oil followed by incubation in Ringer solution.

	n total eggs	n survival (%)
Paraffin	20	20 (100%)
Mineral oil (Sigma)	100	77 (77%)
Halocarbon oil	20	20 (100%)

Injection of the eggs was done in a similar way as *Drosophila* eggs (Santamaria, 1986; Boyle *et al.*, 1993) and did not pose difficulties. In summary, cytoplasm was drawn from an untreated *N. vitripennis* egg and injected into the posterior pole of a dehydrated *N. vitripennis* egg using a microcapillary needle (Eppendorf Femtotips). Hatching rates of eggs injected with conspecific cytoplasm varied between 0-30%.

In vivo incubation of the larvae

After injection and incubation, larvae (AMf6) were recovered from the oil and placed on an opened fly pupa that was parasitized by females from line Ti277c on the same day. The larvae were incubated on parasitized fly pupae because *N. vitripennis* females inject venom in the pupae which kills them and modifies the host's haemolymph throughout the parasitoids larval development (Rivers and Denlinger, 1993). Five *N. vitripennis* larvae were placed on a fly pupa by making a small window at the anterior end of the host puparium.

This window was sealed with a small piece of parafilm that prevented desiccation of both fly pupa and *N. vitripennis* larvae. As control, 5 non treated *N. vitripennis* larvae were transferred from one fly pupa to another. However, mortality was 100% for larvae from injected eggs while 31% of the control larvae survived.

In vitro incubation of the larvae

Injection experiments with *Tribolium confusum* showed that oil, used to cover the eggs, was toxic to the larvae (Chang and Wade, 1994). Therefore, the oil was removed from the *N. vitripennis* eggs just before hatching (embryonic stage). They were placed on a filter paper, washed with the detergent Tween-20 and rinsed with Ringer solution. Subsequently, the embryos were placed on fly pupae and allowed to hatch and develop but they still did not survive. To allow the embryos to hatch before placement on the fly pupa, they were incubated in artificial media. Initially two different artificial media were tested: 1) Hinks medium (Hinks *et al.*, 1970) which was previously used to establish a cell line from *N. vitripennis* (Wahrman and Zhu, 1993) and Minimum essential (ME) medium (GibcoBRL 31095-029). To prevent fungal growth we added the fungicide Nipagin which is commonly used in *Drosophila* media. But Nipagin was found to be toxic to the *N. vitripennis* larvae even in low concentrations (0.12 µg/ml). No other fungicides were tested or used in subsequent experiments. To prevent bacterial contamination, the antibiotics Penicillin and Streptomycin were also tested (Table 4).

Hatching rates of *N. vitripennis* eggs in artificial media were relatively high and not influenced by the different antibiotic concentrations tested. However, after hatching, the majority of larvae died within a few hours in ME medium or within one day in Hinks medium (Table 4). Previous studies have shown that host haemolymph can be necessary for the development of parasitoid larvae into adults (Irie *et al.*, 1987). Because *N. vitripennis* larvae survived longer in Hinks medium, this medium was supplemented with fly pupa haemolymph (not parasitized) in a 1:1 ratio. There was a tendency towards lower hatching rates in Hinks medium supplemented with haemolymph and a higher survival time of *N. vitripennis* larvae compared to incubation in Hinks or ME media only (Table 4).

Table 4. Hatching rates of *N. vitripennis* eggs incubated in Hinks medium, Haemolymph(Hh)-Hinks mixture (1:1) or Minimal essential medium (ME) containing various concentrations of Ampicillin (A) and Streptomycin (S) (nE= number of eggs tested; nH= number of hatched eggs; % hatching percentage, S1,2,4,6 survival percentage after 1,2,4 and 6 days).

	A / S[1]	0.0				0.125				0.25				0.50			
		nE	nH	%	S1,2,4,6	nE	nH	%	S1,2,4,6	nE	nH	%	S1,2,4,6	nE	nH	%	S1,2,4,6
Hinks	0.0	5	4	80	0.	5	5	100	0.	5	3	60	0.	4	4	100	50,0.
	0.1	6	5	83	40,0.	3	3	100	67,0.	5	4	80	25,0.	7	4	57	0.
	0.2	5	5	100	0.	5	3	60	67,0.	5	4	80	50,0.	5	5	100	20,0.
	0.4	6	4	67	25,0.	6	6	100	33,0.	5	4	80	0.	5	5	100	33,0.
+Hh	0.0	5	4	80	100,100, 0.	5	2	40	100,100, 100,100.	5	2	40	100,100, 100,100.	4	4	100	50,50, 50,50.
ME	0.0	8	5	63	0.	5	3	60	0.	5	5	100	0.	7	6	86	0.

[1] concentration in µg/ml

Hatching rates were also determined in Hinks medium media supplemented with haemolymph of parasitized and non-parasitized fly pupae from *Calliphora sp.* and an alternative host *Musca domestica* (Table 5). Hatching rates in Hinks media supplemented with haemolymph were lower than the previous experiment in media without haemolymph. Only slight differences in hatching rates were detected between medium supplemented with haemolymph from *M. domestica* or *Calliphora sp.*. There was also a trend towards higher hatching rates in Hinks medium supplemented with haemolymph from parasitized host pupae than for media supplemented with haemolymph from unparasitized pupae.

Table 5. Hatching rates of *N. vitripennis* eggs incubated in a Haemolymph-Hinks mixture (1:1) with 0.4 µg/ml Ampicillin. Haemolymph types used: non parasitized or parasitized *Calliphora sp.* (C. non., C. par.) and non parasitized or parasitized *M. domestica* (M. non., M. par.). (nE= number of eggs tested; nH= number of hatched eggs; % hatching percentage).

	trial 1			trial 2			trial 3			trial 4		
	nE	nH	%	nE	nH	%	nE	nH	%	nE	nH	%
C. non	239	105	44	216	104	48	100	48	48	48	27	56
C. par.	-	-	-	-	-	-	100	71	71	120	81	68
M. non.	-	-	-	-	-	-	-	-	-	24	7	24
M. par.	-	-	-	-	-	-	-	-	-	24	15	63

On the different experiments, no statistical tests on hatching or survival rates were performed because the haemolymph originated from fly pupa with differing quality within experiments (differences in age, size and batch). Larvae were incubated in the Hinks-haemolymph mixture for various lengths of time, to determine the optimal incubation

time before their placement on a parasitized host (Table 6). The percentage of larvae developing into adults, after incubation in the medium supplemented with haemolymph, was highest for the one day incubation period and no larvae reached adulthood after an incubation period of three days.

No obvious differences in number of larvae reaching adulthood were seen between larvae incubated in medium supplemented with parasitized or non-parasitized fly pupae haemolymph. The controls (Table 6) showed coverage of *N. vitripennis* eggs with oil to be very harmful even when they were washed.

Table 6. Frequency of survival to adulthood of treated larvae placed on a parasitized fly pupae. The larval treatment consisted of an incubation period of 1,2,3,5 or 7 days in a (1:1) mixture of haemolymph and Hinks medium with 0.4 µg/ml Ampicillin. Haemolymph types used: non parasitized or parasitized *Calliphora sp.* (C. non., C. par.) and non parasitized or parasitized *M. domestica* (M. non., M. par.). (nL= number of larvae tested; nE= number of emerged adults; % adult survival percentage).

Time	C. non.			C. par.			M. non.			M. par.		
	nL	nE	%	nL	nE	%	nL	nE	%	nL	nE	%
1 day	54	11	20	40	7	18	4	2	50	8	3	38
2 days	39	1	3	35	0	0	4	0	0	4	0	0
3 days	16	0	0	6	0	0	-	-	-	-	-	-
5 days	16	0	0	-	-	-						
7 days	7	0	0									

Time	Control 1[a]			Control 2[b]		
	nL	nE	%	nL	nE	%
0 days	42	13	31	102	0	0
1 day	45	14	42	24	1	4
2 days	17	2	12	24	0	0
3 days	3	3	100	-	-	-

a: *Nasonia* eggs/larvae were isolated from pupae and directly transferred to a new host.
b: *Nasonia* eggs were covered with paraffin, incubated in Ringer solution, washed and then placed on a new host.

Conclusions and Discussion

Microinjection protocols of *Drosophila* and *Tribolium* were unsuitable for *N. vitripennis*. Therefore we developed a new injection protocol in which we determined the optimum conditions for all steps. Our protocol clearly shows that microinjection is possible for *N. vitripennis*.

The optimum dehydration time of the *N. vitripennis* eggs was approximately one hour. For successful germline transmission or *Wolbachia* infection, injection has to be performed

before pole cell formation, which occurs after three hours for *N. vitripennis* (Bull, 1982). Our protocol meets this requirement, because the time period necessary for egg isolation, dehydration and injection is within this time limit. Experiments using artificial media were done because *N. vitripennis* larvae were unable to develop when they were directly returned to *Calliphora* sp. pupae. Incubation of eggs in Hinks medium or ME medium resulted in a high hatching rate and showed the fungicide Nipagin to be lethal to the larvae. No obvious differences in egg hatching rates were observed among varying concentrations of the antibiotics ampicillin and streptomycin treatments. These antibiotics were selected because they are not lethal to the endosymbiont *Wolbachia* (Stouthamer *et al.*, 1990a). Hatching rates were similar with both Hinks and ME media, but larvae survived for a maximum of 24 h.

The incubation conditions were improved by supplementing Hinks medium with fly pupa haemolymph in a 1:1 ratio. Hatching rates were lower in medium with haemolymph compared to the two media without haemolymph. Normally, eggs are laid inside the pupal shell on the exterior of the fly pupa. Some compounds directly from the haemolymph or originating by degradation of the haemolymph may affect the egg hatching rate. To determine whether hatching rates were dependent on parasitization of the host, Hinks medium was supplemented with parasitized or unparasitized haemolymph. These experiments showed a trend of higher hatching rates in medium supplemented with parasitized haemolymph. Despite the lower hatching rates in the Hinks medium with haemolymph, the larvae were able to develop into adults in contrast to the medium without haemolymph. The transfer of *N. vitripennis* larvae to parasitized *Calliphora* sp. pupae was most successful when the larvae were allowed to develop for 24h in Hinks medium supplemented with fly pupa haemolymph (1:1). This optimal incubation period of only 24 hours is difficult to explain because after this period, larvae were still feeding on the medium. Normally, after parasitization of the fly pupa, the haemolymph changes specifically during the parasitoids larval development (Rivers and Denlinger, 1993). However, this change may not occur *in vitro* and may explain why no larvae reached adulthood after 24h. In addition, due to the incubation conditions, oxidation or other chemical reactions could lead to a deterioration of the haemolymph quality. Experiments where the medium is refreshed daily could resolve this problem. The haemolymph medium was influenced by the quality of the pupae used to extract the haemolymph from. These fluctuations in haemolymph quality makes the comparison of the different experiments and treatments difficult. The benefit of washing the eggs was not tested, but it seems plausible that less contact with a toxic substance should increase larval survival.

Our results show that our protocol allows successful microinjection of *N. vitripennis* eggs and can be used to do advanced genetic studies in this species.

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Female fitness in *Wolbachia*-fixed and *Wolbachia*-mixed *Trichogramma* populations

Abstract – In haplodiploid species, the normal mode of reproduction is arrhenotoky: males arise from unfertilized eggs and females from fertilized eggs. A less common mode of reproduction is thelytoky in which all unfertilized eggs give rise to female offspring. In Hymenoptera, thelytoky can be induced by *Wolbachia*, a α proteobacterium. The infection can be fully established in a population so that only thelytokous females are present (= fixed population). But there are also cases where thelytokous females coexist with arrhenotokous ones (= mixed population). In mixed populations, thelytokous females are still able to mate and produce offspring sexually. In fixed populations, the degree of co-evolution between insect host and symbiont *Wolbachia* will increase and it is theorized that this will result in a reduction of negative impact of the symbiont on its host. This hypothesis was tested with wasps of the genus *Trichogramma* because both mixed and fixed populations occur within this genus. Thelytokous females of two isofemale lines from fixed populations and four isofemale lines from mixed populations were 'cured' from *Wolbachia* infection using antibiotics. Several fitness parameters were measured for these *Wolbachia* infected lines and their cured counterparts. Daughter production was significantly higher for the thelytokous fixed lines compared to the arrhenotokous ones. This contrasts with three mixed lines where the daughter production was lower for the infected lines compared to the cured lines. Only a slight fecundity effect of *Wolbachia* in the fixed lines was found for total offspring numbers while a severe negative impact of *Wolbachia* was found in the mixed lines. The *Wolbachia* transmission efficiency (% females) was generally higher for the fixed thelytokous lines with the exception of one mixed line of *T. deion*. Finally, negative *Wolbachia* effects were detected for longevity, pupa and embryo mortality for several *Trichogramma* lines. Our results are discussed in the context of a previously published model which describes the dynamics of *Wolbachia*-infected *Trichogramma* wasps in mixed populations.

Introduction

The role of cytoplasmic genes as an important component of hereditary material has long been neglected (Cosmides and Tooby, 1981). While nuclear genes are passed from both parents to the offspring, cytoplasmic or extranuclear genes are inherited mainly from the mother. This implies that males constitute a dead end for cytoplasmic genes. Therefore it is advantageous for cytoplasmic factors to bias the offspring sex ratio of their host towards the

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production of daughters. For the nuclear genes, however, reproductive fitness is also gained through males. These differences in evolutionary interests can create a conflict between nuclear and cytoplasmic genes (Cosmides and Tooby, 1981).

Several cytoplasmic sex ratio distorters, which interfere with the host mode of reproduction, have been described (for a review see Hurst, 1993) and one of the most conspicuous examples is the intracellular symbiont *Wolbachia*. This bacterium is widespread in arthropods, cytoplasmically inherited and can modify the reproductive phenotype of its host in various ways such as through cytoplasmic incompatibility (CI), induction of thelytoky (T) or by feminization of the male host (for a review see Werren and O'Neill, 1997). In CI, the bacterium manipulates its host's sperm in such a way that it can only fertilize egg cells from mothers infected with a similar strain of *Wolbachia*. If the sperm enters an uninfected egg, the zygote dies in diploid species or develops into a male in haplodiploid species (Yen and Barr, 1973; Breeuwer and Werren, 1990). *Wolbachia*-induced thelytoky (T) is a form of parthenogenesis that has presently only been found in various species of the order Hymenoptera (Rousset *et al.*, 1992b; Stouthamer *et al.*, 1993; Van Meer *et al.*, 1995; Zchori-Fein *et al.*, 1995). In this order, unfertilized eggs normally develop into males (arrhenotoky), but *Wolbachia* infection leads to a disruption of the chromosome segregation in the first mitotic division (anaphase) of the haploid egg. As a result, haploid eggs become diploid and develop into homozygous thelytokous females (Stouthamer and Kazmer, 1994). Thelytokous strains can be maintained without the involvement of males. When thelytokous females get older, male offspring can be produced probably because the *Wolbachia* titer declines with the mother's age. Finally, feminization is found in isopods where *Wolbachia* can transform genetic males into functional 'neofemales' (Rousset *et al.*, 1992b; Juchault *et al.*, 1994). These three effects result in the spread of the *Wolbachia* infection through the population.

A nuclear-cytoplasmic conflict can exist between thelytoky-inducing *Wolbachia* (T-*Wolbachia*) and their host. The severity of this conflict is a function of the infection status of the population. Two different situations can be recognized: the population consists of a mixture of infected and uninfected conspecifics ('mixed'), or the population consists of only infected females ('fixed') (Stouthamer, 1997).

