THE ROYAL SOCIETY

Received 15 August 2003
Accepted 5 November 2003
Published online 27 January 2004

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

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

Keywords: *Wolbachia*; parthenogenesis; intraspecific horizontal transfer; interspecific horizontal transfer; *Trichogramma*

1. INTRODUCTION

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

It is generally assumed that vertically inherited symbionts cospeciate with their host but this is certainly not the case for *Wolbachia*. Rather, phylogenetic evidence has shown that horizontal transfer of these bacteria must have occurred in the course of evolution because closely related bacterial strains can be found in unrelated hosts (O'Neill *et al.* 1992; Rousset *et al.* 1992; Stouthamer *et al.* 1993; Werren *et al.* 1995*b*). Similarly, micro-injection studies successfully transferred *Wolbachia* into naive hosts both intra- and interspecifically (Boyle *et al.* 1993; Braig *et al.* 1994; Grenier *et al.* 1998). Recently, Fujii *et al.* (2001) were able to show that, after transfection, a single *Wolbachia* strain can induce different phenotypes in different hosts. In general, however, it is very difficult to maintain high infection rates over many generations in newly infected lines (Van Meer & Stouthamer 1999; Pintureau *et al.* 2000*b*; McGraw *et al.* 2002).

In nature, horizontal transfer can occur only when a donor and a recipient host are in close contact because *Wolbachia* is assumed to be incapable of surviving outside the host's tissues. *Wolbachia* were first semi-naturally transferred in woodlice through blood–blood contact (Rigaud & Juchault 1995). Such transfers might occur in nature when individuals become injured during crowding. Close contact is certainly the case in host–parasitoid associations. Heath *et al.* (1999) were able to show a natural host–parasitoid transfer from an infected host *Drosophila simulans* (where *Wolbachia* induces CI) to a parasitoid wasp (*Leptopillina boulardi*). Furthermore, two recent phylogenetic studies revealed the possibility that horizontal transfers were frequent in such communities (Schilthuizen & Stouthamer 1997; Vavre *et al.* 1999). High rates of natural horizontal transfers between conspecifics were first shown in the parasitoid wasp, *Trichogramma kaykai* (Huigens *et al.* 2000). When PI *Wolbachia*-infected and uninfected *T. kaykai* larvae shared the same host, some of the originally uninfected larvae acquired the infection. Newly infected females thereafter produce daughters from their unfertilized eggs. However, it is still unknown how common such horizontal transfers are. Here, we investigate intra- and interspecific natural horizontal transfers in several *Trichogramma* species.

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

Wolbachia in *Trichogramma* are unique compared with almost all other *Wolbachia*–host associations because '*Trichogramma–Wolbachia*' form a monophyletic group based on several *Wolbachia*-specific genes (Stouthamer *et al.* 1999*a*). By contrast, *Wolbachia* associated with other species as in, for instance, the genus *Drosophila*, do not form such a monophyletic group. Phylogenetic analysis of the *Wolbachia–Trichogramma* association showed an obvious discordance between the *Trichogramma* and *Wolbachia* phylogenies, which is most probably explained by horizontal transfer of *Wolbachia* (Schilthuizen & Stouthamer 1997). Such horizontal transfers might take place when different species use the same host egg (Huigens *et al.* 2000). Horizontal transfer may result in double or triple infections when wasps infected with different *Wolbachia* oviposit and develop in the same host egg. Although multiple infections have not yet been described in *Trichogramma*, they are known from several other host species, mainly those associated with CI–*Wolbachia* (Werren 1997). Multiple infections open the way for recombination between different *Wolbachia.* Such genetic exchange has been shown in the *Wolbachia* surface protein (*wsp*) gene in several strains of *Wolbachia* (Jiggins *et al.* 2001; Werren & Bartos 2001).

Here, we study natural intra- and interspecific horizontal transfer of PI *Wolbachia* in three situations: (i) superparasitism, in which larvae of an infected and an uninfected mother of a single species share the same host egg; (ii) multiparasitism, in which larvae of an infected and an uninfected mother of different species share the same host egg; and (iii) multiparasitism, in which the larvae of two infected mothers of different species share the same host egg.

