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Infection polymorphism and cytoplasmic incompatibility in Hymenoptera-*Wolbachia* associations

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Most cases of *Wolbachia* infection so far documented in haplodiploid Hymenoptera are associated with parthenogenesis induction. Only three examples of *Wolbachia*-mediated cytoplasmic incompatibility (CI) have been reported, resulting either in haploidisation of fertilised eggs, which develop into viable males, or in their death. To better document this variability, we studied two new *Wolbachia*-wasp associations involving *Drosophila* parasitoids. In *Trichopria* cf. *drosophilae*, individuals are infected by two different *Wolbachia* variants, populations are nearly totally infected, and *Wolbachia* induces incomplete CI resulting in death of the fertilised eggs. On the other hand, *Pachycrepoideus dubius* harbours only one bacterial variant, populations are polymorphic for infection, and *Wolbachia* has no detectable effect. These two cases show that the range of variation in *Wolbachia*'s effects in Hymenoptera is as wide as in diploids, extending from complete CI to an undetectable effect. Cases so far studied show some parallel between the strength of incompatibility, the number of *Wolbachia* variants infecting each wasp, and the natural infection frequency. These empirical data support theoretical models predicting evolution of CI towards lower levels, resulting in the decline and ultimate loss of infection, and place multiple infections as being an important factor in the evolution of host-*Wolbachia* associations.
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Introduction

The endosymbiont *Wolbachia* is a widespread maternally-inherited bacterium found in numerous Arthropods (Werren and O'Neill, 1997) and also in Nematodes (Bandi *et al*, 1998). Its most common effects are feminisation of genetic males in isopods (Rigaud, 1997), parthenogenesis induction (PI) in haplodiploid Hymenoptera and one thrip (Stouthamer, 1997; Arakaki *et al*, 2001), male killing (Hurst *et al*, 1999; Fialho and Stevens, 2000) and cytoplasmic incompatibility (CI) in many species (Hoffmann and Turelli, 1997). The latter effect occurs in crosses between infected males and uninfected females (unidirectional incompatibility) or between males and females harbouring different *Wolbachia* variants (bidirectional incompatibility) (Hoffmann and Turelli, 1997). *Wolbachia* can also increase fecundity in *Trichogramma* (Girin and Boulétreau, 1995; Vavre *et al*, 1999b) or be obligatory for egg production in *Asobara tabida* (Dedeine *et al*, 2001). All these effects put infected individuals at a reproductive advantage over uninfected ones, and allow the spread of the infection in the host population.

Theoretical models of CI-*Wolbachia* predict that the frequency of infection within a population should correspond to a balance between the bacterial transmission efficiency, the CI intensity, and the cost suffered by infected individuals (review in Hoffmann and Turelli, 1997; Vavre *et al*, 2000). The actual infection cost is sometimes clear, but most often the cost is low or undetectable (Poinsot and Merçot, 1997; Stouthamer *et al*, 1999; Fleury *et al*, 2000); transmission rate ranges from 90 to 100% (Werren, 1997); and CI level shows high range of variation (Hoffmann and Turelli, 1997). Some parallel between the diversity of CI phenotypes and the natural infection frequency is thus expected.

Hymenoptera are of special interest for studying such relationships, since their haplodiploid reproduction allows variation in CI effects. In these species, haploid eggs develop into males, and incompatible eggs either die or develop into males if CI restores complete haploidy, with all possible intermediate cases (Vavre *et al*, 2001). Indeed, CI probably involves modification of male chromosomes by *Wolbachia* (*mod* function), such that when sperm from infected males fertilise oöcytes that are either uninfected, or infected by another bacterial variant, paternal chromosomes become improperly condensed and are lost (incompatible cross). If such sperm fertilise oöcytes bearing the same bacterial variant as their own, modified paternal chromosomes are rescued (*resc* function) and the cross is compatible (Breeuwer and Werren, 1993). In the pteromalid *Nasonia*, complete destruction of male chromosomes leads incompatible fertilised eggs to revert to haploidy and to develop into normal males (Breeuwer and Werren, 1990). No extra-mortality occurs and incompatible crosses produce all-male offspring without reduction in number. This CI type will be referred to as the 'Male Development' CI type, or MD type. In the *Drosophila* parasitoid *Leptopilina hetero-*