In mixed populations mating can take place between infected thelytokous females and uninfected males. The mated thelytokous females produce daughter offspring sexually from fertilized eggs and homozygous females from unfertilized eggs (Stouthamer and Kazmer, 1994). When the frequency of infection rises in a population, the population sex ratio becomes increasingly female biased. In this situation, few males can mate with many females. Therefore, increased reproductive fitness can be gained by the nuclear genes that favour

production of males. This nuclear-cytoplasmic conflict could lead to the evolution of nuclear genes that suppress the effect of *Wolbachia* and thus restore the female's ability to produce male offspring. Suppressor genes have been found against feminizing *Wolbachia* in the isopod *Armadillidium vulgare* (Rigaud and Juchault, 1992) but have not yet been found for T-*Wolbachia*, although there are suspected cases (Stouthamer, 1997). To compensate for the effects of the suppressor genes, T-*Wolbachia* could respond e.g. by overexpression of proteins responsible for thelytoky-induction or by increasing their density in the host. These compensatory effects could lead to a negative fitness effect of T-*Wolbachia* on their hosts. Fecundity experiments have shown that in mixed populations of the egg parasitoids *Trichogramma deion* (Irvine) and *Trichogramma pretiosum* (Nuevo Leon) there is indeed a negative effect of T-*Wolbachia* on daughter and total offspring production (Stouthamer and Luck, 1993; Horjus and Stouthamer, 1995).

Once the T-*Wolbachia* infection has reached fixation in a population, a reduction of negative impact of the symbiont ('benevolence') on its host can be expected over time when host and symbiont co-evolve and horizontal transmission is rare (Ewald, 1987; Bull *et al.*, 1991; Lipsitch *et al.*, 1995). Female-symbiont combinations that are most fit will spread through the population. An additional implication of fixation of the infection is that since sexual recombination is absent, slightly deleterious mutations will accumulate in expressed genes of the host's genome (Muller's ratchet) but accumulate at a much higher rate in those genes which are not expressed any longer. For example, in arrhenotokous populations there is a continuous selection on genes coding for sexual reproduction, i.e. specific male traits (mating behavior or sperm production) or female traits (mating behavior, sperm storage, fertilization, pheromone production). But this selection either disappears, or some costly sexual traits may be selected against in fixed populations (Pijls *et al.*, 1996). Indirect evidence for this kind of phenomena has been found in fixed thelytokous populations of the parasitoid *Apoanagyrus diversicornis* (Pijls *et al.*, 1996) and *Trichogramma cordubensis* (Silva and Stouthamer, 1997), where females were less attractive or reluctant to mate with conspecific males. Secondly, cured females from a fixed population of the parasitoid *Encarsia formosa* did occasionally mate and cured females from a fixed population of *Muscidifurax uniraptor* did not mate. In both cases no female offspring was produced (Zchori Fein *et al.*, 1992; Van den Assem and Povel, 1973). From these fixed populations of *M. uniraptor*, effects of the symbiont on host fecundity were studied and no negative impact of T-*Wolbachia* was found (Horjus and Stouthamer, 1995). This result supports the hypothesis of a reduced impact of the symbiont on its host after a relatively long time of co-evolution.

Besides this study of *Wolbachia* impact on host fitness in fixed lines, one other example exists in which a thelytokous population of *Trichogramma pretiosum* (Hawaii), which is probably fixed, was tested simultaneously with a cured arrhenotokous counterpart. A negative impact of *Wolbachia* on total offspring was found but daughter production did not differ significantly between the arrhenotokous and thelytokous lines (Stouthamer and Luck, 1993).

From the examples above, it becomes clear that the number of studies on T-*Wolbachia* impact on host fitness is limited and that several questions remain unanswered. First of all, is the negative impact of *Wolbachia* on their host consistently linked to the infection status (fixed or mixed) of the population? And secondly, can benevolence of T-*Wolbachia* be found in fixed populations that have relatively recently become fixed for infection? To address these issues, we studied fitness effects of T-*Wolbachia* on several host species. Wasps of the genus *Trichogramma* provide an excellent opportunity to address these questions because several mixed and fixed populations exist within this genus, although the number of fixed *Trichogramma* populations known is limited. The fixation for *Wolbachia* infection in *Trichogramma* is probably relatively recent in comparison with *E. formosa* and *M. uniraptor* because arrhenotokous *Trichogramma* lines can still be established in contrast to the latter species.

Materials and methods

Cultures – Thelytokous lines were started with single females. Wasps were reared in glass vials (150 mm length, 15 mm diameter), on UV irradiated eggs of *Ephestia kuehniella* Zeller (Lepidoptera, Pyralidae) and were incubated at 23 ± 1° C, L:D = 16:8 and 70 ± 10% RH. *Ephestia kuehniella* eggs were provided by Koppert Biological Systems (Berkel en Rodenrijs). The generation time of *Trichogramma* lines under these conditions was approximately 12-13 days. The *Trichogramma* species, their infection status, geographic origin and abbreviations are listed in table 1.

Curing of the Trichogramma lines – The thelytokous strains OL, AW, LC, JT and SW were cured by feeding the females honey with 0.5% w/v tetracycline for at least 3 generations. After being cured, the wasps were reared for at least 4 generations without antibiotics before they were used in the experiment so that no residual effect of tetracycline treatment would influence the fitness results. The line CO was cured in 1994 with the antibiotic rifampicin (1% w/v).

To verify that lines were cured of their infection, the following tests were performed (i) 20 virgin females were isolated and allowed to produce offspring (all male broods indicate complete curing of the infection); (ii) PCR with specific *Wolbachia wsp* primers (Braig *et al.*, 1998) on the cured and infected strains.

Table 1. *Trichogramma* species and lines used in this study with their geographic origin and their infection status.

Species	Isofemale line code (abbreviation)	*Wolbachia* Infection Status	Geographic origin	Reference
T. cordubensis	28B143b (CO)	Fixed	Divor, Portugal, 1993	Silva and Stouthamer, 1997
T. oleae	1A (OL)	Fixed	Yugoslavia	Voegelé and Pointel, 1979
T. kaykai	AW 7-5 (AW)	Mixed	Sky valley, Riverside CA, USA	Schilthuizen *et al.*, 1998
T. kaykai	JT 6-3 (JT)	Mixed	Joshua Tree, San Bernardino, CA, USA.	-
T. kaykai	LC 110 (LC)	Mixed	El Paso Mountains, CA, USA	Schilthuizen *et al.*, 1998
T. deion	SW 436-1 (SW)	Mixed	Mojave desert, CA, USA	Schilthuizen and Stouthamer, 1997

PCR – Twenty-five *Trichogramma* wasps were homogenized with a sterile pestle in 50 µL STE (100mM NaCl/10mM Tris/1mM EDTA, pH 8.0) (O'Neill *et al.*, 1992) and incubated with 1 µL proteinase K (20mg/ml) for one hour at 37°C , followed by 5 min at 95° C. Samples were spun for 2 min in a centrifuge and 1µL of the supernatant was used as template in subsequent PCR reactions. The *Wolbachia* outer membrane protein gene (*wsp*) was amplified using specific primers (Braig *et al.*, 1998). For PCR, a temperature profile of 94°C for 3 min (1 cycle); 94°C for 1 min, 55°C for 1 min and 72°C for 1 min (40 cycles) was utilized.

Fitness experiment – *Trichogramma* females emerging from *Ephestia* eggs are small. Because fitness differences between lines might be more pronounced using larger wasps, we reared *Trichogramma* on the larger *Mamestra brassicae* (Lepidoptera, Noctuidae) host eggs for one generation. *M. brassicae* was reared on cabbage plants (*Brassica oleracea* cv. Icarus) and incubation conditions were 22°C ± 1°C and a photoregime of L16:8D. The fitness experiment, its preparation and incubation of progeny, was carried out in at 23 ± 1° C, L:D = 16:8 and 70 ± 10% RH.

Thirty female wasps, less than 24h old and emerging from *Ephestia kuehniella* eggs, were individually placed in glass vials with approximately twenty *Mamestra brassicae* eggs for one

day. After 5 days, when the parasitized eggs had turned black, they were isolated and incubated in separate vials. To limit size differences between the females, only adults that emerged from host eggs producing two individuals were selected for the experiment. Per line, forty-two females were used. Due to a shortage of *Mamestra brassicae* eggs, only thirty-two thelytokous females of SW could be set up. Virgin females from the cured lines were allowed to mate with a male. After four hours, the males were removed. In the glass vial, a small droplet of honey was added and the wasps were offered an egg card with approximately 120 *Ephestia kuehniella* eggs. The first five days the egg masses were changed daily, after that they were changed every other day. The removed egg cards were placed in a new vial and incubated. Four days after the first offspring started to emerge, the vials were frozen (-80°C) and offspring was subsequently counted. Per female we determined the total number of host eggs parasitized (i.e. that had turned black) during her lifespan, longevity, the number of daughters and embryo or pre-adult mortality, and the sex ratio of the offspring. Individuals from thelytokous lines, which were partially haploid and diploid (gynandromorphs), were counted as males. The sex ratio is expressed as the percentage daughters of the total offspring. Because greenhouse studies showed that the lines CO and SW can parasitize hosts for not longer than five days (I.M.M. Silva, unpublished results) we calculated daughter and total offspring for 5 days and their lifespan respectively. Embryo and pupal mortality was determined by opening the parasitized unhatched eggs. For simplicity, all individuals with no body pigmentation were categorized as embryos. Only for line LC, embryo and pupa mortality was not distinguished. Because the size of a female has been shown to correlate with her fecundity (Waage and Ng, 1984), her hind tibia was measured using an optical micrometer mounted in an eyepiece of a compound microscope.

All experiments were performed within a 3 month period and the infected and corresponding cured lines were always tested simultaneously. The lines OL and SW were tested in two different time periods.

Statistical analysis – Data were analyzed with SAS (version 6.12) statistical software using a generalized linear model with the following distributions: binomial distribution for embryo and pupal mortality, normal distribution for longevity, Poisson distribution for number of daughters and total offspring production. Tibia length, time period (block factor) and infection status (thelytoky or arrhenotoky) were explanatory variables. No time period effects were detected for OL and SW for any of the parameters studied and therefore the results of the separate time periods are not included in the results shown. Most tests were two-sided. Because the experiments for the different lines were not carried out simultaneously, no comparative statistical tests were performed between the lines with exception of the tibia length.

Results

Curing of the lines

All lines were cured after treatment with tetracycline or rifampicin for three generations. PCR with the *Wolbachia* specific *wsp* primers on infected and cured lines resulted in amplification of the proper product only in the infected lines. In addition, cured virgin females from all lines produced male offspring only (data not shown).

Offspring production and sex ratio

The fitness parameters daughter production and total offspring were calculated for five days and for the whole lifespan (Table 2). Daughter production was significantly different between the thelytokous and arrhenotokous lines where thelytokous wasps from the fixed lines CO and OL produced more daughters in the first 5 days than their cured arrhenotokous counterparts. The opposite was found for the mixed lines AW, JT, LC and SW where thelytokous females produced less daughters than arrhenotokous females. Similar results were obtained for lifespan daughter production with the exception of line SW where the difference between arrhenotokous and thelytokous line was not significant.

For total offspring production in the five first days, a negative *Wolbachia* effect was found for the mixed lines AW, JT, LC and SW. For the fixed lines no significant difference was detected for CO (Figure 1; Table 2). For OL an interaction occurred between *Wolbachia* and tibia length (Figure 1) and a positive *Wolbachia* effect on total offspring production was detected for wasps with a tibia length smaller than 0.1815 mm (day 1-5) or 0.1827 mm (day 1-end). Approximately 75% of the tested OL wasps meet this criterion. For lifespan total offspring production, results were similar to the first 5 days with exception of CO where a negative *Wolbachia* effect was found.

Mean sex ratios of the thelytokous lines, which are a measure for *Wolbachia* transmission, were highest for the fixed lines CO and OL for the first five days and for the whole lifespan. Of the mixed lines, only line SW showed the same pattern as the fixed lines (Table 3).

A significant *Trichogramma*-species effect was found for the tibia length and *Wolbachia* infected lines had smaller tibia lengths than the uninfected ones. This could be explained by a negative effect of *Wolbachia* on their host. However, an alternative explanation is that mothers from arrhenotokous lines were derived from *Mamestra* eggs from which in many cases, one female and one male emerged. The situation is different for the thelytokous lines where only two females emerged from the egg.

Table 2. Mean values of daughter and total offspring of day 1-5 or whole lifespan with standard deviation (SD) and mean tibia length of the different thelytokous (T) and arrhenotokous (A) lines. (n= number of females tested; mT= mean tibia length; m♀= mean daughter offspring; m♀♂= mean total offspring).

Line		mT (mm)	m♀ ± SD Day 1-5	n	P	m♀ ± SD Day 1-end	n	P	m♀♂ ± SD Day 1-5	n	P	m♀♂ ± SD Day 1-end	n	P
CO	T	0.1894	85.7 ± 14.5	40	**	113.9 ± 30.3	36	*	86.9 ± 14.7	40	n.s	123.7 ± 37.0	36	**
	A	0.1899	71.3 ± 18.3	41		98.3 ± 22.9	39		81.2 ± 21.1	41		152.5 ± 45.7	39	
OL	T	0.1767	88.1 ± 11.0	41	***	159.3 ± 20.9	37	***	91.1 ± 9.1	41	** §	236.6 ± 26.5	37	** §
	A	0.1809	61.0 ± 22.7	38		69.1 ± 30.5	34		87.0 ± 16.2	40		223.8 ± 51.7	36	
AW	T	0.1922	48.9 ± 15.2	41	**	56.5 ± 17.9	33	***	54.5 ± 15.6	41	***	87.7 ± 24.9	33	***
	A	0.1960	68.6 ± 20.2	38		98.9 ± 32.7	34		86.4 ± 21.7	39		133.8 ± 31.9	34	
JT	T	0.1918	27.9 ± 6.3	40	***	31.9 ± 8.2	31	***	37.2 ± 7.9	40	***	77.2 ± 23.4	31	***
	A	0.1971	60.2 ± 16.4	39		82.0 ± 28.4	32		82.0 ± 14.1	42		152.2 ± 39.9	36	
LC	T	0.1954	39.7 ± 10.3	37	***	47.5 ± 11.8	33	***	52.2 ± 12.9	37	***	99.9 ± 27.3	33	***
	A	0.1961	73.2 ± 17.3	39		91.2 ± 30.0	32		95.8 ± 14.3	39		173.8 ± 32.8	32	
SW	T	0.1874	50.6 ± 13.8	32	**	101.3 ± 34.6	28	n.s.	53.8 ± 15.2	32	***	114.0 ± 39.4	28	***
	A	0.1899	61.3 ± 9.7	40		108.0 ± 23.5	35		80.1 ± 9.0	40		185.6 ± 43.3	35	

n.s.: not significant; $*p<0.05$, $**p<0.005$, $***p<0.0005$. § interaction between tibia length and *Wolbachia* infection.

Because males are generally smaller than females (Kazmer and Luck, 1995), more host resources would be left for females co-residing with a male in a host egg, resulting in larger females.

Table 3. Mean sex ratio (M.s.r.) with standard deviation (SD) of the different thelytokous (T) and arrhenotokous (A) lines (n = number of females tested).

Line	Mode of reproduction	Day 1-5 M.s.r. ± SD	n	Day 1-end M.s.r. ± SD	n
CO	T	1.00 ± 0.00	40	0.97 ± 0.04	41
	A	0.90 ± 0.06	36	0.71 ± 0.18	39
OL	T	1.00 ± 0.00	40	0.74 ± 0.08	37
	A	0.72 ± 0.22	38	0.34 ± 0.16	34
AW	T	0.92 ± 0.07	41	0.68 ± 0.12	34
	A	0.80 ± 0.15	38	0.77 ± 0.16	34
JT	T	0.86 ± 0.06	40	0.51 ± 0.14	31
	A	0.75 ± 0.14	39	0.56 ± 0.16	33
LC	T	0.86 ± 0.06	37	0.60 ± 0.11	33
	A	0.80 ± 0.14	39	0.56 ± 0.19	32
SW	T	0.99 ± 0.01	28	0.95 ± 0.04	28
	A	0.78 ± 0.06	40	0.61 ± 0.09	35

Longevity and pupal/embryo mortality

A negative *Wolbachia* effect on longevity was detected for the fixed lines CO and OL (Table 4). This effect was more pronounced for CO where non-infected females lived eight days longer. A negative *Wolbachia* effect was detected for pupa and embryo mortality in all lines except for AW (pupal mortality) and for CO (embryo mortality) (Table 5). For LC, embryo and pupa mortality was not distinguished but total mortality was significantly higher for the thelytokous line as well. The differences in mortality rates between arrhenotokous and thelytokous lines however, are marginal. Only the thelytokous line OL showed considerably higher embryo mortality (>5 times) in comparison with the arrhenotokous line. In general, mortality rates are initially low, increase after approximately 5 days and fluctuate in the following days (data not shown).