2. MATERIAL AND METHODS

(a) Trichogramma *cultures*

Intra- and interspecific horizontal transfer experiments were conducted using iso-female lines of four *Trichogramma* species: *T. kaykai*, *T. deion*, *T. pretiosum* and *T. atopovirilia*. *Trichogramma kaykai* and *T. deion* lines were initiated with wasps collected from parasitized eggs of the butterfly *Apodemia mormo deserti* in the Mojave Desert, CA, USA. In both species infected and uninfected females coexist. A study of the *ftsZ* gene of *Wolbachia* showed that *Wolbachia* in *T. kaykai* probably originated from a single infection (Schilthuizen *et al.* 1998). The two infected (LC 19-1 and LC 10-1) and three uninfected *T. kaykai* lines (LC 105A, LC 19-1 cured and LC 110 cured) all originate from Last Chance Canyon, El Paso Mountains, Kern County, CA, USA. We used four *T. deion* lines: an infected (SW 436-1) and an uninfected line (SW 649) from Sidewinder Mountains, San Bernardino County, CA, USA; an uninfected line (LC 151) from Last Chance Canyon, El Paso Mountains, Kern County, CA, USA; and an infected line (223) initiated with wasps collected at Sanderson, TX, USA. The infected *T. pretiosum* line (Tpre-13) was collected in Santa Catarina, Brazil (host species unknown). The infected (Tato-01) and uninfected (Tato-02) *T. atopovirilia* were collected in Minas Gerais state, Brazil, and in Colombia, respectively (host species unknown). The infection status in the indigenous populations of *T. pretiosum* and *T. atopovirilia* is unknown. These *Trichogramma* lines were cultured on eggs of the moth *Ephestia kuehniella* for many generations before the experiments were conducted.

(b) *Intra- and interspecific horizontal transfer*

Intraspecific horizontal transfer of *Wolbachia* was attempted by creating hosts that contained eggs of two conspecific females, one of which was infected (donor) and the other uninfected (recipient). The intraspecific horizontal transfer was attempted among three species, *T. kaykai*, *T. deion* and *T. atopovirilia.* Here, *T. kaykai* was used as a control because horizontal transfer was known to occur in this species (Huigens *et al.* 2000).

Interspecific horizontal transfer was determined by allowing: (i) an infected *T. kaykai* and an uninfected *T. deion*; (ii) an infected *T. deion* and an uninfected *T. kaykai*; or (iii) an infected *T. pretiosum* and an infected *T. atopovirilia* female to parasitize the same host egg. This latter experiment involving multiparasitism might result in the female offspring carrying two different *Wolbachia* strains, i.e. a double infection.

(c) *Test for horizontal transfer of* Wolbachia

A moth egg (the host egg), either *Trichoplusia ni* or *Mamestra brassicae*, both uninfected hosts, was offered first to a female, 'line A' and, 2 h later, to a second female, 'line B'. The latter female was either a conspecific (superparasitism) or a congener (multiparasitism). In eggs of both lepidopteran species, a *Trichogramma* female usually lays a clutch of two to four eggs. In half of the cases, a female from 'line A' was offered the host egg first and in the other half the order was reversed. We observed and recorded the number of *Trichogramma* eggs oviposited in a moth egg by each female using behavioural criteria described by Suzuki *et al.* (1984). If only F_1 females emerged from a super- or multiparasitized host egg, we exposed these virgin F_1 females individually to host eggs and recorded the sex of their progeny.

The F_1 females were linked to their parental female by using a molecular marker and, in the multiparasitism experiments, female body colour. To determine the origin of the F_1 females in cases involving superparasitism, we used a microsatellite DNA repeat TTG 49 for *T. kaykai*, a TAC 47 microsatellite repeat for *T. deion*, and specific primer for DNA amplification of the ITS2 region for *T. atopovirilia.* In infected *T. atopovirilia* one DNA fragment is amplified whereas in uninfected *T. atopovirilia* two fragments of different sizes are amplified. In the cases involving multiparasitism, we could easily distinguish the F_1 females of each species by the female's body colour. *Trichogramma kaykai* females have a yellow body colour whereas those of *T. deion* are brown. The *T. pretiosum* and *T. atopovirilia* females used in these experiments are yellow and black, respectively.

The F_1 females from the recipient line were tested for the presence of *Wolbachia* by PCR using *wsp* primers (Braig *et al.* 1998). To confirm that the horizontal transfer had occurred, amplified *wsp* genes were sequenced in the donor lines and in the newly infected females. The presence of daughters in the offspring of an F_1 virgin, originating from a recipient line, indicated a horizontal transfer of *Wolbachia* and subsequent parthenogenetic reproduction.