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toma (Vavre *et al*, 2000), all incompatible fertilised eggs fail to develop and incompatible crosses produce reduced offspring consisting only in males issued from unfertilised eggs ('female mortality' CI type). This FM CI type is similar to that of the haplodiploid mite *Tetranychus*, where some of the incompatible fertilised eggs fail to develop (Breeuwer, 1997). In *Cotesia sesamiae*, *Wolbachia* also induces CI, probably of the MD type, even though some mortality can also be suspected (Ngi-Song *et al*, 1998).

Despite different CI phenotypes being expected in Hymenoptera, few data are available to estimate the extent of this diversity. The present study investigates the effect of *Wolbachia* in two haplodiploid, non-thelytokous *Drosophila* parasitoids: the diapriid *Trichopria* cf. *drosophilae* and the pteromalid *Pachycrepoideus dubius* (Vavre *et al*, 1999a). The results extend the diversity of *Wolbachia*-induced cytoplasmic incompatibility in Hymenoptera, and show parallel variation in the number of *Wolbachia* variants, level of incompatibility, and natural infection frequency.

Materials and methods

Strains

Trichopria (Hymenoptera: Diapriidae) females were collected in several places in south-eastern France, where they are uncommon. They belong to a species close to *T. drosophilae* (D Notton, Museum of Reading, UK, personal communication), and will be referred to as *T.* cf. *drosophilae*. This species develops as a solitary pupal endoparasitoid of various *Drosophila* species (Carton *et al*, 1986). It is infected by two *Wolbachia* variants (Vavre *et al*, 1999a) which differ from that described by Werren *et al* (1995) and Van Meer *et al* (1999) in *T. drosophilae*.

Crosses involved a naturally infected strain (Tw line) originating from Pierrefeu (French Riviera), and an uninfected one (To) derived from the former using rifampicin treatment following Vavre *et al* (2000). This cured strain proved stable and no restoration of infection has been observed after 20 generations. Experimental crosses were performed six generations after the end of antibiotic treatment to avoid any direct effect of antibiotics.

Pachycrepoideus dubius (Hymenoptera: Pteromalidae) can develop in pupae of numerous Diptera including *Drosophila* spp. where it behaves as a solitary ectoparasitoid (Carton *et al*, 1986). It is infected by a single *Wolbachia* variant closely related to that inducing thelytoky in *Muscidifurax uniraptor* (Vavre *et al*, 1999a). Infected (Pw) and uninfected (Po) lines were isolated from a single naturally polymorphic population originating from Lirac (southern France). Field-trapped females were used to establish isofemale lines and were checked for infection. Twenty infected and 20 uninfected lines were kept for five generations (five females at each generation), then tested again for infection, and finally pooled to establish two strains derived either from naturally infected, or uninfected wasps. The mixing of 20 different lines prevented genetic drift during early rearing generations.

In the laboratory, all parasitoids develop on a naturally *Wolbachia*-free *Drosophila melanogaster* strain originating from Lyon. Rearing and experiments were performed at 25°C, L.D. 12:12 and 70% R.H. In all experiments, *Drosophila* larvae were fed a standard diet (David and Clavel, 1965), adult wasps were fed honey.

Wolbachia detection

DNA extraction and PCR reaction were carried out according to Vavre *et al* (2000). Two sets of primers were used for PCR amplification: one specific for the *Wolbachia FtsZ* gene, and one specific for the insect ITS2 region to eliminate any false negative result due to amplification failure.

Crosses

In both *T.* cf. *drosophilae* and *P. dubius*, effects of *Wolbachia* were tested using all four possible crosses between infected and uninfected strains. For each cross, 15 pairs (48-h-old males and 12-h-old females) were isolated in Petri dishes and their behaviour observed to determine if they mated. After mating, females were transferred to Petri dishes containing either 60 (for *Trichopria*) or 30 (for *Pachycrepoideus*) *Drosophila* pupae, with the number differing considering the respective biology and daily fecundity of wasp species. For 3 days, females were daily transferred to Petri dishes with new hosts, and 10 control Petri dishes were kept unparasitised to estimate the natural mortality of the flies. Adult *Drosophila* and wasps of both sexes emerging from each dish were counted and used to estimate three parameters (defined in Vavre *et al*, 2000).