Discussion

In this study we determined whether the infection status of a *Trichogramma* population is correlated with the influence *Wolbachia* has on various host life-history characters. We hypothesize that in populations where the infected individuals coexist with uninfected

conspecifics and where sexual reproduction of infected females still takes place, a nuclear-cytoplasmic conflict exists over the offspring sex ratio. This conflict may result in an "arms race" between the nuclear genes of the wasp and *Wolbachia*. Effects of this conflict can be inefficient transmission of *Wolbachia* or a reduced offspring production of the infected wasp. Once a population has gone to fixation for the infection and thus no more sexual reproduction takes place, we expect that "benevolence" will evolve, i.e. those nuclear-cytoplasmic (wasp-*Wolbachia*) combinations that produce the most infected offspring will spread through the population. We determined whether this predicted pattern holds by comparing various female fitness characters of thelytokous lines and cured arrhenotokous lines of two populations where the infection has gone to fixation and of four populations where both infected and uninfected individuals coexist.

The dynamics of the fraction of infected *Trichogramma* individuals in a mixed population have been modeled by Stouthamer (1997). The equilibrium infection frequency among females in a population can be calculated as:

$$F = (w\alpha - x) / (w\alpha + (1-\alpha)\, wx - x)$$

w = total offspring production of an infected female divided by the total offspring of an uninfected female
α = the frequency of infected daughters in the offspring of infected mothers
x = fertilization rate of the eggs

This equilibrium is valid when $w\alpha > x$ and assuming that both infected and uninfected females mate. The nuclear-cytoplasmic conflict will disappear when males are no longer present in the populations. Thus α needs to be 1 while simultaneously w is larger than the fertilization proportion of eggs (x). Subsequently, we expect selection for increased w in fixed populations.

In the populations we studied, we can distinguish three different groups of *Wolbachia*-host interactions using the parameters α and w. Group one consists of the fixed thelytokous lines CO and OL which produce higher numbers of daughters ($w\alpha > x$), and relatively similar offspring numbers compared to its arrhenotokous counterpart (w close to one). The second group consists of the thelytokous line of the mixed population SW which produced equal numbers of females ($w\alpha \approx x$) but showed reduced offspring numbers compared to their cured counterparts ($w < 1$). Finally, the third group consists of the *T. kaykai* lines AW, LC and JT from mixed populations where low values were found for both α and w. The species from mixed populations of *T. pretiosum* (Nuevo Leon) and *T. deion* (Irvine) (Stouthamer and Luck, 1993) belong also to the third group while *T. pretiosum* (Hawaii) belongs to the second.

Table 4. Longevity in days (age) with standard deviation (SD) of the different thelytokous (T) and arrhenotokous (A) lines (n = number of females tested).

Line	Mode of reproduction	n	Age±SD	P
CO	T	36	18.1±6.8	
	A	39	25.6± 6.9	***
OL	T	37	26.5±4.0	
	A	36	28.6±5.1	*
AW	T	34	28.7±3.8	
	A	36	26.7±6.2	*n.s.*
JT	T	31	24.5±6.6	
	A	36	25.4±.5.0	*n.s.*
LC	T	37	24.7±3.6	
	A	33	25.6±3.8	*n.s.*
SW	T	28	26.8±5.2	
	A	35	26.1±4.5	*n.s.*

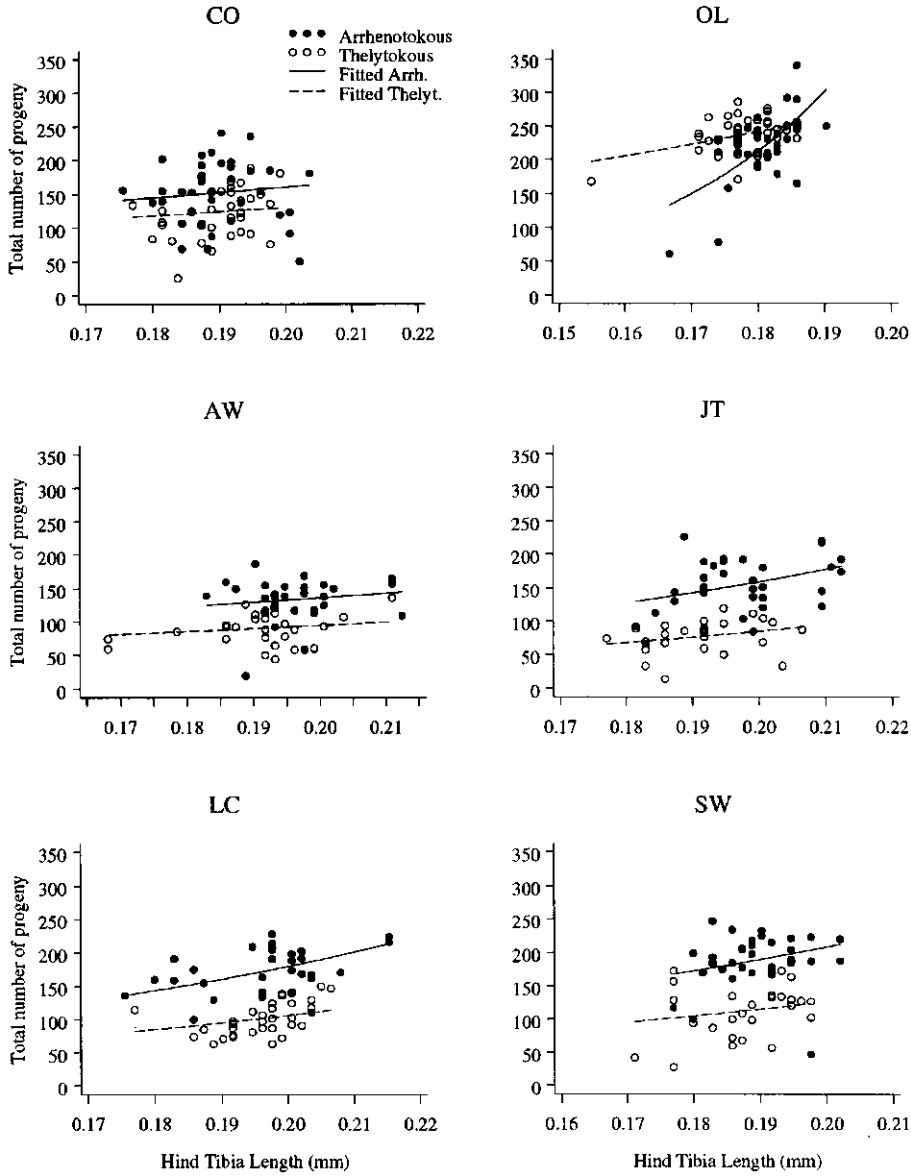
*n.s:: not significant; *p < 0.05, **p < 0.005, ***p < 0.0005.*

Table 5. Mean Pupal and Embryo mortality with standard deviation (SD) of the different thelytokous (T) and arrhenotokous (A) lines (n = number of females tested).

Line		n	Mean Pupal mortality ± SD	P	Mean Embryo mortality± SD	P
CO	T	39	2.17 ± 2.15	*	2.44 ± 2.10	*n.s.*
	A	36	1.62 ± 2.14		2.31 ± 1.88	
OL	T	36	2.36 ± 1.99	**	10.22 ± 4.02	***
	A	36	1.19 ± 0.95		2.03 ± 2.03	
AW	T	35	1.85 ± 1.42	*n.s.*	1.35 ± 1.70	***
	A	34	1.74 ± 2.07		0.71 ± 1.10	
JT	T	36	3.16 ± 1.99	***	7.39 ± 4.09	***
	A	31	1.63 ± 1.33		3.11 ± 3.73	
LC [a]	T	33	16.3 ± 9.07	***		
	A	37	8.15 ± 4.94			
SW	T	28	2.46 ± 3.22	***	4.14 ± 2.53	***
	A	35	1.74 ± 1.92		3.09 ± 3.22	

*n.s: not significant; *p < 0.05, **p < 0.005, ***p < 0.0005*; [a] pupae and embryos were not discriminated with this line and numbers indicate the total mean of unhatched eggs.

Figure 1. Relationships between size of the mother (measured as hind tibia length) and lifetime total offspring production.



From the last species only thelytokous individuals were collected but it is unknown whether this line is fixed (Stouthamer and Luck, 1993).

Besides differences in α and w, differences were also detected in the longevity between the tested lines. It was expected that *Wolbachia* would have less negative effect on their host in fixed lines than in mixed lines. But the opposite was found: thelytokous CO and OL lived significantly shorter than their arrhenotokous counterparts with only slight differences for OL and a difference of on average eight days for CO. Because the reduced longevity of the thelytokous lines CO and OL is not linked to a severe negative impact on offspring production compared to the cured lines, it remains unclear what causes this difference. It may be that *Wolbachia* change the host's physiology in such a way that it reallocates the host resources resulting in higher offspring numbers but shorter longevity (G.J. Driessen, pers. comm.).

No obvious differences were detected in pupa and embryo mortality rates between thelytokous fixed lines or mixed lines. Instead, mortality rates were generally higher for the thelytokous lines compared to the cured arrhenotokous lines. Mortality rates were initially low but started to increase when females were a few days old. In this period, daughter production began to decline and male production increased including the production of gynandromorphs. The cytological processes responsible for the production of gynandromorphs (Stouthamer, 1997) may cause the increased mortality. In addition, because mortality varied among the thelytokous lines, host or symbiont genotype may play a role as well.

The differences found in offspring numbers between thelytokous lines of mixed populations and their conspecific arrhenotokous lines do not necessarily reflect the field situation. A previous study of Stouthamer and Luck (1993) showed that despite the negative effect of *Wolbachia* on *T. pretiosum* and *T. deion* (Irvine), the thelytokous females produced more daughters when the number of host eggs available was limited. But so far, it is unclear how many potential hosts *Trichogramma* females encounter in the field. It is therefore difficult to extrapolate our results to the field situation. Because there can be a negative effect of *Wolbachia* on offspring production, *Wolbachia* may also have a negative impact on additional life history parameters such as host searching behavior. But a greenhouse experiment with the same SW line used as in our experiment showed no differences in the number of patches parasitized on tomato plants between the thelytokous and arrhenotokous line. The arrhenotokous line parasitized more eggs per patch than the thelytokous line (Hoogenboom *et al.*, 1998) which corresponds with our offspring results of line SW.

Our results and the results of Stouthamer and Luck (1993) show that fixed *Trichogramma* lines have both a high transmission fidelity ($\alpha \approx 1$) and hardly show any negative impact of the *Wolbachia* infection on the offspring production ($w \approx 1$), while the opposite is true for the mixed lines with the exception of the mixed line SW and the line *T. pretiosum* Hawaii (Stouthamer and Luck, 1993), which have a low w but high α. These last two lines may represent examples where the infection is going to fixation or alternatively, a situation in which *Wolbachia* has temporarily overcome the host's countermeasures. The results are consistent with the idea that benevolence should evolve when the infection has gone to fixation, whereas the nuclear-cytoplasmic conflict in mixed populations results in a reduced *Wolbachia* transmission and/or a negative impact of the infection on offspring production. However, a less likely explanation may also be given. Infection will go to fixation in those populations that became initially infected with a *Wolbachia* that has hardly any influence on the host offspring production and has a high transmission rate, whereas those *Wolbachia*-host interactions where the transmission efficiency is low and the fitness cost of carrying a *Wolbachia* is high results in, at best, a mixed population. Because physiology can differ substantially between different arthropod hosts, it is questionable whether primary benevolent symbiont-host combinations exist. The study of Grenier *et al.* (1998) already showed that even within the genus *Trichogramma* not all symbiont-host combinations are initially successful: *Trichogramma dendrolimi*, infected with *Wolbachia* of *Trichogramma pretiosum* only showed extreme low levels of thelytoky. A series of micro-injection experiments of *Wolbachia* strains (from fixed and mixed lines) into *Trichogramma* lines could show whether benevolent *Wolbachia* - novel host combinations exist. A second set of experiments could test the assumption that 'benevolence' will evolve once the infection has gone to fixation. This can be done in the laboratory by artificial selection of *Wolbachia* infected lines for increased transmission ($\alpha = 1$). Then, over time, these lines should be tested to determine whether w is increasing. In conclusion, we can state that our results are consistent with the idea that *Wolbachia* has less negative impact in fixed populations than in mixed populations. The mechanisms responsible for these differences need to be studied next.

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Biological control potential of *Wolbachia*-infected *versus* uninfected wasps: laboratory and greenhouse evaluation of *Trichogramma cordubensis* and *T. deion* strains

Abstract – The effects of thelytoky-inducing *Wolbachia* (α-proteobacteria) on *Trichogramma cordubensis* and *T. deion* (Hymenoptera, Trichogrammatidae) were studied in laboratory and greenhouse conditions. One infected (thelytokous, all female) line of each wasp species was compared with its conspecific uninfected (arrhenotokous, sexual) counterpart for several fecundity and dispersal traits. Arrhenotokous lines had a higher fecundity than their thelytokous counterparts, which suggests that *Wolbachia* negatively affect the fecundity of *Trichogramma* females. The arrhenotokous females dispersed more in the laboratory than their thelytokous counterparts. In the greenhouse, the opposite effect or no difference between lines was found, indicating that the laboratory set-up used to measure dispersal is not useful to predict relative dispersal of the females in the greenhouse. Calculations show that by releasing hundred adult wasps of both lines, thus including arrhenotokous males in the sexual line, more eggs are parasitized by the thelytokous wasps. Therefore, in spite of their lower individual female fecundity, thelytokous lines have a better potential for biological control than their arrhenotokous counterparts.

Introduction

Hymenoptera are haplo-diploid insects which are generally arrhenotokous, *i.e.* unfertilized eggs develop into haploid males while fertilized eggs yield diploid females. Thelytokous forms, in which unfertilized eggs give rise to diploid females are also known in several families of Hymenoptera. Thelytoky is often associated with the presence of endosymbiotic bacteria of the genus *Wolbachia* (a-proteobacteria) (Stouthamer, 1997). Infection with thelytoky-inducing *Wolbachia* (T-*Wolbachia)* leads to a disruption of the chromosome segregation in the first mitotic division (anaphase) of the haploid egg. As a result, the egg becomes diploid and develops into a homozygous thelytokous female (Stouthamer and Kazmer, 1994). T-*Wolbachia* can also affect their host's fecundity, longevity and pre-adult mortality, which has been reported for *Trichogramma* spp. (Hym., Trichogrammatidae) by Stouthamer and Luck (1993) and van Meer *et al.* (submitted).

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Trichogramma are minute wasps which are widely used in biological control programs against lepidopteran pests. A few species (mainly arrhenotokous forms) have been frequently used as biological control agents (Li, 1994; Smith, 1996). From the ca. 180 species described (Pinto, 1998), at least 14 have thelytokous forms (Pinto and Stouthamer, 1994; Stouthamer, 1997). When *Trichogramma* infected with T-*Wolbachia* are 'cured' from the bacterial infection by antibiotic treatment or exposure to high temperatures, they are rendered permanently sexual (Stouthamer *et al.*, 1990a).