In the test for a double infection involving infected *T. pretiosum* and infected *T. atopovirilia* larvae sharing the same host egg, we used amplified *wsp* fragments of *Wolbachia* that differed between species using the restriction enzymes *Mbo*I and *Mbo*II. A combination of the different restriction patterns in F_1 *T. pretiosum* or *T. atopovirilia* females confirms a double infection.

(d) *Molecular techniques*

DNA extraction was performed using one wasp homogenized in 50 µl of 5% Chelex-100 and 2 µl of proteinase K (20 mg ml^{-1}) and incubated for at least 4 h at 56 °C, followed by 10 min at 95 °C. PCR reactions were performed in a total volume of 25 µl using a Techne thermocycler, 2.5 µl of DNA template, 2.5 µl of 10 × PCR-buffer, 0.5 µl of dNTPs (each in a 10 mM concentration), 0.5 µl of forward and reverse primers, 0.07 µl of TAQ polymerase (5 units μl^{-1}) and 18.43 µl of sterile distilled water. Primers sequences and cycling programmes were: (i) *wsp*-forward primer 5′-TGGTCCAATAAGTGA TGAAGAAAC-3′ and *wsp*-reverse 5′-AAAAATTAAACG CTACTCCA-3′ (Braig *et al.* 1998), cycling programme: 3 min at 94 °C followed by 40 cycles of 1 min at 94 °C, 1 min at 50 °C and 1 min at 72 °C with 5 min at 72 °C after the last cycle; (ii) TTG 49-forward primer 5′-GTAGTCTGGTTTTCGATT CCCA-3′ and TTG 49-reverse primer 5′-TCCCCGACC TATCGATTTTCC-3′ (R. Stouthamer, unpublished data), cycling programme: 5 min at 94 °C followed by 45 cycles of 1 min at 94 °C, 1 min at 63 °C and 1 min at 72 °C with 5 min at 72 °C after the last cycle; (iii) TAC 47-forward primer 5′-CTACGGCGACAATTGCCAC-3′ and TAC 47-reverse primer 5′-CATCTTGGTCGAACCGAGCAG-3′ (R. Stouthamer, unpublished data), cycling programme: 5 min at 94 °C followed by 30 cycles of 1 min at 94 °C, 1 min at 65 °C and 1 min at 72 °C with 5 min at 72 °C after the last cycle; and (iv) ITS2-forward primer 5′-TGTGAACTGCAGGACACATG-3′ and ITS2-reverse primer 5′-GTCTTGCCTGCTCTGCTC TGAG-3′ (Stouthamer *et al.* 1999*b*), cycling programme: 3 min at 94 °C followed by 33 cycles of 40 s at 94 °C, 45 s at 53 °C and 45 s at 72 °C with 5 min at 72 °C after the last cycle. All PCR products were run on a standard 1.5% agarose gel.

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

Restrictions of the *wsp* genes using the enzymes MboI and MboII were performed to confirm double infection in *T. atopovirilia* and *T. pretiosum* F_1 females after they had shared the same host egg. The sizes of the different digestion products were estimated using the Webcutter v. 2.0 program (Heiman 1997). To perform a restriction of the *wsp* fragments, 10 µl volume (5 µl of PCR product, 1 µl of (10×) reaction buffer, 1 µl of restriction enzyme and 3 µl of distilled water) was used and incubated for 1 h at 37 °C. The digestion products were run on a 1.5% agarose gel. In *T. pretiosum*, the enzyme *Mbo*I generated two cutting sites and three restriction fragments (266, 203 and 131 bp) of the *wsp* fragment and one cutting site and two restriction fragments (397 and 203 bp) in *T. atopovirilia*. With *Mbo*II, the restriction of the *wsp* fragment resulted in one cutting site and two restriction fragments (318 and 282 bp) in *T. pretiosum* and two cutting sites and three restriction fragments (218, 204 and 76 bp) in *T. atopovirilia*. For both enzymes, a restriction of the amplified *wsp* product from DNA template that was a mixture of infected *T. atopovirilia* DNA and infected *T. pretiosum* DNA clearly showed a combination of the restriction patterns of both *wsp* fragments.

