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Wolbachia affects oviposition and mating behaviour of its spider mite host

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Introduction

Wolbachia are bacterial endosymbionts of several insect, mite, isopod and nematode species (for a review, see Stouthamer et al., 1999). Wolbachia are transmitted vertically: infected mothers pass these bacteria to their offspring through the cytoplasm of the egg (Stouthamer et al., 1999). These bacteria can induce a reproductive alteration of their hosts termed cytoplasmic incompatibility (CI). In CI, uninfected (U) females mated to males infected with Wolbachia bacteria produce unviable offspring, whereas the reciprocal cross is fully compatible. Theoretical analysis shows that CI confers an advantage to Wolbachia in infected (W) females below that of W-females (Hofmann et al., 1990) and, consequently, the proportion of hosts infected increases. Wolbachia in males act spitefully: they are not transmitted to offspring themselves but indirectly benefit related Wolbachia in females (Hurst, 1991). However, CI is a mechanism that benefits the bacteria but, apparently, not the host. The host could benefit from avoiding incompatible mates. Parasite load and disease resistance are known to be involved in mate choice. Can Wolbachia also be implicated in reproductive behaviour? We used the two-spotted spider mite – Wolbachia symbiosis to address this question. Our results suggest that uninfected females preferentially mate to uninfected males while infected females aggregate their offspring, thereby promoting sib mating. Our data agrees with other results that hosts of Wolbachia do not necessarily behave as innocent bystanders – host mechanisms that avoid CI can evolve.

Keywords:
assortative mating; cytoplasmic incompatibility; reproductive behaviour; spider mite; vertically transmitted symbiont; Wolbachia.

Abstract

Wolbachia bacteria are transmitted from mother to offspring via the cytoplasm of the egg. When mated to males infected with Wolbachia bacteria, uninfected females produce unviable offspring, a phenomenon called cytoplasmic incompatibility (CI). Current theory predicts that ‘sterilization’ of uninfected females by infected males confers a fitness advantage to Wolbachia in infected females. When the infection is above a threshold frequency in a panmictic population, CI reduces the fitness of uninfected females below that of infected females and, consequently, the proportion of infected hosts increases. CI is a mechanism that benefits the bacteria but, apparently, not the host. The host could benefit from avoiding incompatible mates. Parasite load and disease resistance are known to be involved in mate choice. Can Wolbachia also be implicated in reproductive behaviour? We used the two-spotted spider mite – Wolbachia symbiosis to address this question. Our results suggest that uninfected females preferentially mate to uninfected males while infected females aggregate their offspring, thereby promoting sib mating. Our data agrees with other results that hosts of Wolbachia do not necessarily behave as innocent bystanders – host mechanisms that avoid CI can evolve.

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mate with the first. If, in a population host to a Wolbachia infection, assortative mating is to evolve two important prerequisites are. (i) that natural populations infected with CI-W can be polymorphic for the infection and (ii) that this polymorphic state lasts for a sufficient amount of time. We will examine each in turn.

The only epidemic by CI-W documented describes the spread of a Wolbachia infection in Drosophila simulans in CA, USA (Turelli & Hoffmann, 1991). The infection spread (or is spreading) rapidly but after 6 years all populations sampled were polymorphic (Turelli & Hoffmann, 1991). Studies of such nature are lacking for other species known to be infected with CI-W. However, a few papers report estimates of infection frequencies in natural host-populations based on one-instance sampling events. We have summarized the results of these studies in Table 1. Half of the host species that have been sampled are polymorphic for the infection at the among-population level. More importantly, within-population polymorphism is also common. In fact, excluding the field survey of populations of D. recens and of Callosobruchus chinensis, where infection was assessed after keeping stocks in the laboratory for some generations, only one species, Leptopilina heterotoma, seems to be completely infected.