Arrhenotokous and thelytokous forms of the same *Trichogramma* species are often sympatric ('mixed' populations) but in at least two species (*T. cordubensis* and *T. oleae*) only thelytokous forms are known ('fixed' populations) (Stouthamer, 1997). The hypothesis that adverse effects of *Wolbachia* are less severe for fixed populations of *Trichogramma* than for mixed ones has been supported by laboratory studies, in which a surplus of hosts was available (van Meer *et al.*, submitted). Whether arrhenotokous or thelytokous wasps do better in reducing their host population may also depend on host availability, assuming that both forms are equally effective in finding hosts (Stouthamer, 1993; Stouthamer and Luck, 1993).

The best choice for the mode of reproduction of parasitoids in biological control has been discussed and advantages of either mode of reproduction in this context have been listed (Aeschlimann, 1990; Stouthamer, 1993). Thelytokous forms may be preferred because in the asexual lines a low density of mates is not a limiting factor for reproduction, so they may establish better at low host densities. Furthermore, they are cheaper to produce in mass rearings because no resources are "wasted" for the production of males. On the other hand, arrhenotokous forms may be able to better adapt to new environments, due to genetic recombination, and may have a higher rate of population increase at high host densities.

Infection with T-*Wolbachia* has in some cases a severe negative effect on the host fecundity (Stouthamer and Luck, 1993; van Meer *et al.*, submitted). Therefore, additional effects on other important biological control traits such as host searching ability may also be expected. Traits considered to be important for the success of parasitoids as biological control agents have been discussed in general (*e.g.* van Lenteren, 1986 a,b) and for *Trichogramma* in particular. For *Trichogramma*, these traits are: fecundity, longevity, dispersal, adult size (Kot, 1979; Greenberg 1991; Kazmer and Luck, 1995), emergence rate of the parasitized eggs, percentage of non-deformed females, fecundity on the factitious host and on the natural host (Greenberg, 1991; Cerutti and Bigler, 1995). In addition, walking speed and turning rate have been shown to positively correlate with parasitism in the field (Bigler *et al.*, 1988).

Assessment of the overall quality of the wasps during pre-release studies is laborious. A less time-consuming approach is to split 'quality' into parameters which are then quantified (Cerutti and Bigler, 1995). However, a single trait will hardly ever predict the overall performance accurately and therefore, the best combination of a set of laboratory methods must be developed. These measurements should be quick, simple and reliable in order to be applied in a production unit (Bigler, 1994).

One such approach is to measure the parasitoids fecundity and dispersal. Fecundity is important for mass production of the wasps and may be relevant in field situations, too. Dispersal is crucial in field (or greenhouse) situations, since the wasps must be able to find eggs situated at some distance from the release sites. To study dispersal, indirect methods, based on the distance of parasitized host eggs to the wasps release site, are frequently used. When these methods are used, not only dispersal is involved but also host-habitat location, host location and host acceptance and suitability (Suverkropp, 1997). Differences in dispersal between two *Trichogramma cordubensis* lines in a laboratory chamber corresponded to the relative dispersal of those lines (assessed by parasitism) in the greenhouse (Honda *et al.*, submitted). If such a relation is shown to be a general rule, dispersal in this chamber could be a very useful trait in pre-release studies.

To determine if the presence of *Wolbachia* influences the biological control potential of their hosts, the fecundity and dispersal of thelytokous lines of *Trichogramma deion* (mixed population) and of *T. cordubensis* (fixed population) were compared with those of their arrhenotokous counterparts by testing: (a) fecundity in glass vials, when females are given a surplus of host eggs; (b) combined fecundity and dispersal in a laboratory chamber; (c) combined fecundity and dispersal in a greenhouse.

Materials and methods

Cultures – *Trichogramma* thelytokous lines were started with single females from field-collected parasitized lepidopteran eggs. Arrhenotokous lines were obtained by feeding thelytokous lines antibiotics, as described by Stouthamer *et al.* (1990a). The procedure used to check whether lines were completely cured is described in van Meer *et al.* (submitted). These lines had been cured for at least 4 generations prior to our experiments. *Trichogramma cordubensis* Vargas and Cabello (1985) were collected from parasitized *Helicoverpa armigera* Hübner (Lepidoptera, Noctuidae) eggs, on tomato plants (*Lycopersicon esculentum* Miller), in Divor (Alentejo, Portugal) in 1993. *Trichogramma deion* Pinto and Oatman (SW) were collected as parasitized *Apodemia mormo* Felder & Felder

(Lepidoptera, Lycaenidae) eggs, on the desert trumpet *Eriogonum inflatum*, near Barstow (Sidewinder mountains, California, USA) in 1996. Voucher specimens are kept in the collection of J.D. Pinto (Univ. California, Riverside, USA).

Wasps were reared in glass vials (150 mm length, 15 mm diameter), on *Ephestia kuehniella* (Lepidoptera, Pyralidae) UV-killed eggs, at 23±1°C, RH 50±20%, 16h light/8h dark. One day old honey fed wasps were given fresh egg cards. The egg cards were parasitized for at least one day. After ca. 13 days the newly emerged wasps were used for the rearing or for experiments. Thelytokous and arrhenotokous *T. cordubensis* and *T. deion* lines are designated as COT, COA, SWT and SWA, respectively.

Fecundity in glass vials: surplus of hosts and no searching required – Individual one-day old females were placed in glass vials (75 mm length x 10 mm diameter), with an egg mass (6 mm diameter, ca. 130 eggs) and a drop of honey. The vials were closed with cotton wool. The females of the thelytokous line were unmated. The arrhenotokous females had emerged together with males and had thus been allowed to mate. The egg masses were changed daily, for five days. The experiment was carried out at 23±1°C, 70±10% RH, L:D=16:8, 8000 lux. After parasitization, the egg masses were placed in a clean vial and incubated at 23±1°C. We determined the total number of host eggs parasitized (which turned black) per female per day. Sample sizes for COT, COA, SWT and SWA were 28, 29, 54 and 57, respectively. Conspecific thelytokous and arrhenotokous lines were tested simultaneously. For *T. cordubensis*, the size of the mothers was determined by measuring their hind tibia length. The size of the mothers was not measured for *T. deion*.

Dispersal and parasitism in a laboratory chamber – We used the chamber described by Honda *et al.* (submitted), with the modifications mentioned below (Figure 1). It consisted of a solid aluminum block (63.9 x 23.2 cm) containing a continuous winding channel measuring 8 m in length which was routed out of the metal. The channel consisted of 2 x 20 connected sub-channels, each 20 cm in length. To split the chamber in two, a small piece of rubber at the end of channel 20 closed off the connection between adjacent channels. A glass plate, placed over the aluminum block and secured with clamps, closed the chamber. At the both ends of the chamber an entrance hole (15 mm diameter) allowed the release of *Trichogramma* individuals into the chamber. The wasps emerged from two parasitized *E. kuehniella* egg masses in a glass vial (75 mm length x 10 mm diameter). Just before the release, these egg masses were removed from the vial. The vial opening was then inserted in the entrance hole of the chamber, allowing the 1-3 days old wasps to disperse into the chamber. The experiment was carried out at 23±1°C, 75±10% RH, L:D=16:8, 24000 lux. Two releases (one at each side) were performed simultaneously.

Trichogramma lines were alternated between sides to avoid biases. Three different types of releases were performed: (a) release of a thelytokous line at one side of the chamber and of its arrhenotokous counterpart at the other side; (b) release of the same line at both sides of the chamber to test the chamber for symmetry; (c) release of thelytokous females at one side of the chamber and of thelytokous females together with arrhenotokous males at the other side. This last kind of release was only performed with *T. cordubensis*. For arrhenotokous lines, females were always released together with conspecific males which had emerged together with the females. To arrest and eventually attract the wasps to the end of the chamber (see Noldus, 1989; Kaiser *et al.*, 1989, Renou *et al.*, 1989) and to determine the number of eggs parasitized per female, we placed *Ephestia kuehniella* eggs (eight egg masses, ca. 130 eggs each) in channel 17 of each side. Twenty hours after the release, individuals were anaesthetized with CO_2 and the number of females and males in each channel was recorded. After each release the chamber was cleaned with ethanol and dried. The number of parasitized eggs was recorded for 8 replicates, for both *T. cordubensis* and *T. deion*.

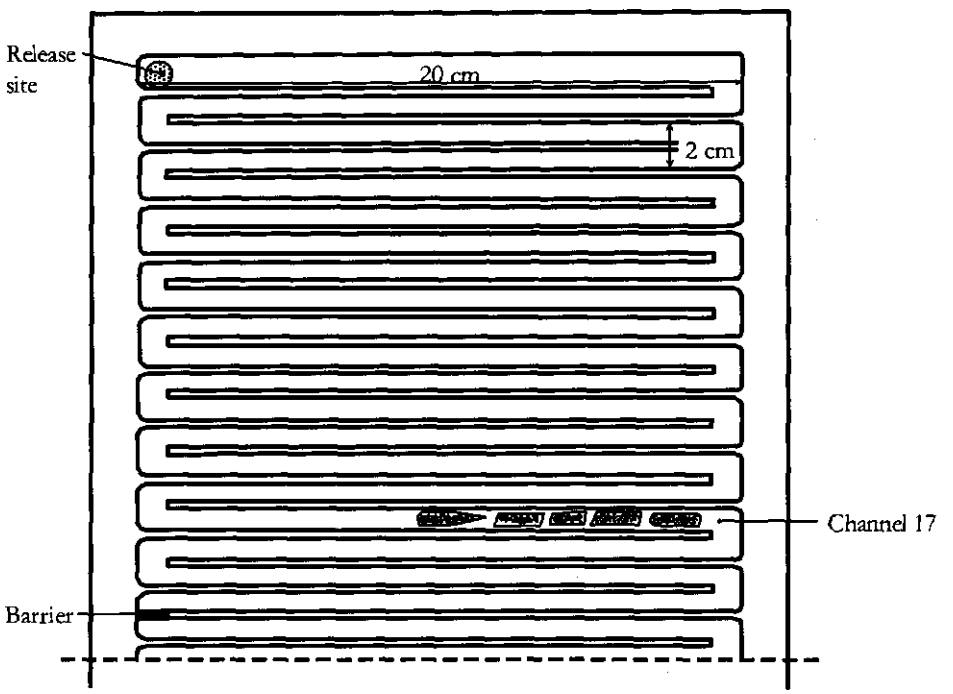

Figure 1. Section of aluminium block (top view) showing the channel cut into metal block. Only one of the two release sites is shown. Wasps were allowed to walk in the chamber for 20 h and to parasitize *Ephestia kuehniella* eggs present in subchannel 17, ca. 3.4 m from the entrance. Total length 2 x 4 m (subchannel dimensions 1 cm x 1 cm x 20 cm).

Parasitism and dispersal in greenhouse – Releases were performed in 2 adjacent compartments of 10.8 m^2 each. In each compartment 13 tomato plants (*Lycopersicon esculentum*) of the cultivar Money Maker, were placed in an U-shape (Figure 4 and 5). Plants were 40-50 cm high, had ca. 15 leaves each and the total leaf area per plant was ca. 1500 cm^2 (Garg and Mandahar, 1972). For uniformity, flowers were removed from the plants. Each plant touched the two adjacent plants. Twelve *Ephestia kuehniella* egg masses were placed on each plant, except on the plant at the mid-point of the U, where the wasps were released. An egg mass consisted of eggs fixed on doublesided adhesive tape in a circle (6 mm diameter, ca. 130 eggs each), surrounded by white paper. Two egg masses per leaf were tape-glued on 6 leaves of each plant, one egg mass at the uppersurface and the other at the undersurface of the same leaflet.

Three experiments were performed in which conspecific arrhenotokous and thelytokous *Trichogramma* were released simultaneously. *Trichogramma cordubensis* releases (experiment 1) were performed from February to May 1997. *Trichogramma deion* releases were performed from June to August 1997 (experiment 2) and in September–October 1997 (experiment 3). In experiments 1 and 2 the thelytokous and arrhenotokous lines were released in separate, adjacent, compartments. In experiment 3 the arrhenotokous and the thelytokous lines were released together in both compartments. Fifteen well parasitized egg masses (ca. 80 parasitized eggs each) were selected, from which ten were randomly chosen for the release; the remaining egg masses were placed in the freezer (-80°C) at the time of release, for later determination of the sex ratio. Wasps were released from a glass vial (150 mm length, 15 mm diameter), after removing the egg masses from which they had emerged. The release was performed at the side most distant from the door at the midpoint of the U shape on the plant without egg masses. The vial containing the wasps to be released was placed in the soil of the flowerpot, its opening leaning against the stem.

The number of females released was estimated *a posteriori*, by counting the number of host eggs from which parasitoids emerged and by assessing the sex ratio of the non-released subsamples. Mean numbers of females released for experiments 1 and 2 are given in figure 4 and 5 respectively. In experiment 3 an average of 394 $\pm$ 60.1 thelytokous and 310 $\pm$ 93.0 arrhenotokous females were released. The trials were repeated 10 times, for *T. cordubensis* (experiment 1), 6 times for *T. deion* (experiment 2) and 4 times (x 2 compartments) for *T. deion* (experiment 3).

Climatic conditions were measured during the experiments and varied between: 16-35°C, 55-85% R.H. (experiment 1); 16-50°C, 30-100% R.H. (experiment 2) and 18-52°C, 49-98% R.H. (experiment 3). In all experiments, day temperature usually remained above 20°C and periods above 35°C did not exceed 4 hours a day. Additional metereological data were

obtained from the Department of Meteorology of the Wageningen Agricultural University.

The egg masses were collected five days after the release and were incubated at 23±1°C, 60±10% R.H., 16h light/8h dark. If the eggs were to be counted more than 5 days later, the egg masses were subsequently incubated at 15±1°C, 60±10% R.H., 16h light/8h dark. The parasitized (black) eggs were counted for each egg mass, except for replicate one of experiment 3, as mentioned below in this section. Dispersal in the greenhouse was measured indirectly, based on the parasitized eggs. Between replicates, fourty fresh *E. kuehniella* egg masses were placed at the same location where the tomato plants were during the experiment. These egg masses were never found parasitized, indicating that after 5 days no more ovipositing wasps were present. In addition, live wasps were never found during collection of the egg masses. In experiment 3, in which the thelytokous and the arrhenotokous lines of *T. deion* were released together in both compartments, we determined whether an egg mass had been parasitized by the thelytokous line, the arrhenotokous line, or both. For that purpose, individual females emerging from egg masses parasitized in the greenhouse were given an unparasitized *E. kuehniella* egg mass and the sex ratio of their progeny was determined. When individual greenhouse parasitized eggs had been isolated (done only for a fraction of the eggs in replicate one) we categorized all-female broods as being produced by a thelytokous mother and all-male broods as having an arrhenotokous mother. However, most wasps emerged from eggs which were not isolated. In these cases, mixed (male and female) progeny was often obtained, from arrhenotokous mated mothers. When an egg mass had been parasitized by both wasp lines, the number of parasitized eggs per line was not determined. In all other cases, except in replicate one of experiment 3, the number of parasitized eggs per egg mass was recorded. For 23 out of the 273 parasitized egg masses it was impossible to determine to which wasp line parasitism should be attributed, because only a few eggs were parasitized or the eggs had been eaten by *E. kuehniella* larvae. These egg masses were not included in the statistical analyses.

Statistical analyses – The statistical software SAS (version 6.12) was used in all analyses. In the case that a generalized linear model (*e.g.* Crawley, 1993) was appropriate, we used the procedure Genmod. A correction for possible overdispersion with respect to the Poisson distribution was made. Linear models (including the two sample situation) were fitted with Proc. GLM, while paired sample t-tests were calculated with the procedure Means. Wilcoxon two-sample tests were performed through the Npar1way procedure. Below, detailed information is given for each experiment. For all analyses an $\alpha=0.05$ was chosen.