(1) Sex ratio (SR) is the proportion of males among adult offspring of each pair. SR is important to detect CI since both the male development and the female mortality CI types induce male bias in sex ratio, whereas the absolute number of males and females allow us to distinguish between the two CI types.

(2) Death by parasitism (DP) is the difference between the number of flies emerged from unparasitised and parasitised dishes, and measures the number of *Drosophila* killed by wasps. Death can be due either to the oviposition itself, or to the parasitic development of the wasp. Assuming that no host pupa recovers from parasitism (Carton *et al*, 1986), DP estimates the number of parasitised hosts. For each female, DP was summed over the 3 days.

(3) Parasite success (PS) is the ratio of emerging wasps to parasitised hosts as estimated by DP. PS is calculated for each female.

At the end of experiments, all individuals used in crosses were checked for infection. All proved infected in Tw and Pw strains, and uninfected in To and Po.

Results

Infection status and *Wolbachia* effects in *Trichopria* cf *drosophilae*

Out of 74 field-collected *Trichopria* females (five populations), 70 proved infected (Table 1) demonstrating a high natural infection rate in this species (94.5%). This is consistent with the complete infection of three laboratory strains established from French populations (25 individuals checked in each strain). However, it does demonstrate that some uninfected wasps are present in the wild.

Control crosses (Tw (infected) female × Tw male and To (uninfected) female × To male) produce offspring with identical sex ratios (Table 2), demonstrating that *Wolbachia* does not affect sex determination. Moreover, the offspring number is the same in both crosses, suggesting that *Wolbachia* does not affect the fertility of infected females, and has a low physiological cost.

Table 1 Infection status of field-collected individuals of *Trichopria* cf. *drosophilae* and *Pachycrepoideus dubius* (infected/checked individuals)

Collection site (department)	*T.* cf. *drosophilae*	*P. dubius*
Villette/Vienne (38)	–	3/6
Péage de Roussillon (38)	–	2/12
Vénérieu (38)	15/15	–
Istres (13)	5/5	6/6
Lirac (30)	16/16	12/39
Aniane (34)	7/8	10/31
Avignon (84)	27/30	–
Total	70/74	33/94

In contrast, results of reciprocal crosses between infected and uninfected individuals depart strongly from each other (Table 2). While the cross between uninfected males and infected females does not differ from infected or uninfected control crosses, the reciprocal cross between infected males and uninfected females gives a highly male-biased sex ratio (87% males *vs* 52% in control), indicating CI. Death by parasitism (DP) is the same in the four crosses (Table 2), indicating that all females parasitised an equal number of hosts, whereas parasite success (PS) and total offspring production are far lower in the incompatible cross (Table 2), proving high mortality due to CI. The number of males is the same in the four crosses (Table 2), and thus the highly male-biased sex ratio (SR) in incompatible crosses is due to mortality among females (about 25 are lacking). Reduction in PS (about 35%) precisely corresponds to this decrease in female number. Altogether these data demonstrate that *Wolbachia* induces a female mortality CI type in *T.* cf. *drosophilae*, and from the difference between female numbers in compatible and incompatible crosses, mortality among fertilised eggs can be estimated to be about 82%.

All females issuing from incompatible crosses (76) proved *Wolbachia*-free. Therefore, their normal development resulted from escaping CI, and not from spontaneous restoration of infection. CI is incomplete, and about 18% of fertilised eggs develop normally.

Infection status and *Wolbachia* effects in *Pachycrepoideus dubius*

Most *P. dubius* populations are polymorphic for infection (Table 1), but no valid comparison can be drawn among populations due to the low number of individuals collected. Over all populations, infection frequency is only 35%, contrasting with the almost complete infection in *T.* cf. *drosophilae* and *L. heterotoma* (Vavre *et al*, 2000).