(e) *Statistical analysis*

Multiple χ^2-tests with 2×2 contingency tables were used to test whether the efficiency of horizontal transfer of *Wolbachia* and induction of parthenogenesis differed between the multi- and superparasitism combinations.

3. RESULTS

Both intra- and interspecific horizontal transfer of PI *Wolbachia* occurred but inconsistently. Depending on the super- or multiparasitism combination, 0–39% of the females acquired an infection inside the host egg (table 1). When PI was observed in newly infected virgins, these females never produced only daughters, in concordance with the results of an earlier study by Huigens *et al.* (2000). Sequencing the *wsp* fragments confirmed that *Wolbachia* in the newly infected *T. kaykai* and *T. deion* lines originated from the donor lines. However, the horizontal transfer rate may be underestimated in our tests because the bacterial density may occur below the threshold value necessary for detection by PCR with *wsp* primers in some of the recipient F_1 females.

(a) *Intraspecific horizontal transfer of PI* **Wolbachia**

Both of the originally uninfected *T. kaykai* and *T. deion* larvae acquired an infection after sharing a host egg with an infected conspecific. However, subsequent parthenogenesis is manifested more frequently in newly infected *T. kaykai* females than in newly infected *T. deion* females. In *T. kaykai*, 39% of the originally uninfected F_1 females became infected inside the host egg (17 out of 44 tested positive with *wsp*) and 88% of them produced some daughters from their unfertilized eggs. These results are similar to those previously described by Huigens *et al.* (2000). In *T. deion*, 36 superparasitized host eggs resulted in 11 all-female F_1 broods, consisting of 17 females that potentially were newly infected. Twenty-nine per cent of these F_1 virgin females were infected (5 out of 17) and one of them produced a few daughters. Intraspecific horizontal transfer in *T. kaykai* and *T. deion* occurred independently of the order in which the two *Trichogramma* females were allowed to oviposit in a host egg (respectively: $\chi^2_{0.05,1} = 0.021$, $p = 0.886$, $n = 37$ for *T. kaykai*; and $\chi^2_{0.05,1} = 2.21$, $p = 0.137$, $n = 11$ for *T. deion*). We did not detect intraspecific horizontal transfer in *T. atopovirilia*: 47 all-female broods resulted in 80 F_1 females that potentially

Table 1. Inter- and intraspecific horizontal transfer of PI *Wolbachia* in *Trichogramma* sp.
(M, *Mamestra brassicae*; T, *Trichoplusia ni*. Bold entries indicate infection.)

donor species	recipient species	host egg (number)	recipient females tested	horizontal transfer*	PI in newly infected females**
T. kaykai	*T. kaykai*	M (37)	44	39[a]	88[b]
T. deion	*T. deion*	T (11)	17	29[ab]	20[a]
T. atopovirilia	*T. atopovirilia*	M (47)	80	0[c]	0[c]
T. kaykai	*T. deion*	M (21)	26	12[ab]	0[a]
T. kaykai	*T. deion*	T (12)	13	8[ab]	0[a]
T. deion	*T. kaykai*	M (35)	59	19[b]	0[a]
T. deion	*T. kaykai*	T (21)	30	20[ab]	83[b]
T. pretiosum	***T. atopovirilia***	M (30)	95	0[c]	0[c]
T. atopovirilia	***T. pretiosum***	M (30)	120	0[c]	0[c]

* ($p < 0.05$); ** ($p < 0.01$).
[a, b, ab] Within columns, these indicate significance between rows.
[c] Data not statistically analysed.

were newly infected but none of them was infected or produced daughters as virgins (table 1).