(a) Fecundity in glass vials: surplus of hosts and no searching required. The number of eggs parasitized per female (first day, total of first three days, total of five days) was compared between thelytokous and arrhenotokous lines as follows: for *T. deion*, a Wilcoxon two-sample test was used; for *T. cordubensis*, a generalized linear model was fitted, with Poisson distribution, log link function, and using tibia length as a covariable. A t-test was used to compare hind tibia lengths of mothers between lines.

(b) Dispersal and parasitism in a laboratory chamber. By means of a linear model incorporating the explanatory variables *Trichogramma* line, gender, repetitions in time and release side, we studied the following response variables:

- Average distance of females to the release site, using channel number as distance unit. The square root of the total number of individuals released was used in the model as weight.
- Average distance of females and males to the release, calculated as above. By means of a generalized linear model with Poisson distribution and log link function and the explanatory variables *Trichogramma* line, repetitions in time and release side, we studied the following response variable:
  - (i) Number of parasitized eggs, assuming the expected number of parasitized eggs to be proportional to the number of females arriving at channel 17 (where the egg masses were present) or further. Factors release side and repetitions, which showed no significant effect, were subsequently excluded from the model.
  - (ii) Number of parasitized eggs per female released. A Wilcoxon two-sample test was used.

c. Parasitism and dispersal in greenhouse – For all three experiments, temperature and relative humidity were initially used as explanatory variables. For experiment 1, air pressure, light intensity and percentage of hours of sunshine per day were tested as well. No significant effects were found for these variables, so they were excluded from the analyses. For experiments 1 and 2, we studied the following response variables by means of a linear model incorporating the explanatory variables *Trichogramma* line, repetitions in time, greenhouse compartment:

- Number of parasitized eggs per female, using the square root of the total number of females released as weight.
- Number of parasitized egg masses per female, calculated as above.
- Average distance of parasitized eggs to the release plant. Distance was calculated as the average plant number (see Figure 4 and 5) where parasitized eggs were found. The square root of the total number of parasitized eggs was used in the model as weight.

- Average distance of parasitized egg masses to the release plant, calculated as above.
- Average height on the plant of parasitized eggs. Height was calculated as the average plant height where the parasitized eggs were found. Lowest leaves with eggs were assigned number one, highest leaves with eggs were assigned number 6. The square root of the total number of parasitized eggs was used in the model as weight.
- Average height on the plant of parasitized egg masses, calculated as above.
- Average number of parasitized eggs per parasitized egg mass.
- Proportion of parasitized eggs at the uppersurface of the leaves of the total number of parasitized eggs: comparison of the wasp lines with each other and of each of the lines with 0.5.

For experiment 3, the same parameters were determined, but the differences between the averages of each line were analysed (instead of the averages itself), by means of a paired sample t-test.

Results

Fecundity in glass vials: surplus of hosts and no searching required.

In both *Trichogramma* species, the arrhenotokous females parasitized significantly more eggs than their thelytokous counterparts, in all cases (i.e, first day, total first three days and total five days) (Figure 2). Hind tibia lengths differed significantly between *T. cordubensis* lines ($p = 0.015$), with the mean values ($\pm$ sd) of 0.167 mm ($\pm$ 0.0083) for the arrhenotokous line and 0.162 mm ($\pm$ 0.0075) for the thelytokous line. Note that a significant difference in number of parasitized eggs was found between *T. cordubensis* lines after correction for tibia length.

Dispersal and parasitism in a laboratory chamber

Percentage distributions show the dispersal pattern of females of arrhenotokous and thelytokous forms of *T. cordubensis* and of *T. deion* in the chamber (Figure 3). For both species, arrhenotokous females showed significantly more dispersal (as expressed by average distance to release site) than their thelytokous counterparts and than their conspecific males. Thelytokous *T. cordubensis* females which were released with arrhenotokous males did not differ significantly in dispersal from thelytokous conspecific females which were released alone. For *T. cordubensis*, arrhenotokous females, which dispersed more than their thelytokous counterparts, parasitized more eggs per female as well. For *T. deion* the difference in parasitism between lines was not significant (Table 1).

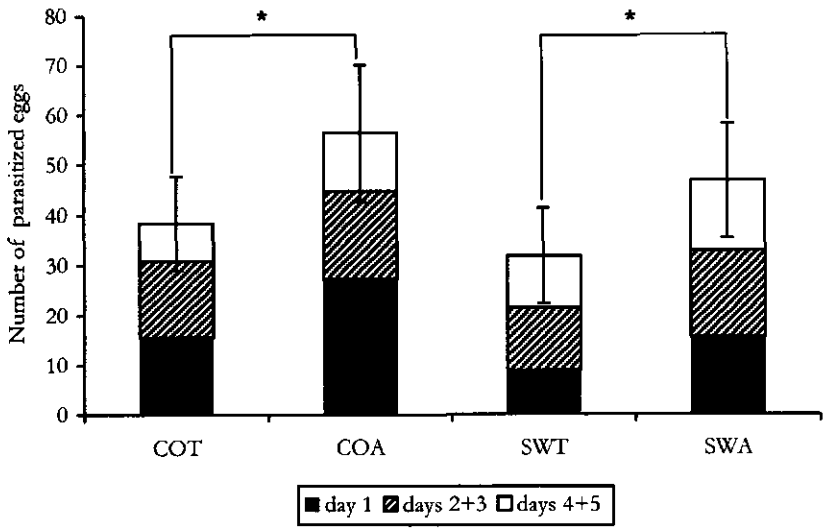

Figure 2. Parasitism of *Ephestia kuehniella* eggs by thelytokous and arrhenotokous *T. cordubensis* (COT and COA) and *T. deion* (SWT and SWA) in glass vials: surplus of hosts and no search needed. Columns represent mean number of parasitized eggs per *Trichogramma* female (day 1, days 2+3 and days 4+5). Standard deviation bars refer to cumulated number of parasitized eggs (days 1 to 5). For sample sizes see Materials and Methods. *: values for the arrhenotokous line are significantly higher than for their conspecific thelytokous line ($p < 0.05$).

Parasitism and dispersal in greenhouse

In all three experiments, the number of eggs parasitized per female was higher for the arrhenotokous lines, but this difference was found to be significant only in *T. deion* released in separate compartments (experiment 2, Table 2). No significant differences between thelytokous and arrhenotokous lines in the number of egg masses parasitized per female were found.

In all three experiments, the average distance of parasitized eggs and egg masses to the release point was higher for the thelytokous line, with significant differences found for *T. cordubensis* (experiment 1) and *T. deion* lines released together in the same compartments (experiment 3). Regarding the vertical distribution of parasitized eggs, thelytokous *T. cordubensis* parasitized egg masses significantly higher on the plant than their arrhenotokous counterparts, while no such a difference was found for *T. deion*. In all cases, average number of parasitized eggs per egg mass was significantly higher for the arrhenotokous lines. Significantly more eggs were parasitized by *T. cordubensis* at the

uppersurface of leaves than at the undersurface. For *T. deion* no such difference could be found. No significant differences in this last parameter were found between arrhenotokous and thelytokous lines.

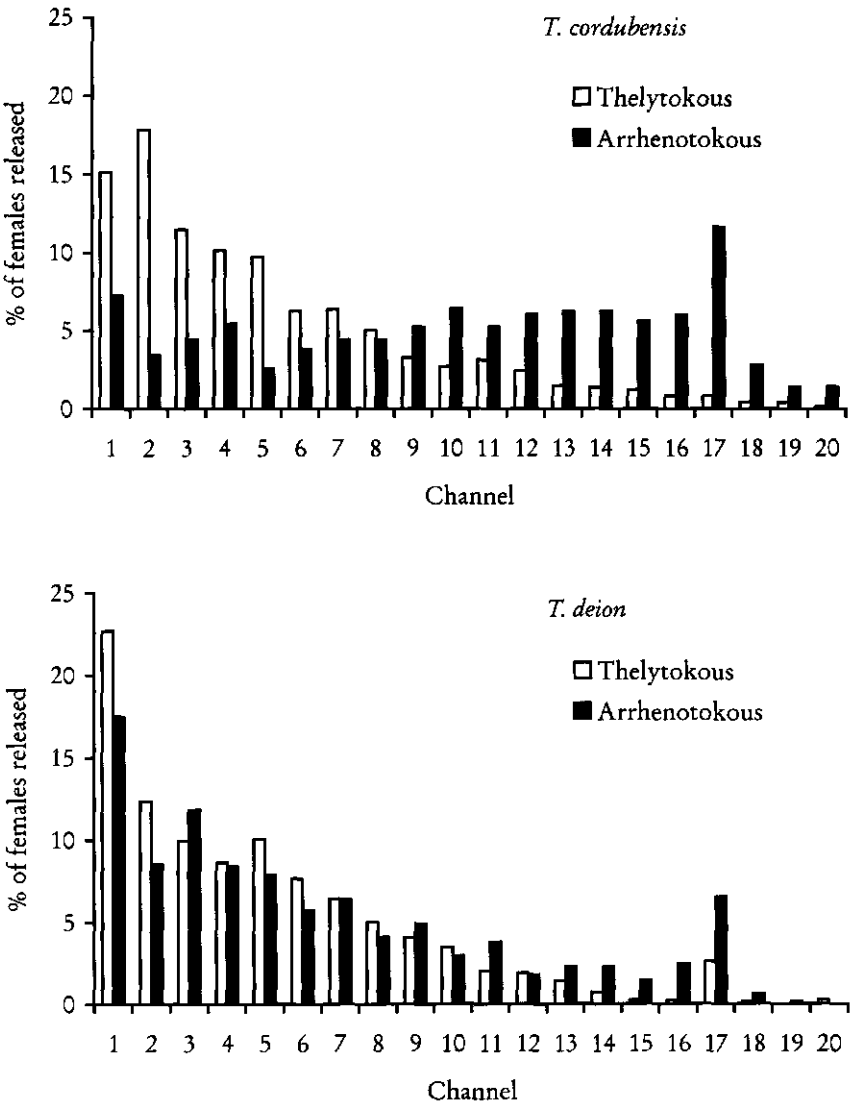

Figure 3. Percentage of thelytokous (white bars) and arrhenotokous (black bars) females of (i) *Trichogramma cordubensis* (COT and COA) and (ii) *T. deion* (SWT and SWA) found per channel in the laboratory chamber after being allowed to walk for 20 h; wasps were released in channel 1. *Ephestia kuehniella* eggs were present in channel 17.

Table 1. Dispersal and parasitism of thelytokous and arrhenotokous lines of *Trichogramma cordubensis* (COT and COA, respectively) and of *T. deion* (SWT and SWA, respectively) in the laboratory chamber (descriptive statistics).

Gender and wasp line released to which the results refer to	Mean (± sd) wasps released	Released together with	Number of replicates‡	Mean channel (± sd) reached after 20 h	Mean (± sd) number of eggs parasitized per ♀ channel ≥ 17 (n=8)	Mean (± sd) number of eggs parasitized per ♀ (n=8)
♀♀ COT	89.5 ± 26.47	-	17	5.2 ± 1.42	2.2 ± 3.30	0.06 ± 0.09
♀♀ COA	56.6 ± 23.87	♂♂ COA	12	9.3 ± 2.01	21.2 ± 7.60	2.88 ± 1.65
♂♂ COA	34.3 ± 15.46	♀♀ COA	12	6.1 ± 1.87	-	-
♀♀ COT	88.3 ± 8.14	♂♂ COA	3	5.3 ± 0.90	-	-
♂♂ COA	28.3 ± 15.89	♀♀ COT	3	7.0 ± 1.30	-	-
♀♀ SWT	117.3 ± 34.45	-	10	5.2 ± 0.92	9.1 ± 6.52	0.31 ± 0.26
♀♀ SWA	75.9 ± 32.30	♂♂ SWA	8	6.3 ± 0.85	12.7 ± 4.76	0.75 ± .050
♂♂ SWA	51.4 ± 22.97	♀♀ SWA	8	4.8 ± 2.04	-	-

Significant differences between conspecific populations are indicated by a * ($p < 0.05$); ‡:considering each side of the maze independently; -: not tested or no biological meaning; ns: not significant; n: number of replicates used for number of eggs parasitized.

Discussion

To determine the effect that an infection with *Wolbachia* may have on the biological control efficiency of *Trichogramma*, we compared fecundity and dispersal of thelytokous (i.e, infected) and arrhenotokous (i.e., uninfected) *T. cordubensis* and *T. deion* wasps in a series of experiments, both in the laboratory and in the greenhouse.

We first determined the fecundity of thelytokous and arrhenotokous females, when they were offered daily a surplus of host eggs in glass vials. Arrhenotokous females of *T. deion* had a higher fecundity than their thelytokous counterparts. These results agree with previous findings of Stouthamer and Luck (1993) and van Meer *et al.* (submitted) for *T. deion*, *T. pretiosum* and *T. kaykai* and are compatible with the hypothesis of a negative effect of *Wolbachia* on female fecundity for mixed populations. On the other hand, we expected that a negative effect of *Wolbachia* would be less or non-existent in *Trichogramma* from fixed populations. However, in our laboratory fecundity experiment, arrhenotokous *T. cordubensis* females parasitized significantly more eggs than their thelytokous conspecifics. Previous work on the same *T. cordubensis* lines had shown a negative effect of *Wolbachia* on lifetime fecundity, but not in five days fecundity (van Meer *et al.*, submitted). The main difference between the two fecundity experiments is that the wasps we tested were smaller than the ones tested by van Meer *et al.* (submitted). These authors used *Mamestra brassicae* L. (Lepidoptera, Noctuidae) as rearing host, instead of *E. kuehniella*. In our experiment, arrhenotokous wasps were, on average, larger than thelytokous wasps. Larger *Trichogramma* wasps have often higher fecundity than small ones (e.g. Waage and Ng, 1984). But the size of mothers explains only in part the difference in fecundity obtained between arrhenotokous and thelytokous *T. cordubensis*. A possible explanation for the difference between our results and the ones of van Meer *et al.* (submitted) in relative parasitism of thelytokous and arrhenotokous *T. cordubensis* is that the *Wolbachia* exert a more pronounced negative effect on smaller wasps. More fecundity experiments should be performed using, simultaneously, hosts of different species and sizes, including hosts of different species but with the same size, to clarify the effects of host-species, host-size and infection status on the wasps' size and fecundity.

In the second set of experiments, in a laboratory chamber, arrhenotokous *T. cordubensis* and *T. deion* females dispersed more than their thelytokous counterparts. Also, previous work done on *T. deion* (isofemale lines Seven Pines California and Texas) has shown a higher dispersal of arrhenotokous females in the laboratory chamber, when compared with the thelytokous ones (Vereijssen *et al.*, 1997). These authors have observed that immediately after the release, thelytokous females remained near the release site, while

Table 2. Several greenhouse experiments parameters, regarding thelytokous and arrhenotokous *Trichogramma cordubensis* (COT and COA, experiment 1) and *T. deion* (SWT and SWA) released apart (experiment 2) and released together (experiment 3). For experiments 1 and 2 values are result of GLM model, for experiment 3 data were not transformed. Average distance of eggs and egg masses to the release plant are given in plant numbers units (Figure 4 and 5). Average height on the plant of parasitized eggs is given in leaf numbers: lowest leaf with eggs is number one, highest leaf with eggs is number six.