The effect of *Wolbachia* in *P. dubius* was studied using the same protocol as in *T.* cf. *drosophilae*. Neither death by parasitism (DP), nor parasite success (PS), nor total offspring production show significant variation between crosses (Table 3). Thus it seems that *Wolbachia* has no effect and is not able to induce CI in *P. dubius*. However, a slight difference appears in sex ratio ($P = 0.06$), due to a lower female offspring number in the cross between Po females and Pw males, while male production is unchanged. Together with a non significant decrease in DP, PS and total offspring in the 'incompatible' cross, this suggests some weak CI of the FM type.

Finally, we can conclude that *Wolbachia* has almost no effect on the reproduction of *P. dubius*, even if a very low CI of the FM type can be suspected.

Discussion

Up to now, most descriptions of *Wolbachia* effect in haplodiploid Hymenoptera have involved parthenogenesis induction, with only three cases of CI, resulting in various phenotypes, being described (Breeuwer and Werren, 1990; Ngi-Song *et al*, 1998; Vavre *et al*, 2000). The scarcity of reported CI cases is likely to be due partly to some sampling bias, since PI is far easier to detect than CI, as was pointed out by Cook and Butcher (1999). We describe here two other situations where *Wolbachia* either induces partial CI of the female mortality (FM) type, in *T.* cf *drosophilae,* or has almost no effect, in *P. dubius*. It is likely that many more examples of non-parthenogenetic *Wolbachia* will be forthcoming, and further studies, especially in arrhenotokous species, will help us to clearly appreciate the real distribution of *Wolbachia* effects in this group, and to better assess the variability of effects other than parthenogenesis induction.

The current hypothesis accounting for the difference between the male development (MD) and the female mortality (FM) CI types puts forward the destruction of paternal chromosomes, which would be complete in the MD type, thus restoring haploidy and allowing male development, or incomplete in the FM type, resulting in aneuploidy and the death of embryos (Breeuwer, 1997; Vavre *et al*, 2000). CI thus appears to be more severe in the MD type than in the FM type. This could be due to a weaker *mod* function in the FM type, achieved either through a lower bacterial density, or through a reduction

Table 2 Results of crosses between infected (Tw) and uninfected (To) individuals (female × male) in *Trichopria* cf. *drosophilae* (mean and SD)

	To × *To* (*n* = 15)	*Tw* × *Tw* (*n* = 15)	*Tw* × *To* (*n* = 15)	*To* × *Tw* (*n* = 15)	$F_{3,56}$	*P*
DP	70.80 (8.03)[a]	68.73 (8.37)[a]	66.80 (7.80)[a]	70.63 (7.35)[a]	0.83	0.48
PS (%)	0.92 (0.08)[a]	0.92 (0.09)[a]	0.94 (0.06)[a]	0.57 (0.14)[b]	30.68	$<10^{-4}$
Offspring	65.73 (6.62)[a]	63.20 (8.18)[a]	62.87 (6.44)[a]	39.93 (8.63)[b]	36.85	$<10^{-4}$
Males	33.80 (9.51)[a]	29.40 (10.19)[a]	32.73 (9.23)[a]	34.86 (8.80)[a]	0.92	0.44
Females	31.93 (11.21)[a]	33.80 (12.31)[a]	30.13 (10.75)[a]	5.07 (5.17)[b]	24.53	$<10^{-4}$
SR (%)	0.52 (0.15)[a]	0.47 (0.16)[a]	0.52 (0.15)[a]	0.87 (0.12)[b]	26.94	$<10^{-4}$

DP = death by parasitism; PS = parasite success; SR = offspring sex-ratio (% males). One-way analysis of variance done on arcsin ($\sqrt{P}$) for PS and SR. Means marked with the same letter within a row are not significantly different ($p = 0.05$) by a least significant difference test.