(b) *Interspecific horizontal transfer of PI* Wolbachia

Interspecific horizontal transfer occurred from *T. kaykai* to *T. deion* and vice versa. Only newly infected *T. kaykai* females exhibited the parthenogenesis phenotype. In *T. kaykai*, 19% of the F_1 females acquired *Wolbachia* from *T. deion* (17 out of 89) and 29% of them produced at least one daughter. Only 10% (four out of 39) of the *T. deion* F_1 females acquired the *Wolbachia* from *T. kaykai*, but none of newly infected virgins produced daughters. The host egg species, *T. ni* or *M. brassicae*, did not affect the likelihood of an interspecific horizontal transfer or the percentage of F_1 females that acquired an infection inside the host egg: from *T. deion* to *T. kaykai*: $\chi^2_{0.05,1} = 0.36$, $p = 0.436$, $n = 56$; from *T. kaykai* to *T. deion*: $\chi^2_{0.05,1} = 0.25$, $p = 0.614$, $n = 33$. As with the intraspecific transfers involving these two species, interspecific horizontal transfer from *T. kaykai* to *T. deion* occurred independently of the order in which the two *Trichogramma* females were allowed to parasitize the host egg ($\chi^2_{0.05,1} = 1.01$, $p = 0.316$, $n = 33$). However, horizontal transfer occurred significantly more often from *T. deion* to *T. kaykai* when *T. deion* was the first female to oviposit ($\chi^2_{0.05,1} = 12.08$, $p < 0.001$, $n = 56$).

We did not find evidence for double infections in infected F_1 *T. pretiosum* and *T. atopovirilia* females after they shared the same host egg. All 120 *T. pretiosum* females carried only the '*pretiosum Wolbachia*' and all 95 *T. atopovirilia* females carried only the '*atopovirilia Wolbachia*' (table 1).

(c) *Subsequent vertical transmission after interspecific horizontal transfer*

We determined whether vertical transmission of *Wolbachia* and manifestation of parthenogenesis had occurred in subsequent generations in two *T. kaykai* lines. These lines were newly infected with a '*deion Wolbachia*'. In both cases we detected an infection and parthenogenesis in the F_2 generation but not in subsequent generations: the infection seemed to have been lost by the F_3 generation. This was indicated by a decreased sex ratio (percentage of females) and a failure to observe parthenogenesis in the F_3 and subsequent generations. In one line, we tested 15 infected virgins in the F_5 generation females but they produced only sons. In a second line, we tested 10 virgins in the F_4 generation females and 10 virgins in the F_9 generation females: all these virgins produced only sons.

4. DISCUSSION

To our knowledge, our results are the first to show a natural, interspecific horizontal transfer of a PI *Wolbachia*. It explains, in part, the discordance between the *Wolbachia* and *Trichogramma* phylogenies (Schilthuizen & Stouthamer 1997). Both intra- and interspecific transfer seem highly probable in nature, because these wasps are host generalists and multiparasitism has been documented (Pinto 1999). Our study confirms the frequent intraspecific horizontal transfer that has been previously observed in *T. kaykai* (Huigens *et al.* 2000). The interspecific horizontal transfer of PI *Wolbachia* between *T. kaykai* and *T. deion*, which are sympatric species, also seems likely to occur in nature. These two species are found together in a single host egg in the Mojave Desert. At Randsburg Road, Kern County, CA, USA, 3% of the parasitized *Manduca sexta* eggs were multiparasitized by both species (M. E. Huigens, unpublished data).

We certainly do not always find successful horizontal transfer in our experiments. For horizontal transfer to be successful, *Wolbachia* first need to attain a high density in the ovaries of a newly infected female and subsequently be transferred vertically and induce parthenogenesis. Unsuccessful horizontal transfer is most probably caused by an incompatibility between *Wolbachia* and the host's nuclear/cytoplasmic background (Heath *et al.* 1999; Vavre *et al.* 1999). *Wolbachia* might be unable to adapt to a new nuclear background when it confronts different nuclear backgrounds infrequently. Certainly in *T. kaykai*, and most probably in *T. deion*, *Wolbachia* is frequently confronted with a new nuclear background. In *T. kaykai*, 6–26% of the females are infected (Stouthamer *et al.* 2001) and in *T. deion* only two out of 229 broods were the offspring of an infected female. We know that most infected *T. kaykai* females also mate with uninfected males in the

population (Kazmer 1992). *Wolbachia* from populations in which there is frequent gene flow into the infected population are therefore selected to adapt more easily to new nuclear backgrounds. After their horizontal transfer, such PI *Wolbachia* can be transmitted vertically and induce parthenogenesis in a new host whereas PI *Wolbachia* from fixed populations cannot adapt to such a new situation. The fact that both the '*deion Wolbachia'* and the *'kaykai Wolbachia'* can be transmitted to another species supports this idea. The infected *T. atopovirilia* and *T. pretiosum* lines used in our experiments might be from a population fixed for the infection, i.e. all females in the population are infected, explaining why these *Wolbachia* are not easily transmitted horizontally. At least, they do not attain densities detectable with the PCR method we used. In the future, double infections resulting from infected females sharing the same host egg should be tested with *Wolbachia*–host associations where infected and uninfected individuals coexist, e.g. infected *T. kaykai* and infected *T. deion*.