	Experiment 1 (n = 10)		Experiment 2 (n=6)		Experiment 3 (n=4x2)
Trichogramma lines ⇒	COT (mean ± SEM)	COA (mean ± SEM)	SWT (mean ± SEM)	SWA (mean ±SEM)	SWT – SWA (diff ±SE)
number of parasitized eggs/_	1.9 ± 0.42	2.4 ± 0.43	0.84 ± 0.06	1.12* ± 0.07	-0.46 ± 0.20
number of paras. egg masses/_	0.09 ± 0.01	0.06±0.01	0.06 ± 0.003	0.05 ± 0.003	-0.01 ± 0.009
eggs distance	2.4* ± 0.15	1.3 ± 0.15	2.4 ± 0.11	2.1 ± 0.10	0.12 ± 0.27
egg masses distance	2.7* ± 0.16	1.5 ± 0.18	2.6 ± 0.12	2.4 ± 0.13	0.35* ± 0.12
eggs height on plant	4.4* ± 0.13	2.8 ± 0.13	4.1 ± 0.13	4.0 ± 0.13	-0.46 ± 0.38
egg masses height on plant	4.1* ± 0.12	3.2 ± 0.13	4.0 ± 0.07	4.0 ± 0.08	0.12 ± 0.22
number of eggs/egg mass	20.2 ± 2.15	36.8* ± 2.24	15.1 ± 0.77	23.2* ± 0.81	-5.95* ± 0.56
Proportion eggs upperside leaf	0.59# ± 0.03	0.64# ± 0.03	0.57 ± 0.03	0.48 ± 0.03	0.12 ± 0.05

*: values for the conspecific thelytokous and the arrhenotokous line within an experiment are significantly different ($p < 0.05$); #: values are significantly different from 0.5. ($p < 0.05$), no significant differences between lines were detected; n: number of replicates.

arrhenotokous females dispersed much quicker. We also observed this phenomenon for *T. cordubensis* during our laboratory chamber experiments. Our releases of thelytokous *T. cordubensis* females with conspecific males in the laboratory chamber indicate that the presence of males is not the cause of the differences in dispersal found between females of the two *T. cordubensis* lines. Therefore, the infection with *Wolbachia* could be responsible for a lower dispersal of the females in the laboratory chamber. The difference in number of parasitized eggs per released female was particularly pronounced between the lines of *T. cordubensis*. While in glass vials the *T. cordubensis* arrhenotokous females parasitized ca. 1.5 times more eggs than their thelytokous counterparts during day one, in the chamber the arrhenotokous females parasitized almost 50 times more eggs than their thelytokous counterparts. This difference between lines is partly due to a difference in dispersal (affecting the number of females reaching the eggs) and partly due to a difference in fecundity between the two lines (Table 1). For both wasp species, males dispersed less than females, which agrees with findings for other *Trichogramma* species in field conditions (Stern *et al.*, 1965; Suverkropp, 1997).

Table 3. Parasitism by thelytokous and arrhenotokous *Trichogramma cordubensis* and *T. deion* wasps per hundred individuals released. Mean sex ratio (% females), mean number of parasitized eggs per female and number of eggs parasitized per 100 wasps released, calculated as sex ratio x mean number of parasitized eggs per female x 100.

Species	Mean sex ratio	Mean number of parasitized eggs per female	Number of eggs parasitized per 100 wasps
COT	1.00	1.90	190
COA	0.55	2.40	132
SWT	1.00	0.84	84
SWA	0.61	1.12	68

In the third type of experiments, *i.e.*, the greenhouse releases, arrhenotokous females of both species parasitized more eggs than their thelytokous counterparts. The number of eggs parasitized per female was not in all cases significantly different between the lines but the number of eggs parasitized per egg mass was always significantly higher for the arrhenotokous lines. This indicates that arrhenotokous lines were more effective in parasitizing aggregated host eggs. No differences were found between thelytokous and arrhenotokous lines in the number of egg masses parasitized per female, suggesting that lines do not quantitatively differ in their ability to find host egg masses.

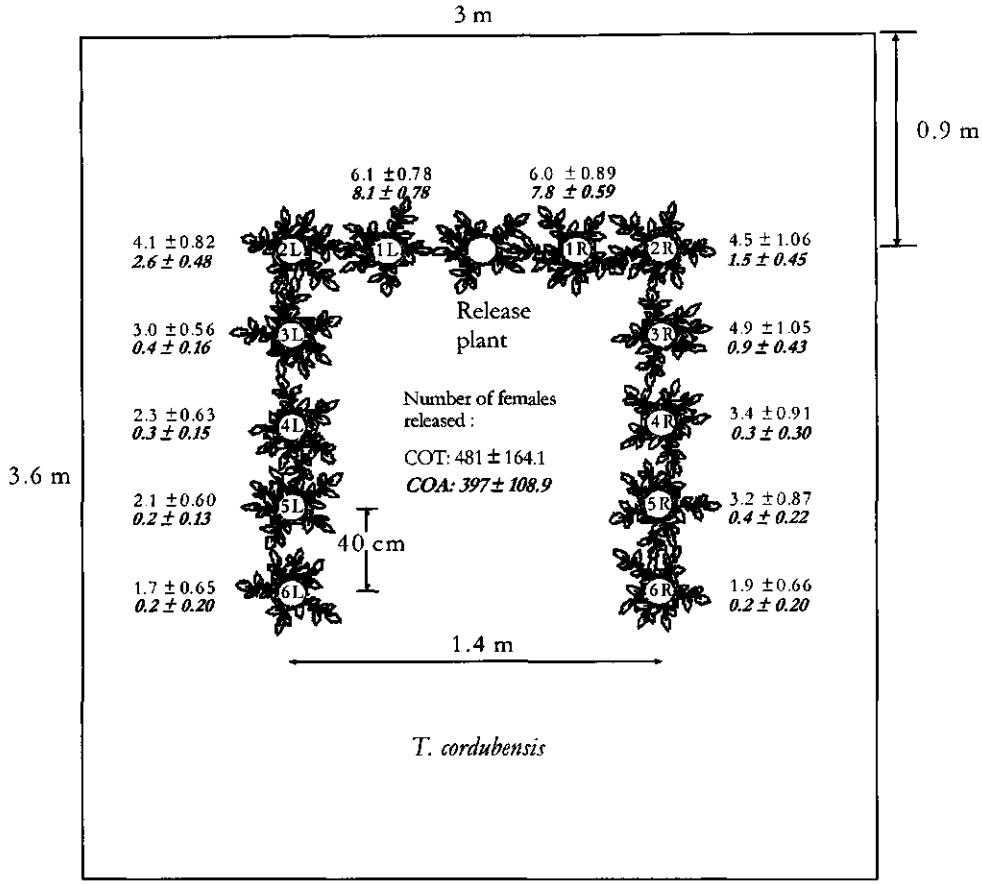

Figure 4. Position of tomato plants and parasitism patterns of *Trichogramma cordubensis* populations (COT and COA) on 12 tomato plants in paired greenhouse releases (experiment 1). Numbers represent mean numbers of sentinel egg masses parasitized (± SEM) at each tomato plant.

When we calculate the number of eggs which would be parasitized when we release one hundred individuals of each line, taking into account the average fecundity per wasp (from our greenhouse results) and the sex ratio (of the wasps we released in the greenhouse), we see that a higher parasitism is achieved by the thelytokous wasps (see Table 3).

The number of females that we released in the greenhouse was high when compared with the general recommendation for heavy host infestations, which is 20 individuals/m^2 (Lenteren *et al.*, 1997). We released, on average, from 46/m^2 (arrhenotokous females, experiment 3), to 133/m^2 (thelytokous females, experiment 1). Yet parasitism was low, particularly at the plants far from the release point.

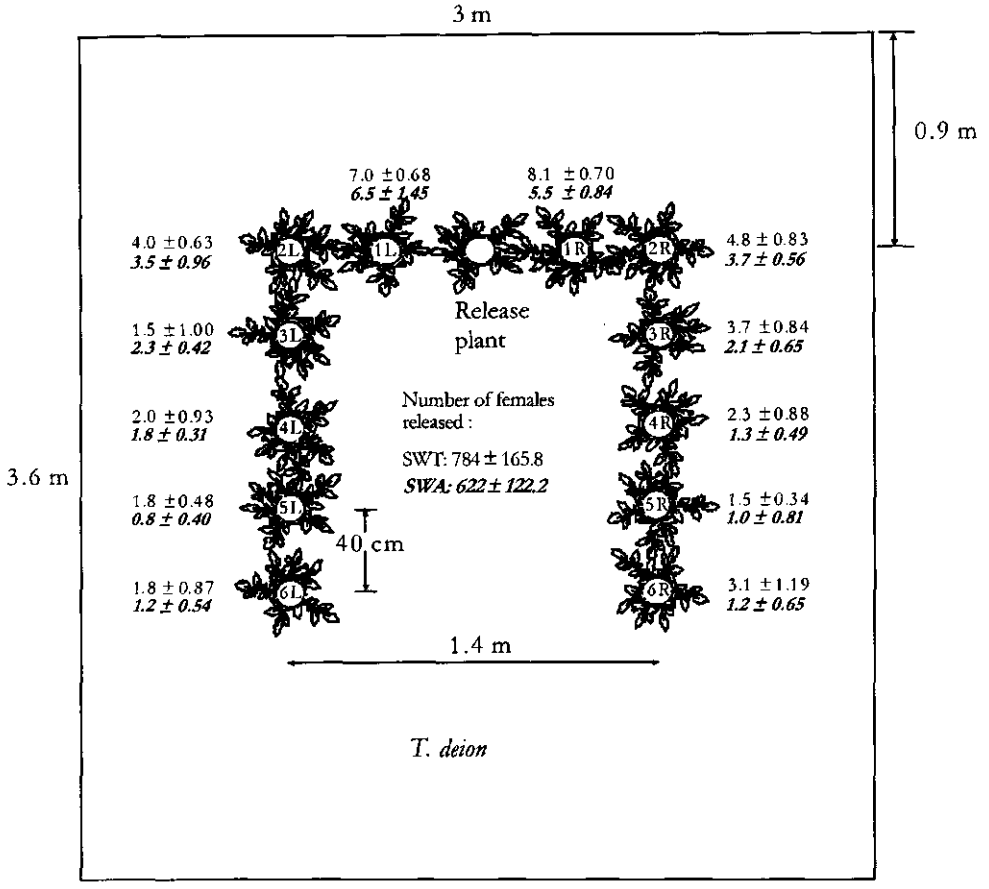

Figure 5. Position of tomato plants and parasitism patterns of *Trichogramma deion* (SWT and *SWA*) on 12 tomato plants in paired greenhouse releases (experiment 2). Numbers represent mean numbers of sentinel egg masses parasitized (± SEM) at each tomato plant.

Extreme climatic conditions at times in the greenhouse and absence of nutrition for the released parasitoids could be responsible for the results obtained. Also the plant species and structure, the lepidopteran host species, the distribution of the eggs on the plants, the presence or absence of moth scales on the leaves and the release method may play an important role on the number of hosts parasitized (Bigler *et al.*, 1997). On the other hand, the fact that there were egg masses close to the release site could lead to arrestment behaviour of the wasps and demotivate further dispersal.

For *Trichogramma cordubensis*, we found that the thelytokous line significantly dispersed more than the arrhenotokous line. The difference found in the vertical distribution of

parasitized eggs between *T. cordubensis* lines may be explained by a larger vertical dispersal of the thelytokous line. Plants touched each other usually at the level of leaf 2 or 3 (counting from the bottom). A mean height of the parasitized eggs at leaf 4 may indicate that, after walking from a plant to the other through the leaves, females of the thelytokous line disperse more, by moving up in the plant. For *T. deion*, no significant differences were found in vertical distribution and with exception of egg masses distance in experiment 3, in which dispersal of the thelytokous line was significantly higher, no differences were found in dispersal.

In the chamber, arrhenotokous females of both *T. cordubensis* and *T. deion* dispersed more than their thelytokous counterparts, but the opposite effect or no differences were found in the greenhouse. So, contrary to the findings of Honda *et al.* (submitted), the dispersal in the chamber did not give an indication of relative dispersal of arrhenotokous and thelytokous females in the greenhouse. The presence of plants and the climatic conditions in the greenhouse could have provided the necessary stimuli for a relatively high dispersal of the thelytokous *T. cordubensis* wasps. We suggest that flight may play a role in the differences found between lines in the greenhouse experiment. *Trichogramma* individuals could not fly in the chamber but they could fly in the greenhouse. Suverkropp (1997), studying dispersal of *T. brassicae* on maize, found that flight was important in the movement from one plant to another, while walking was the most important way of locomotion within a plant. In a preliminary set of *T. cordubensis* releases in a cage (ca. 200 individuals per release, 5 replicates per line) thelytokous and arrhenotokous females wasps were compared in their ability to fly. While for the thelytokous line 41-50% of the released females were caught on the sticky trap, which could be reached by flying only, for the arrhenotokous line only 0-3% of the released wasps were trapped (M. Roskam, unpublished). The different mating status of the thelytokous and arrhenotokous females may have played a role in the differences just mentioned. However, in contrast to these results, it has been reported that unmated *Trichogramma minutum* (arrhenotokous) females had less propensity to fly than mated ones (Forsse *et al.*, 1992). More experiments are needed to clarify the reasons for differences in dispersal between thelytokous and arrhenotokous lines and the role that mating status, flight and laboratory rearing play in it.

In inoculative biological control, thelytokous females can be advantageous simply because they always produce daughters and do not need males for mating, while for arrhenotokous lines daughter production can decrease drastically in situations of low host density. For inoculative biological control at high host densities, the choice between

thelytokous and arrhenotokous wasps should depend on the rate of population increase, adaptation to the environment and production costs.

In inundative biological control, adaptation to environment and rate of population increase play a much less important role. When instead of egg masses, single and scattered eggs of the pest are present, the fecundity disadvantage of the thelytokous females becomes insignificant. We assume that each female is able to locate only a few hosts over her lifetime and that thelytokous and arrhenotokous wasps do not differ in the ability of finding eggs. Under such circumstances, thelytokous females will be able to parasitize equal numbers of hosts as arrhenotokous females. Thelytokous females are advantageous because of their lower production costs (no host eggs "wasted" in males). But also in the case pests produce egg masses, despite the lower individual female fecundity of the thelytokous lines, more eggs are parasitized per 100 wasps released. Hence, thelytokous wasps are advantageous because they are relatively more economic biological control agents. The range of dispersal of the wasps is crucial when a large area is to be controlled and relatively few release points are used. Also in this point the thelytokous line of *T. cordubensis* is advantageous.

In conclusion, arrhenotokous *T. cordubensis* and *T. deion* parasitized more eggs per female than their thelytokous counterparts in glass vials, in the chamber and in the greenhouse. These differences between conspecific lines were significant except for *T. cordubensis* and once for *T. deion* in the greenhouse (experiments 1 and 3) and for *T. deion* in the chamber. These results suggest that a negative effect of *Wolbachia* on the fecundity of their female hosts exists. The effect of *Wolbachia* on *Trichogramma* dispersal appears to be positive or neutral under greenhouse conditions, although it was negative in the laboratory. Thelytokous females seem to be more appropriate to release against scattered isolated eggs because they may disperse more and they produce females only. Even though arrhenotokous females are more efficient in parasitizing large egg masses, calculations show that the use of thelytokous females is more advantageous, because production costs are lower.

The artificial transformation of arrhenotokous strains to thelytokous ones by microinjecting arrhenotokous females with *Wolbachia* (van Meer *et al.*, 1996; Grenier *et al.*, 1998) may prove to be useful not only to answer evolutionary questions about the relation between *Wolbachia* and their hosts, but it could also have an application in biological control projects. To assess its practical usefulness, more studies are needed on the direct and indirect impact of these thelytokous-made wasps on target and non-target species (Howarth, 1991; Simberloff and Stiling, 1996) and on the final costs of producing these wasps.

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Summary and conclusions

Bacteria of the genus *Wolbachia* (α-Proteobacteria, Rickettsia) are widespread in arthropods and can induce cytoplasmic incompatibility (CI), thelytoky (T), feminization (F) or fecundity increase in their host. The subjects of this thesis are focused on *Wolbachia* inducing thelytoky (T-*Wolbachia*). T-*Wolbachia* infect several hymenopteran parasitoids which enables infected females to produce daughters from unfertilised eggs. Thelytokous strains can be maintained without the involvement of males. T-*Wolbachia* may represent a tool to improve biological control because only female parasitoids attack target pest species. Advantages of thelytokous reproduction of parasitic wasps in biological control programs may include:

(1) lower costs of mass rearing;

(2) faster population growth after release and

(3) easier establishment of thelytokous wasps in pest populations.