Table 3 Results of crosses between infected (Pw) and uninfected (Po) individuals (female × male) in *Pachycrepoideus dubius* (mean and SD)

	Po × *Po* (*n* = 13)	*Pw* × *Pw* (*n* = 15)	*Pw* × *Po* (*n* = 14)	*Po* × *Pw* (*n* = 14)	$F_{3,52}$	*P*
DP	58.22 (3.38)[a]	57.24 (5.46)[a]	55.91 (8.32)[a]	54.41 (5.06)[a]	1.09	0.36
PS (%)	0.93 (0.06)[a]	0.97 (0.04)[a]	0.96 (0.05)[a]	0.94 (0.06)[a]	1.31	0.28
Offspring	54.77 (6.04)[a]	57.33 (6.71)[a]	55.50 (10.31)[a]	52.57 (6.80)[a]	0.95	0.42
Males	8.08 (2.06)[a]	7.00 (2.59)[a]	8.00 (2.57)[a]	8.29 (2.02)[a]	0.88	0.46
Females	46.69 (5.35)[a,b]	50.33 (5.97)[a]	47.5 (8.78)[a,b]	44.29 (5.97)[b]	2.03	0.12
SR (%)	0.147 (0.037)[a,b]	0.121 (0.041)[a]	0.143 (0.035)[a,b]	0.158 (0.032)[b]	2.63	0.06

DP = death by parasitism; PS = parasite success; SR = offspring sex-ratio (% males). One-way analysis of variance done on arcsin ($\sqrt{P}$) for PS and SR. Means marked with the same letter within a row are not significantly different ($p = 0.05$) by a least significant difference test.

in the *mod* function itself. This is consistent with the high frequency of partial CI in the FM type (three out of four cases, Breeuwer, 1997; Vavre *et al*, 2000; this study). Thus, variation in bacterial density could be responsible not only for variation in CI levels (Boyle *et al*, 1993; Breeuwer and Werren, 1993; Sinkins *et al*, 1995), but also for difference between the MD and FM CI types in Hymenoptera. Clearly, cytogenetic studies of early developmental events, in correlation with *Wolbachia* density, are needed to assess the basis of CI-*Wolbachia* diversity in Hymenoptera.

With respect to *Wolbachia* effects, comparison of the two species here studied with *L. heterotoma* is of special interest. The three species display cytoplasmic incompatibility of the FM type, but while in *L. heterotoma* all incompatible eggs fail to develop (Vavre *et al*, 2000), only 80% die in *Trichopria*, and none (or very few) in *Pachycrepoideus*. Considering the natural infection frequency in the three species, these observations closely fit theoretical predictions on the relationship between the intensity of CI and the natural infection frequency: no *Wolbachia*-free females can be found in *L. heteroroma* (Vavre *et al*, 2000), a few in *Trichopria*, and many in *Pachycrepoideus*.

Theoretical and empirical studies have predicted that evolution of CI could lead to a loss of infection in host-*Wolbachia* associations (Hurst and Mac Vean, 1996; Vavre, 2000; Werren and Windsor, 2000), provided *Wolbachia* reduces or loses its ability to manipulate host reproduction. The species here studied could thus reflect different evolutionary states of host-*Wolbachia* association.

In this evolutionary framework, the parallel between the multiple infections here observed (triple in *L. heterotoma*, double in *T.* cf *drosophilae*, single in *P. dubius*; Vavre *et al*, 1999a) and CI level could be explained by competition among *Wolbachia* variants within a host, that could lead to higher *Wolbachia* density (Van Baalen and Sabelis, 1995; Frank, 1996), and to selection towards higher CI level (Frank, 1998). It thus appears that multiple infection could be associated with stronger effects, making an association more stable, and reducing the risk of the loss of infection.

A similar pattern and scenario have been proposed in diploid *D. simulans* (Hoffmann *et al*, 1996; Bourtzis *et al*, 1998; Merçot and Poinsot, 1998; James and Ballard, 2000) and *D. mauritiana* (Giordano *et al*, 1995). It is true that, in the associations described here, both host and *Wolbachia* genotypes vary, and this makes it difficult to identify which partner is responsible for the CI difference. However, *L. heteroroma*, *Trichopria* and *Pachycrepoideus* could illustrate possible evolutionary steps of the CI effects of *Wolbachia*, showing a decrease in CI level, and the association of very weak CI with a low natural infection frequency, perhaps preceding infection loss.

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