It is thus clear that polymorphic population states do occur, but is polymorphism a transient state? Theoretical models show that, when imperfect transmission of the symbiont is assumed, the infection can spread until a stable polymorphic population state is reached (Fine, 1978; Hoffmann et al., 1990; Turelli, 1994). Under such conditions, a host trait that suppresses the effects of CI will be positively selected (Turelli, 1994; Vala et al., 2002), reinforcing the stable polymorphism. Documented factors causing less than perfect maternal transmission efficiency include, exposure to high temperatures (e.g. Hoffmann et al., 1986; Van Opijnen & Breeuwer, 1999), malnutrition or mass rearing (e.g. Clancy & Hoffmann, 1998; Hoffmann et al., 1998) and diapause (Perrot-Minnot et al., 1996). Environmental curing because of natural occurrence of antibiotics has also been suggested (Stevens & Wicklow, 1992; Stouthamer et al., 1999). Depending on characteristics of the natural history of the host species, one or more of these factors may be common. Hence, polymorphism regarding infection by CI Wolbachia can be a permanent, rather than transient, population state.

The herbivorous two-spotted spider mite, Tetranychus urticae Koch, exhibits all the characteristics documented to decrease maternal transmission efficiency of Wolbachia. Colonies are founded by dispersing adult females that settle on uninfested plants to oviposit. Colonies grow until the plant is overexploited at which time females disperse again. Consequently, immature stages developing at the end of the colony lifetime experience crowding because of large population densities and malnutrition because of plant over-exploitation. Field populations of the two-spotted spider mite will occasionally be exposed to temperatures above 30 °C in the summer, whereas greenhouse populations of the species experience these temperatures more regularly. Finally, adult females of the species over-winter in the soil in diapauser form. Perhaps not surprisingly, therefore, a survey of natural coastal populations of T. urticae in The Netherlands showed that, at least at the among-population level, the species is polymorphic (see last row of Table 1).

The life history of T. urticae potentially allows for assortative mating with respect to infection type (infected/uninfected). Populations of T. urticae are patchily distributed over their host plants. Larvae and immature individuals have limited mobility and do not move further than a few millimeters from their hatching place unless food conditions are poor (McEnroe, 1969; Mitchell, 1973). Populations of this mite exhibit local

### Table 1 Published surveys on natural populations of host species infected by CI-Wolbachia. $n_p$, number of populations sampled; $n_{i}$, number of individuals sampled per population; NA: North America; Au Australia; Ja Japan; Eu Europe; NL The Netherlands. *Levels of infection that may be inaccurate as lines were kept in the laboratory for some generations before infection assessment (PCR).

<table>
<thead>
<tr>
<th>Host species</th>
<th>Populations infected ($n_p$, location)</th>
<th>Infection % within populations ($\bar{n}<em>{i, \text{min}} - \bar{n}</em>{i, \text{max}}$)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Callosobruchus chinensis</td>
<td>All (6, Ja)</td>
<td>100* (21-66)</td>
<td>Kondo et al. (1999)</td>
</tr>
<tr>
<td>Diabrotica vergifera vergifera</td>
<td>Not all (15, NA)</td>
<td>not reported (4–7)</td>
<td>Giordano et al. (1997)</td>
</tr>
<tr>
<td>Drosophila melanogaster</td>
<td>Not all (12, Au)</td>
<td>Polymorphic, 18–85 ( &gt;14)</td>
<td>Hoffmann et al. (1994)</td>
</tr>
<tr>
<td>D. recens</td>
<td>All (5, USA)</td>
<td>100* (2–3)</td>
<td>Werren &amp; Jaenike (1995)</td>
</tr>
<tr>
<td>Laodelphax striatellus</td>
<td>Not all (9, Ja)</td>
<td>5 pops. 100; 3 pops. polymorphic, 8–93 (3–30)</td>
<td>Hoshizaki &amp; Shimada (1996)</td>
</tr>
<tr>
<td>Leptopilina heterotoma</td>
<td>All (8, Eu)</td>
<td>100 (4–39)</td>
<td>Vavre et al. (2000)</td>
</tr>
<tr>
<td>Tetranychus urticae</td>
<td>Not all (48, NL)</td>
<td>not reported (PCR on pooled individuals)</td>
<td>Breeuwer &amp; Jacobs (1996)</td>
</tr>
</tbody>
</table>