Therefore, it was suggested to render sexual reproducing parasitoids thelytokous by infecting them with T-*Wolbachia*. To determine whether this approach was feasible, several experiments were done to improve our understanding of this specific host-symbiont relationship.

Phylogeny

The phylogeny of *Wolbachia* has been studied using 16S rDNA and the cell cycle gene *ftsZ* but sequence variation of those genes is limited. The spacer 2 region (SR2) was amplified to determine if this region would improve phylogenetic resolution (Chapter 2). The SR2 of *Wolbachia* is 66 basepairs (bp) long and shows slightly higher sequence differences between strains than *ftsZ*. Due to the short length of SR2 of *Wolbachia*, little phylogenetic information could be retrieved. A conspicuous finding was that SR2 of the F-*Wolbachia* of *Armadillidium vulgare* contained a 10 bp deletion compared to the other *Wolbachia* SR2 sequences and suggest that F-*Wolbachia* differs considerably from the CI and T-*Wolbachia* strains. Additional phylogenetic research was done using the sequence of an outer membrane protein (*wsp*) of *Wolbachia* (Chapter 3). Previous research in Yale (USA) showed that this gene evolved at a much faster rate than 16S rDNA or *ftsZ* and the *Wolbachia* clade was subdivided into twelve distinct groups based on the proposed 2.5% *wsp* sequence divergence grouping criterion. We extended this former *Wolbachia wsp* data

set with fifteen T-*Wolbachia*, one F-*Wolbachia* and three CI-*Wolbachia*. Our results showed that:

(1) Seven groups could be added to the existing twelve. Although the phylogenetic resolution between strains was improved, the grouping criterion of sequence 2.5% divergence can result in a excessive proliferation of groups when more *Wolbachia wsp* strains will be added. In addition, the design of group specific primers will become more difficult with the increase in the number of closely related groups as found for the *Trichogramma Wolbachia*;

(2) No relation between host phenotype and *Wolbachia* phylogenetic position was found meaning that both CI and T- *Wolbachia* clustered in one group as was found in the phylogenies based on *ftsZ* or 16S. Three different mechanisms have been postulated for this phenomenon: (a) the existence of plasmids or phages associated with *Wolbachia* that carry the genes responsible for the phenotype; (b) the host determines the effect of an aspecific *Wolbachia* infection; (c) genes inducing CI or T are similar and can be derived from each other by a simple mutation;

(3) Based on identical *wsp* sequences of the moth *Ephestia kuehniella* and its parasitoid *Trichogramma*, possible horizontal *Wolbachia* transfer between them was postulated. The exact mechanism of horizontal transfer remains to be clarified;

(4) The *wsp* sequence of F-*Wolbachia* of the isopod *A. vulgare* differed more than 13% from the other sequences, suggesting that they diverged long time ago from the insect *Wolbachia*. This is consistent with our previous result found for SR2 of the same species in which a 10 bp deletion was found compared to all other CI- and T-*Wolbachia* SR2.

Micro-injection

The *Wolbachia* phylogeny shows that T-*Wolbachia* are not monophyletic and several hypotheses were postulated for this phenomenon (see Chapter 3). One of the hypothesis is that T-*Wolbachia* in one host species would induce CI in another species. To test this, a horizontal transfer experiment of *Wolbachia* by micro-injection was carried out (Chapter 4). T-*Wolbachia* from the fly pupa parasitoid *Muscidifurax uniraptor* (*w*Uni) was transferred to an uninfected fruitfly *Drosophila simulans*. The latter species can harbour different strains of *Wolbachia* (e.g. *w*Ri) and has been used for many *Wolbachia* transfer studies. *Drosophila simulans* has successfully been infected with *Wolbachia* from *Drosophila mauritiana*, *Drosophila melanogaster* and even the mosquito *Aedes albopictus*.

The *ftsZ* sequences (728 bp) of *w*Ri and *w*Uni only differ in 2 basepairs suggesting they are closely related. The infection could be detected in the new host for several generations by PCR, however no specific effects on the host were detected and the bacteria were not stably maintained. This lack of successful transfer can be explained by (a) the fact that *Wolbachia* of *D. simulans* and *M uniraptor* were not at all closely related according to new *wsp* data because they belonged to two different groups and/or (b) *Wolbachia* may have adapted too much to *M. uniraptor* host for successful transfer to a new species. Evidence for this hypothesis may be the fact that *M. uniraptor* wasps can not reproduce sexually anymore (see also Chapter 6).

Our next goal was to do a similar T-*Wolbachia* transfer experiment between hymenopteran species to test whether arrhenotokous wasps could be rendered thelytokous. No suitable micro-injection protocol for parasitoids was available and initial injection-experiments with the fly pupa parasitoid *Muscidifurax raptor* failed. Therefore, we shifted to the gregarious fly pupa parasitoid *Nasonia vitripennis* because with this species, large number of eggs could be collected relatively easy. This facilitated the testing of the different steps of the micro-injection procedure. Different micro-injection protocols of *Drosophila* spp. and *Tribolium confusum* were combined and optimal conditions for each of the different steps were determined (Chapter 5). In addition, an *in vitro* incubation step for the larvae had to be included. The final protocol enables us to do *Wolbachia* transfer studies in this species.

Host-Wolbachia *interactions*

Different fitness parameters of T-*Wolbachia* infected and non-infected *Trichogramma* species were studied (Chapter 6). We distinguished two different groups: 'fixed' populations in which the infection is fully established so that only thelytokous females are present and 'mixed' populations in which thelytokous females coexist with arrhenotokous ones. In mixed populations thelytokous females are still able to mate and to produce daughters sexually. In mixed populations, a potential cytoplasmic-nuclear conflict exists but in fixed populations, this conflict is absent. It is theorised that fixation of the infection results in a reduction of negative impact of the symbiont on its host. This hypothesis was tested with the egg parasitoid *Trichogramma* because both mixed and fixed populations exist within this genus.

Two isofemale lines from fixed populations and 4 isofemale lines from mixed populations were 'cured' of *Wolbachia* infection using antibiotics and different lifespan fitness parameters were measured. Daughter production was significantly higher for the thelytokous fixed lines (16–131% more daughters) compared to the conspecific

arrhenotokous ones in contrast to three mixed lines where the opposite was found (6–61% less daughters). Only slight fecundity effects of *Wolbachia* were found in the fixed lines (varying among 19% less offspring and 6% more offspring) while these effects are clearly negative in the mixed lines (34–49% less offspring). Comparing all thelytokous lines, *Wolbachia* transmission was generally higher for the fixed thelytokous lines with exception of one mixed line of *T. deion*. Unexpectedly, longevity was significantly shorter in infected fixed lines compared to the cured lines and no longevity differences were found for the mixed lines. Finally, for both mixed and fixed lines, negative *Wolbachia* effects were detected for pupal and embryo mortality. Our results are discussed in the context of a previous published model which describes the dynamics of *Wolbachia*-infected *Trichogramma* wasps in mixed populations.

Finally, we determined whether thelytokous wasps do equally well as biological control agents as their arrhenotokous counterparts (Chapter 7). Theoretically, thelytokous wasps may be better biological control agents than the arrhenotokous ones. However, previous studies, including the one described in chapter 6, show that *Wolbachia* can have a negative fecundity impact on their host. Therefore, other fitness parameters such as host searching efficacy, dispersal etc. could also be affected. We assessed whether *Wolbachia* infection had an impact on the 'parasitization efficiency' of the *Trichogramma* species *T. deion* and *T. cordubensis* in greenhouse compartments. Laboratory studies, to assess the effect of *Wolbachia* on host fecundity and dispersal were also done to correlate these results with the greenhouse experiment results. Laboratory results showed:

(1) The fecundity of the thelytokous wasps species was reduced compared to the arrhenotokous counterparts. This corresponds with our results of chapter 6 with the only difference that for a five-day parasitization period, we found a higher fecundity for thelytokous *T. cordubensis* compared to the arrhenotokous line in chapter 6, while in this experiment it is the other way around. The use of different lepidopteran host species on which we reared the *Trichogramma* species (*Mamestra brassicae* in chapter 6 and *Ephestia kuehniella* in this experiment) may explain this difference;

(2) Experiments of *Trichogramma* in a laboratory chamber showed that for both species, the arrhenotokous lines dispersed more than their thelytokous counterparts, suggesting a negative effect of *Wolbachia* on dispersal.

Greenhouse experiments showed for both species that thelytokous wasps parasitize approximately equal number of patches but parasitize fewer eggs per patch than the arrhenotokous females. These results correspond with the laboratory fecundity experiments. However, in contrast to laboratory chamber experiments, thelytokous

T. deion females dispersed equally well as their arrhenotokous counterparts while thelytokous *T. cordubensis* females showed significant more dispersal than the arrhenotokous ones. No explanation was found for these dispersal differences in greenhouse or laboratory chamber experimental set-ups. According to calculations, it is still advantageous to use thelytokous parasitoids for biological control when the negative impact of *Wolbachia* on host fitness is taken into account.

In the next years, the possibilities and restrictions of using T-*Wolbachia* for biological control purposes will become clear. Our developed injection protocol enables us to do T-*Wolbachia* transfer studies in the parasitoid *Nasonia vitripennis*. Initial T-*Wolbachia* transfer studies between two *Trichogramma* species in Lyon (France) were successful. However, the novel infected species only showed low induction of thelytoky. Infection experiments with different strains of T-*Wolbachia* in *Trichogramma* could show which factors are respon2sible (host and/or *Wolbachia*) for successful establishment of the infection and a high expression of thelytoky. Final field tests will reveal whether 'novel' thelytokous parasitoids are indeed better biological control agents than their arrhenotokous counterparts.

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Samenvatting en conclusies

Bacteriën van het geslacht *Wolbachia* infecteren een belangrijk deel van de geleedpotigen (arthropoden). Een *Wolbachia* infectie kan op verschillende manieren de voortplanting van besmette arthropoden beïnvloeden. We onderscheiden:

(1) cytoplasmische incompatibiliteit (CI). CI komt tot uitdrukking als een geïnfecteerd mannetje met een ongeïnfecteerd vrouwtje paart en resulteert in productie van een verminderd aantal nakomelingen t.o.v. alle andere kruisingscombinaties

(2) feminizatie (F), waarbij geïnfecteerde mannetjes worden omgevormd tot functionele vrouwtjes. Dit is met name bestudeert in pissebedden;

(3) verhoging van vruchtbaarheid waarbij geïnfecteerde moeders tot twee maal zoveel nakomelingen produceren dan de niet-geïnfecteerde moeders;

(4) thelytokie (T) waarbij geïnfecteerde moeders dochters kunnen produceren uit onbevruchte eieren. Dit type infectie is gevonden in verscheidene wespsoorten van de orde vliesvleugeligen (Hymenoptera). In de dezelfde orde produceren ongeïnfecteerde vrouwtjes zonen uit onbevruchte eieren en dochters uit bevruchte eieren en dit verschijnsel wordt arrhenotokie genoemd.

Thelytoky-*Wolbachia* komt voor bij verscheidene sluipwespsoorten. Sluipwespen zijn insecten die hun eieren in andere insecten leggen en hun "gastheer" op deze manier doden. Een infectie met T-*Wolbachia* in sluipwespen kan een aantal belangrijke voordelen voor de biologische bestrijding opleveren omdat alleen vrouwelijke sluipwespen effectief zijn in het doden van de schadelijke insecten. Bijvoorbeeld, als een arrhenotoke wesp 5 zonen en 5 dochters produceert, kan een thelytook vrouwtje 10 dochters produceren. Thelytoke wespen zouden t.o.v. arrhenotoke wespen de volgende voordelen hebben voor biologische bestrijding:

(1) geringere kweekkosten per vrouwtje omdat er geen mannetjes worden geproduceerd

(2) hogere populatiegroeisnelheid van de sluipwespen

(3) betere vestigingskansen van de losgelaten natuurlijke vijanden. Als er weinig schadelijke insecten zijn, zal de sluipwespdichtheid laag worden. In deze situatie kunnen arrhenotoke vrouwtjes moeite hebben om mannetjes te vinden om te paren. Thelytoke vrouwtjes hebben dit nadeel niet.

Vanwege deze voordelen zijn verscheidene experimenten uitgevoerd met T-*Wolbachia* die hierna worden samengevat. Deze experimenten zijn verricht om de ecologische en evolutionaire kennis van deze specifieke gastheer-*Wolbachia* relatie te verbreden, en om na te gaan of arrhenotoke sluipwespen met T-*Wolbachia* geïnfecteerd konden worden.

Fylogenie

De verwantschap tussen verschillende *Wolbachia* stammen (fylogenie) was reeds bestudeerd aan de hand van het 16S ribosomale gen en het *ftsZ* gen. Deze genen verschilden echter weinig tussen de verscheidene *Wolbachia* stammen. Om het onderscheidend vermogen tussen verschillende *Wolbachia* stammen te verhogen is, m.b.v. moleculaire technieken, de 'spacer' 2 (SR2) van *Wolbachia* vermeerderd en geanalyseerd (Hoofdstuk 2). SR2 liet een groter verschil zien tussen de stammen dan het eerder onderzochte *ftsZ* gen. Maar omdat SR2 relatief kort is, leverde het niet meer informatie dan het *ftsZ* gen. Een opmerkelijk resultaat was dat van de F-*Wolbachia* SR2 (van de pissebed *Armadillidium vulgare*) een stukje ontbrak t.o.v. de andere *Wolbachia* SR2. Dit suggereert dat F-*Wolbachia* aanzienlijk verschilt van de CI- en T- *Wolbachia* stammen . Het fylogenetisch onderzoek werd vervolgd met analyses van een membraaneiwit "*wsp*" van *Wolbachia* (Hoofdstuk 3). Recent onderzoek in Yale (VS) had aangetoond dat dit gen veel sneller evolueerde dan het 16S rDNA of het *ftsZ* gen. In dit onderzoek werd tevens het genus *Wolbachia* onderverdeeld in twaalf groepen op basis van maximum 2.5 % *wsp*-gen verschillen binnen één groep. Wij hebben de *Wolbachia wsp* dataset uitgebreid met vijftien T-*Wolbachia* en drie CI-*Wolbachia* en één F-*Wolbachia*. Onze resultaten toonden aan dat:

(1) zeven groepen toegevoegd konden worden aan de bestaande twaalf groepen. Het onderscheidend vermogen tussen de *Wolbachia* stammen was aanzienlijk verbeterd maar de 2.5% regel kan leiden tot een excessieve groei van het aantal groepen.

(2) CI- and T- *Wolbachia* vormden geen aparte groepen. Dit komt overeen met eerder beschreven *Wolbachia* fylogenieën gebaseerd op het *ftsZ* gen of 16S rDNA. Er zijn drie verklaringen mogelijk waarom CI- en T-*Wolbachia* geen aparte groepen vormden: (a) het bestaan van plasmiden of virussen van *Wolbachia* die de genen bevatten die verantwoordelijk zijn voor het specifieke effect van *Wolbachia* op haar gastheer. Deze elementen kunnen van één *Wolbachia* stam naar een andere overspringen; (b) de gastheer bepaalt het CI of T effect t.g.v. een niet-specifieke *Wolbachia* infectie. Bijvoorbeeld, een T-*Wolbachia* stam uit een specifieke gastheer induceert CI in een andere gastheer; (c) genen die verantwoordelijk zijn voor het veroorzaken van CI of T zijn nauw verwant en kunnen uit elkaar ontstaan door een eenvoudige mutatie.