Another explanation for the fact that we observe only interspecific horizontal transfer between *T. kaykai* and *T. deion* might be the host phylogeny. These two North American species are closely related (Schilthuizen & Stouthamer 1997; Pinto 1999) in contrast to *T. pretiosum* and *T. atopovirilia* (Pinto 1999; R. P. de Almeida, unpublished results). The same is the case for the *Wolbachia* in the species. Horizontal transfer of PI *Wolbachia* between more distant host species probably occurs in nature (Schilthuizen & Stouthamer 1997) but it is likely to be a rare event, undetectable in our experiments.

Bacterial density is important for successful horizontal transfer of PI *Wolbachia* (Grenier *et al.* 1998). *Wolbachia* density effects have previously been shown on CI expression in *Nasonia* and *Drosophila* (Breeuwer & Werren 1993; Karr 1994; Bourtzis *et al.* 1996). In this study, newly infected virgin females never produced daughters exclusively. Moreover, they also manifested large variations in their offspring's sex ratios. Both of these factors suggest a bacterial density effect (see also Huigens *et al.* 2000). We only tested for horizontal transfer in virgin recipient F_1 females and therefore indirectly selected for the presence of a high bacterial density. If parthenogenesis does not occur in newly infected virgin females because of insufficient bacterial densities then *Wolbachia* will not be transmitted to subsequent generations. Thus, in future work we should test mated recipient F_1 females. This would result in fertilized eggs with a relatively low *Wolbachia* titre to become females and allow the *Wolbachia* to attain a sufficient titre to be transmitted and expressed in subsequent generations.

The question remains, however, why the '*kaykai Wolbachia*' are more easily transmitted intraspecifically than those of *T. deion*. One possible explanation may be that *T. kaykai* may parasitize larger host eggs in the field than *T. deion*. During the period of the year that we can find *T. kaykai* in the field, they are found on eggs of *Apodemia mormo*, from which three to five *T. kaykai* individuals can emerge. *Trichogramma deion* are known to parasitize many species of host eggs. If, in general, these are smaller hosts they will receive fewer wasp eggs, which may result in fewer possibilities for horizontal transfer.

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

In conclusion, intra- and interspecific horizontal transfers of *Wolbachia* should occur in nature between organisms that interact in close confinement. We may have underestimated: (i) the rate of horizontal transfer of PI *Wolbachia* in *Trichogramma* owing to our PCR detection method; and (ii) the subsequent vertical transmission because we detected PI only in the cases where bacterial density is high in newly infected virgins. Subsequent vertical transmission probably limits the successful spread of the newly acquired infections (Rigaud 1997; Cook & Butcher 1999; Heath *et al.* 1999; Pintureau *et al.* 2000*b*), which is also indicated by the loss of infection in two *T. kaykai* lines newly infected with a '*deion Wolbachia'*. Such unsuccessful horizontal transfer is probably caused by incompatibilities between *Wolbachia* and the host's nuclear/cytoplasmic background. Such incompatibilities clearly must exist in nature; otherwise, experimentally derived horizontal transfers would be easily obtained. Research has shown that this is generally not the case. Although subsequent vertical transmission after horizontal transfer may occur at very low rates (almost undetectable in laboratory experiments), given an evolutionary timescale, it becomes frequent enough to explain the discordance between the *Wolbachia*- and host phylogenies.

We thank Fabrice Vavre, Gilsang Jeong and the Entomology PhD discussion group in Wageningen for discussions and ideas. Joop van Lenteren and Nina Fatouros provided useful comments on an earlier version of the manuscript. We thank Patrick Verbaarschot for his assistance with the molecular work and Leo Koopman, André Gidding and Frans van Aggelen for culturing some of the wasp lines. We are also grateful to Americo I. Ciociola Jr for sending *T. atopovirilia* and *T. pretiosum* lines to us. We are very grateful to Earth and Life Sciences Foundation (ALW) (M.E.H. and R.S.), Embrapa (Brazilian Agricultural Research Corporation) PRODETAB (R.P.d.A.) and the graduate school of Production Ecology and Resource Conservation of The Netherlands (M.E.H. and R.P.d.A.) who supported this work.

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