Note: Studies reporting on infection frequencies for species where induction of CI has not been clearly established were not included, nor were infection reports from populations kept in the laboratory for more than 1 year. The latter is justified because polymorphism is unlikely to be maintained under laboratory conditions, as follows. Transmission efficiency may be higher in the laboratory than in the field (Hoffmann et al., 1990), thus CI infections may rapidly become fixed if initially present at frequencies close to, or higher than, the threshold. On the other hand, founder effects may lead to infection exclusion in the lab if field frequencies are under represented in the sample.
mating structure. Females in the last moulting stage are guarded by males and mate immediately after emergence – thus, dispersing females are usually mated. Males are haploid, and develop from unfertilized eggs, and females are diploid and develop from fertilized eggs. Males mate multiply and females may mate again and use sperm from later mates (Cone, 1985). Assortative mating may become manifest in this species as either a preference of males for females or of females for males – females may refuse males (or refuse their sperm – although there is presently no evidence for this). As mating occurs locally, ovipositing females may promote assortative mating of their offspring by choosing to lay eggs in isolated clutches, and/or place them in clutches of eggs from females of the same infection type.

Here, we provide laboratory tests of behavioural traits that are likely to influence panmixia of two-spotted spider mite populations polymorphic for infection by CI-Wolbachia. Specifically, we studied oviposition site selection by Wolbachia-infected and uninfected females, and mate preference of uninfected and infected male and female spider mites. To minimize the possibility that females or males choose for traits other than the presence/absence of Wolbachia two isofemale lines were used. These lines were highly inbred, prior to removal of Wolbachia by heat shock treatment (Van Opijnen & Breeuwer, 1999) and establishment of the uninfected sub-lines.

**Material and methods**

**Mite strains**

Strains of two-spotted spider mites, *T. urticae*, were reared on bean leaves (*Phaseolus vulgaris*) at 22 °C (Breeuwer & Jacobs, 1996). Two strains of mites were used, one collected from Rose (R) and another from Cucumber (C) (Vala et al., 2000). Wolbachia infected R-males are reproductively incompatible with uninfected females (CI) whereas all other crosses are compatible. Infected C- and uninfected mites are compatible. Infected C- and R-females harbour Wolbachia whose wsp and *fts-Z* sequences are identical (Vala et al., 2002).

Isofemale lines from the C and R strains were produced by four consecutive generations of mother × son mating, which gives an expected inbreeding coefficient of 0.98 (Hartl, 1980). In this study two pairs of lines were used (R1 infected and uninfected, and C5 infected and uninfected). For each isofemale line a sub-line was cured by culturing at 32 °C (Van Opijnen & Breeuwer, 1999) for 8–9 generations. Then the lines were transferred back to 22 °C for at least four generations before the experiments (Vala et al., 2002). Previous experiments have shown that curing of a Wolbachia infected inbred isofemale line by antibiotic and heat-treatment produced two uninfected sub-lines which could not be distinguished based on several fitness traits (Vala et al., 2002) – this result indicates that neither procedure yields treatment-specific side-effects. Curing was successful, as assessed by PCR amplification of the *fts-Z* Wolbachia gene (for experimental details see Breeuwer, 1997; Vala et al., 2002). Cytological analysis of infected eggs after the experiments revealed only one morph type of bacteria (Vala, pers. obs.). Hence, it is assumed that the infected and uninfected sub-lines of each isofemale line are genetically similar at the nuclear level, and differ predominantly due to presence/absence of Wolbachia in the cell cytoplasm.