(3) De *wsp* genen van *Wolbachia* uit twee sluipwespsoorten van het geslacht *Trichogramma* zijn nagenoeg hetzelfde als die van *Wolbachia* uit de mot *Ephestia kuehniella* (Lepidoptera). *Trichogramma* wespen kunnen eieren van *E. kuehniella* parasiteren. Het vermoeden bestaat dat horizontale overdracht van *Wolbachia* tussen wesp en mot heeft plaatsgevonden. De wijze waarop deze overdracht bewerkstelligd wordt is nog onduidelijk.

(4) Het F-*Wolbachia wsp* gen van de pissebed *A. vulgare* verschilde meer dan 13% t.o.v. andere *wsp* genen en suggereert dat deze *Wolbachia* stam al lang geleden van andere insect *Wolbachia* stammen (CI en T) is afgesplitst. Dit is in overeenstemming met de resultaten van hoofdstuk 2 die aantoonden dat SR2 van *A. vulgare* relatief veel afweek van de andere SR2 sequenties.

Micro-injectie

T-*Wolbachia* en CI-*Wolbachia* zijn niet specifiek genetisch te onderscheiden en verscheidene hypothesen zijn opgesteld om dit verschijnsel te verklaren (zie ook Hoofdstuk 3). Het zou kunnen dat T-*Wolbachia* van een specifieke gastheer, CI induceert in een andere gastheer. Om dit te toetsen is de T-*Wolbachia* stam van de vliegenpopparasitoïde *Muscidifurax uniraptor* (*w*Uni) overgebracht naar een ongeïnfecteerde fruitvlieg *Drosophila simulans* met behulp van micro-injectie (Hoofdstuk 4). De microinjectie-techniek houdt in dat *Wolbachia* in eieren van ongeïnfecteerde gastheren wordt geïnjecteerd. We hebben ervoor gekozen om T-*Wolbachia* naar *D. simulans* over te zetten omdat deze soort geïnfecteerd kan zijn met verschillende CI-*Wolbachia* stammen (o.a. *w*Ri). Ook zijn in *D. simulans* verscheidene succesvolle infectie experimenten uitgevoerd met *Wolbachia* stammen uit andere fruitvlieg soorten en zelfs uit de mug *Aedes albopictus*. Tenslotte verschillen de *ftsZ* sequenties van *w*Ri of *w*Uni heel weinig. Dit suggereert dat ze nauw verwant zijn.

De nieuwe infectie van *w*Uni in *D. simulans* kon m.b.v. moleculaire technieken aangetoond worden, maar er werden geen effecten op de gastheer gevonden. De nieuwe infectie kon echter na zeven generaties niet meer aangetoond worden. Hiervoor zijn tenminste twee verklaringen mogelijk. Ten eerste toonde nieuwe fylogenie gegevens van *w*Ri en *w*Uni aan, gebaseerd op het *wsp* gen, dat deze stammen niet nauw verwant zijn, maar dat ze in twee verschillende groepen thuishoren. Ten tweede kan de associatie tussen van *w*Uni en de gastheer *M. uniraptor* kan relatief langdurig zijn geweest aangezien *M. uniraptor* wespen zich niet meer sexueel kunnen voortplanten (zie hoofdstuk 6). Door deze langdurige associatie kan *Wolbachia* op een zodanige manier aan haar gastheer aangepast zijn dat ze andere gastheersoorten niet meer kunnen infecteren.

Ons volgende doel was om een soortgelijke infectieproef met T-*Wolbachia* tussen verschillende sluipwespen uit te voeren om te bepalen of arrhenotoke wespen op deze manier thelytook gemaakt konden worden. Eerst werd een protocol ontwikkeld voor de arrhenotoke vliegenpopparasitoïde *Muscidifurax raptor* maar door technische problemen zijn we overgestapt naar de gregaire vliegenpopparasitoïde *Nasonia vitripennis*. *N. vitripennis* heeft als voordeel dat relatief eenvoudig veel eieren verzameld kunnen worden wat het testen van het protocol vergemakkelijkt. Verschillende micro-injectie protocollen van de fruitvlieg *Drosophila* spp. en de kever *Tribolium confusum* zijn gecombineerd en voor elke stap zijn de optimale condities uitgezocht (Hoofdstuk 5). Het resulterende protocol kan men gebruiken om er *Wolbachia* infectie proeven mee uit te voeren in *N. vitripennis*.

Gastheer-Wolbachia *interacties*

In het 6e hoofdstuk is de invloed van verscheidene T-*Wolbachia* stammen op de 'fitness' van een aantal *Trichogramma* lijnen gemeten om te bepalen of deze invloed tussen lijnen verschilt. *Trichogramma* zijn kleine sluipwespen (± 0.3 mm) die eieren van verscheidene schadelijke insecten kunnen parasiteren. De getoetste *Trichogramma* lijnen waren afkomstig uit populaties met een verschillende '*Wolbachia* infectiegraad' waarin twee groepen onderscheiden konden worden. De eerste groep waren 'gefixeerde' populaties waarin alle individuen geïnfecteerd zijn zodat alleen thelytoke vrouwtjes aanwezig zijn. De tweede groep zijn niet-gefixeerde populaties waarin geïnfecteerde en ongeïnfecteerde individuen naast elkaar voorkomen. In deze niet-gefixeerde populaties kunnen thelytoke vrouwtjes paren en sexueel dochters produceren. Afhankelijk of *Wolbachia* afkomstig is uit een gefixeerde of niet-gefixeerde populatie verwachtten we een verschil van *Wolbachia* invloed op haar gastheer.

We verwachtten dat *Wolbachia* uit gefixeerde populaties een minder negatieve invloed op haar gastheer zou hebben ten opzichte van *Wolbachia* uit niet-gefixeerde populaties. Dit verschil zou worden veroorzaakt doordat in niet-gefixeerde populaties een zogenaamd nucleair-cytoplasmatisch conflict bestaat. Dit conflict ontstaat doordat genen uit de kern (nucleaire genen) door beide ouders doorgegeven worden maar *Wolbachia* (en cytoplasmatische genen) wordt alleen geërfd via de moeder. Voor *Wolbachia* is het dus voordelig om de fractie vrouwtjes te verhogen aangezien mannetjes "een doodlopende weg" vormen voor deze symbiont. Als *Wolbachia* zich in een populatie verspreidt, resulteert dat in een verlaging van de fractie mannetjes. Nu wordt het 'voordelig' voor

nucleaire genen om op een of andere manier de werking van *Wolbachia* ongedaan te maken. Immers een klein aantal mannetjes kunnen dan hun nucleaire genen aan veel vrouwtjes doorgeven. Dit mechanisme dat de werking van *Wolbachia* neutraliseert, kan op zijn beurt weer leiden tot een reactie van *Wolbachia* om deze effecten teniet te doen. Dit conflict zou ervoor kunnen zorgen dat *Wolbachia* een negatief effect heeft op de fitness van de gastheer. Factoren die van belang zijn voor verspreiding van *Wolbachia* in een populatie zijn o.a. (a) transmissie efficiëntie (het percentage geïnfecteerde dochters) en (b) de invloed van *Wolbachia* infectie op de gastheer fitness. Deze twee factoren, en wellicht ook andere, zouden door nucleaire genen negatief beïnvloed kunnen worden.

In gefixeerde populaties is de situatie geheel verschillend omdat er geen nucleair-cytoplasmatisch conflict meer aanwezig is aangezien er geen mannetjes meer zijn. In deze omstandigheden zal de *Wolbachia*-gastheer combinatie die het meest succesvol is, d.w.z. die het meeste nakomelingen geeft, zich door de populatie verspreiden. Selectie is dus gericht op *Wolbachia* stammen die het minst negatieve invloed op hun gastheer hebben. Deze hypothese werd getoetst m.b.v. wespenlijnen van het genus *Trichogramma* omdat in dit geslacht niet-gefixeerde en gefixeerde populaties bestaan. Twee "inteeltlijnen" (lijnen die begonnen zijn met een één vrouwtje) van gefixeerde populaties en vier inteeltlijnen van niet-gefixeerde populaties zijn m.b.v. antibiotica genezen van *Wolbachia* infectie. Vervolgens zijn van deze lijnen verscheidene fitness kenmerken gemeten gedurende de gehele levensperiode (zie Tabel 1).

Tabel 1. Verscheidene fitness kenmerken van met *Wolbachia* geïnfecteerde thelytoke (T) lijnen en corresponderende arrhenotoke lijnen (A) afkomstig uit gefixeerde of niet-gefixeerde populaties.

Fitness kenmerk	Niet-gefixeerd (T. vs. A)	Gefixeerd (T. vs. A.)
Dochter productie	6–61% minder dochters	16–131% meer dochters
Totale nakomelingen productie	34–49% minder nakomelingen	19% minder tot 6% meer nakomelingen
Levensduur van de wespen	Geen verschil	Korter

De productie van dochters was significant hoger voor de thelytoke gefixeerde lijnen vergeleken met de corresponderende genezen arrhenotoke lijnen (zie Tabel 1). Het tegenovergestelde resultaat werd gevonden met de vier niet-gefixeerde lijnen (minder dochters voor de thelytoke lijnen vergeleken met de genezen lijnen). In de gefixeerde lijnen is tevens de negatieve invloed van *Wolbachia* op de totale productie van nakomelingen relatief klein t.o.v. de genezen lijnen terwijl dit effect in de niet-gefixeerde lijnen veel groter is.

De levensduur van geïnfecteerde gefixeerde lijnen was onverwacht korter vergeleken met de genezen lijnen terwijl bij de niet-gefixeerde lijnen de geïnfecteerde en niet-geïnfecteerde lijnen even lang leefden. Overdracht van *Wolbachia* overdracht is over het algemeen lager in de niet-gefixeerde lijnen met uitzondering van de lijn *T. deion* SW. De sterfte in het embryo- en popstadium van de gastheer was zowel bij gefixeerde als bij of niet-gefixeerde lijnen hoger in geïnfecteerde lijnen. Onze resultaten komen overeen met de hypothese dat *Wolbachia* een meer negatieve invloed op haar gastheer heeft in niet-gefixeerde populaties dan in gefixeerde populaties met uitzondering van de levensduur van de wesp. Dit is waarschijnlijk het gevolg van een nucleair-cytoplasmatisch conflict in niet-gefixeerde populaties. Een minder aannemelijke alternatieve hypothese is dat een primaire 'succesvolle' *Wolbachia*-gastheercombinatie naar fixatie gaat terwijl minder succesvolle *Wolbachia*-gastheercombinaties zich niet of maar gedeeltelijk in de populatie kunnen vestigen.

In hoofdstuk 7 worden experimenten beschreven waarin getoetst wordt of thelytoke wespen even effectief zijn voor biologische bestrijding als arrhenotoke wespen. Theoretisch gezien kunnen thelytoke wespen voordelen hebben t.o.v. arrhenotoke wespen in biologische bestrijding. Laboratoriumproeven (inclusief hoofdstuk 6) lieten echter zien dat sommige *Wolbachia* stammen de fitness van de gastheer negatief kunnen beïnvloeden. Verder is het onbekend of andere gastheerfactoren, die van belang zijn in het veld (o.a. verspreidingscapaciteit of gastheer-zoekcapaciteit) niet mede beïnvloed zijn. Met behulp van laboratoriumproeven zijn de vruchtbaarheid en verspreidingscapaciteit gemeten van arrhenotoke en thelytoke *Trichogramma deion* en *T. cordubensis* wespen. Tegelijkertijd is bepaald of *Wolbachia* infectie de parasiterings-efficiëntie van deze *Trichogramma* lijnen in kassen beïnvloedt. De parasiterings-efficiëntie werd onderverdeeld in drie verschillende categorieën namelijk (a) verspreiding van de wespen in een kas; (b) hoeveelheid eipaketten die per vrouwtje gevonden werden en (c) hoeveelheid eieren die per losgelaten vrouwtje geparasiteerd werden. De laboratoriumproeven toonden aan dat:

(1) thelytoke wespen een lagere vruchtbaarheid hadden dan de corresponderende arrhenotoke wespen. Dit resultaat komt overeen met de resultaten van hoofdstuk 6, maar met het verschil dat voor een parasiteringsperiode van vijf dagen thelytoke *T. cordubensis* een hogere vruchtbaarheid hadden dan de arrhenotoke lijn (Hoofdstuk 6), terwijl het in deze experimentele opzet net andersom was. De proefopzetten verschilden in het gebruik van de soort vlindereieren waarop de *Trichogramma* wespen zijn gekweekt en dit zou de verschillen kunnen verklaren;

(2) de arrhenotoke lijnen voor beide *Trichogramma* soorten een hogere verspreidingscapaciteit hadden dan de thelytoke lijnen. Dit suggereert een negatief effect van *Wolbachia* op de verspreidingscapaciteit van de gastheer.

De kasexperimenten toonden aan dat voor beide *Trichogramma* soorten de thelytoke wespen evenveel eipaketten parasiteren als de arrhenotoke wespen maar dat de thelytoke wespen minder eieren per eipakket parasiteren dan de arrhenotoke. Deze resultaten komen overeen met de laboratoriumexperimenten. Verder werd er in de kasexperimenten, in tegenstelling tot de laboratoriumproeven, geen verschil gevonden in de verspreiding tussen thelytoke en arrhenotoke *T. deion* vrouwtjes. Thelytoke *T. cordubensis* vrouwtjes verspreidden zelfs beter dan de arrhenotoke vrouwtjes. Aangezien de verspreiding van thelytoke lijnen in de kas hetzelfde of zelfs beter is dan die van de arrhenotoke lijnen, is volgens berekeningen, het nog steeds voordeliger om thelytoke dan arrhenotoke sluipwespen te gebruiken in biologische bestrijding, ondanks de negatieve invloed van *Wolbachia* op de gastheervruchtbaarheid.

De komende jaren zal het duidelijk worden wat de mogelijkheden van T-*Wolbachia* zijn voor de biologische bestrijding. Met het door ons ontwikkelde injectieprotocol kan onderzocht worden of arrhenotoke *Nasonia vitripennis* wespen, door ze te infecteren met een T-*Wolbachia* stam, thelytook gemaakt kunnen worden. *Wolbachia* infectie proeven in *Trichogramma* in Lyon (Frankrijk) hebben al laten zien dat *Wolbachia* overgedragen kan worden tussen verschillende *Trichogramma* soorten. Helaas was de inductie met thelytoky gering in de nieuwe geinfecteerde wespen. Infectie- proeven van verschillende combinaties T-*Wolbachia* stammen en *Trichogramma* soorten zou opheldering kunnen geven welke factoren (gastheer en *Wolbachia*) van belang zijn voor een succesvolle infectie en thelytokie inductie. Uiteindelijk zullen praktijktoetsen moeten uitwijzen of de "nieuw gemaakte" thelytoke wespen daadwerkelijk 'beter' zijn in de biologische bestrijding dan de arrhenotoke wespen.

Curriculum vitae

Op 4 september 1965 werd ik, Marnix Maurice Marie van Meer, geboren te Nispen. In Augustus 1986, behaalde ik, na het doorlopen van MAVO en HAVO, mijn VWO diploma (Avondcollege Breda). In datzelfde jaar ben ik begonnen met een studie Biologie aan de Landbouwuniversiteit Wageningen. Mijn specialisatie was in de gewasbescherming met fysiologische en moleculair biologische oriëntatie. Een drietal afstudeervakken zijn gevolgd in de vakgebieden Virologie, Entomologie en Nematologie. Vervolgens ben ik in 1991 voor zes maanden op stage gegaan bij de 'Institute of Medical Health' in Madang, Papoea Nieuw Guinea, om ervaring op te doen in de medische entomologie. In 1992 is een 2e stage gedaan bij de vakgroep Entomologie in Davis, Californië (V.S.) om onderzoek te doen aan insektenvirussen. In de periode 1993-1998 heb ik als onderzoeker in opleiding (OIO) promotie onderzoek uitgevoerd bij het laboratorium voor Entomologie aan de Universiteit van Wageningen.