**Oviposition-site selection tests**

Because immature stages of spider mites have reduced motility, and adult females usually mate before dispersal, individuals mate in the vicinity of the site where egg-hatch occurs. Therefore if eggs are not homogeneously distributed in space with respect to infection by Wolbachia, this will likely reduce the occurrence of incompatible matings (i.e. mating between uninfected females and infected males). In this experiment we tested whether young (3-4 days after emergence from last moulting stage) infected and uninfected R1-females have a preference for specific oviposition sites and whether their preference differs with respect to infection. In particular, we asked whether they would prefer to add their eggs to an existing infected clutch, to an existing uninfected clutch, start their own egg-clutch elsewhere, or any combination of these.

Two types of experiments were performed simultaneously: R1-females were offered leaf discs with infected and uninfected egg clutches from R1; or R1-females were offered egg clutches from infected and uninfected C5 females. In the first case, when clutches on leaves are by R1-females, test females have a nuclear genome identical to the eggs in both patches on the leaf, and a cytoplasmic genome (presence/absence of Wolbachia) identical to one of the patches. In the second case, when clutches on leaves are by C5 females, only the cytoplasmic genome of the female tested will be identical to one of the two egg clutches present on the leaf. This set of experiment tests whether presence/absence of Wolbachia bacteria alone suffices to elicit a different response in R1-females.

To create infected and uninfected egg clutches, one infected and one uninfected female of the same isofemale line were placed on bean leaf discs (Ø 3 cm) on water-soaked cotton wool. Each female was confined to a small area (approximately 0.5 cm in diameter) using wet cotton wool as a barrier. The two areas were opposite one another at the edges of the leaf discs and placed in a line parallel to but 0.5 cm away from the mid axis. Females and cotton wool barriers were removed after 24 h. The number of eggs laid by each female was
counted and their position was mapped. Clutches had on average six eggs. Next, the test female was introduced on the leaf disc to lay eggs for 24 h. Location and number of newly laid eggs were identified using the ‘egg-map’. Eggs laid by experimental females were assigned to one, the other, or a separate, new, clutch. Because females lay eggs where they feed, the area of feeding scars defines each clutch. The clutch of the newcomer was considered separate when the feeding scars she produced did not overlap with existing scars. Typically, ‘separate clutches’ were located 1–1.5 cm from existing scars.

The statistical analysis, in this and further experiments, is based on Zar (1999) and Siegel & Castellan (2000). Heterogeneity (two-tailed) G-tests were used to test for differences between the proportion of W and U-females doing one of three possibilities: laying their eggs in an infected or an uninfected existing patch, or start a new patch elsewhere, in presence of C5 and R1 patches. If this test was significant ($P < 0.05$), we concluded that females behaved differently in the two situations, whereas if the test was not significant we concluded that they did not (Zar, 1999). The analysis proceeded by analysis of a 3 × 2 contingency table (rows: W-female, U-female; columns: lay eggs to W-patch, lay eggs to U-patch, new patch). If this test was significant subsets of the table were analysed to look for specific differences in allocation of eggs to infected vs. uninfected egg patches or in allocation of eggs to existing patches vs. new patches (by summing up of the females that laid eggs in W-patches and in U-patches). This procedure is legitimate: as many subsets of a contingency table as the number of degrees of freedom may be analysed following a significant G-test (Siegel & Castellan, 2000, p. 194).

Male and female mate-choice tests

The goal of this experiment was to directly test mate preference of infected and uninfected R1-males and females. Young males were individually given a choice between guarding infected or uninfected R-females in the last moulting stage. Females in the last moul were placed opposite one another, on the mid axis of the leaf disc, each at 0.5 cm from the edge of a bean leaf disc (Ø 1.5 cm). Leaf discs were checked 2 h later and guarding was scored when the male was on top of a moulting female (Potter et al., 1976).

Heterogeneity G-tests (two-tailed) were performed on the three possible types of response (guarding W-female, guarding U-female, not guarding) to test for differences between U- and W-males. If this test was significant ($P < 0.05$) analysis proceeded on subsets of the contingency table as above (Siegel & Castellan, 2000) to specifically test for mating preference differences between W- and U-males.

To ensure that mites used in the experiments were virgin, for female preference tests, females and males in the last moul were collected and placed on separate leaves. Five R-females and two R-males, one infected and one uninfected, were placed on leaf discs and mating behaviour was observed and recorded for half an hour (Potter et al., 1976). A 5 : 2, female to male, ratio was chosen to reduce competition between males. Males produce enough sperm to successfully inseminate (at least) ten females (Pijnacker, 1985). Females were removed from the group as they mated. To distinguish the infected from the uninfected male, one male was painted dorsally with a minute droplet of blue water-based paint (in half the samples uninfected males were painted, in the other half infected males were painted).

Because experiments were performed using groups of five females we first employed a G-test to detect heterogeneity between the five-female samples within each female group (W or U). These tests were not significant (for W-females, $G = 8.37$, d.f. $= 18$, $P > 0.9$; and for U-females, $G = 6.49$, d.f. $= 16$, $P > 0.9$) and therefore the analysis proceeded on pooled samples (i.e. taking W- and U-females in two separate groups). A G-test was used to detect differences, between R1 U- and W-females, in the proportion of females showing one of three possible types of response: mating to a W-male, mating to a U-male, or not mating. If this test was significant the analysis proceeded by using G-tests to detect heterogeneity between the five-female samples within each female group (W or U). These tests were not significant (for W-females, $G = 8.37$, d.f. $= 18$, $P > 0.9$; and for U-females, $G = 6.49$, d.f. $= 16$, $P > 0.9$) and therefore the analysis proceeded on pooled samples (i.e. taking W- and U-females in two separate groups). A G-test was used to detect differences, between R1 U- and W-females, in the proportion of females showing one of three possible types of response: mating to a W-male, mating to a U-male, or not mating. If this test was significant the analysis proceeded by using G-tests to determine what caused this effect. Once the category (U-male, W-male, no male) causing the difference had been identified preference of females for a specific mate type was tested using the binomial test (one-tailed). The latter test used a Bonferroni corrected significance level because of the repetitive use of the data (Zar, 1999).

Results

Oviposition site selection tests

Since mating is more likely to occur between individuals born nearby in space and time, we investigated oviposition site selection of infected and uninfected R-females (Fig. 1, top). Ovipositing R1-females were offered a choice between laying eggs on egg clutches from infected or uninfected females, or elsewhere (or any combination of these). In the first set of experiments egg clutches offered on leaf discs had been produced by females from the rose line (Fig. 1a) and in the second by females from the cucumber line (Fig. 1b).

When given a choice, 35–54% of the females deposited all eggs in a patch of their own which – as immature stages have reduced mobility and only females disperse – will promote sib-mating. Roughly one third of the remaining females allocated all eggs to one of the two egg clutches offered. This pattern was similar for R1 W- and U-females regardless of whether patches were by R1 or C5 females, i.e. the distributions in Fig. 1a, b are not significantly different [3D G-test (Zar, 1999); $G = 3.34$, d.f. $= 7$, $P > 0.75$]. For example, when patches
on leaves were by R1-females (Fig. 1a) 25% of R1 W-females, placed all their eggs in infected patches vs. 3% that placed all eggs in clutches by U-females. When patches on leaves were by C5 females (Fig. 1b), 18% of R1 W-females tested placed all their eggs in patches by infected C5 females vs. 3% that placed their eggs in clutches by uninfected females.

Heterogeneity (two-tailed) $G$-tests detected significant differences ($G$-test on pooled samples, $G = 10.26$, d.f. = 2, $P = 0.006$) in the proportion of W- and U-females doing one of the three possibilities (laying their eggs in an infected- or an uninfected-existing patch or start a new patch elsewhere). Furthermore, W- and U-females differ in the way they distribute their eggs over infected and uninfected patches in general ($G$-test on pooled samples, $G = 10.23$, d.f. = 1, $P = 0.002$), but not in allocating eggs to existing vs. new patches ($G$-test on pooled samples, $G = 0.03$, d.f. = 1, $P > 0.75$).

Taking together the eggs laid by all females, 80–90% of the clutches contained eggs of females of the same infection type (Fig. 1, bottom) – thus, clutch heterogeneity with respect to infection is strongly reduced. As noted above, results were similar when clutches available on the leaf-discs were from C5 or R1-females. Sequences of two Wolbachia genes ($fts-Z$ and $wsp$) are identical in the mite populations from cucumber and rose (Vala et al., 2002). Thus we propose that infected R1-females responded to a signal associated with presence of Wolbachia in eggs.

However, both W- and U-females probably perceive cues associated with nuclear genetic differences as well as cues associated with cytoplasmic differences and avoid egg clutches based on those differences. This could explain why the number of females that chose to start a new patch increased slightly when patches present on leaves were by C5 females. This increase resulted in a smaller number of females allocating all eggs to an existing patch. It is clear, however, that R1 W- and U-females allocate their eggs differently to infected and uninfected egg clutches (see above). Based on results so far, we conclude that – independent of host genotype – R1 W- and U-females behave differently with respect to choice of a site for oviposition. These results suggest that females can assess the infection status of the eggs present on the leaf, or of the female ovipositing previously in that patch, and adjust their behaviour in an adaptive way.

**Male and female mate-choice tests**

To examine avoidance of incompatible matings, infected or uninfected R1-males were offered a choice between guarding last moult stages of infected or uninfected R1-females. A heterogeneity $G$-test did not detect significant differences between U- and W-males (Fig. 2, top row), so the data was not analysed further ($G = 4.72$, d.f. = 2, $P = 0.1$).

Choosiness in mites, however, may rather be a property of females (Lesna & Sabelis, 1999). Mate preference of
virgin R-females was tested by offering one infected and one uninfected virgin R-male. The proportions mating (mated to U-male, mated to W-male, unmated) differed for W- and U-females ($G = 6.57, \text{d.f.} = 2, P = 0.039$) – Fig. 2, bottom row. This difference arose not because of difference between the proportion of U- and W-females mating to U- or W-males ($G = 0.58, \text{d.f.} = 1, P > 0.25$), but because of differences in the proportion of U-females mating to U-males vs. mating to W-males or not mating at all ($G = 5.32, \text{d.f.} = 1, P = 0.022$). A binomial test, with Bonferroni corrected significance levels of 0.025, showed that while infected females have no preference for either male type (d.f. = 30, $P > 0.1$), uninfected females significantly preferred uninfected males (d.f. = 42, $P = 0.007$). This is not surprising: infected females are compatible with both types of males, but uninfected females are compatible only with uninfected males. Thus, even when infected and uninfected spider mites co-occur, most matings will be compatible.

Discussion

Not all individuals in a given population are reproductively compatible. Trivers (1972) recognized that the presence of genetically incompatible mates in a population may select for female choice mechanisms. Zeh & Zeh (1996, 1997) proposed that such genetic incompatibility could promote the evolution of female polyandry. They suggest that post-copulatory mechanisms may be available to females that reduce the risk of fertilization by incompatible sperm (through interactions at the molecular/cellular level), whereas it may be difficult to evaluate before copulation the compatibility status of a potential mate (based on phenotype alone). In this paper we investigated if Wolbachia-associated reproductive incompatibility can be avoided by spider-mite females at the precopulatory level. We found that females exhibit precopulatory and oviposition behaviour that increases the chance of compatible mating.

The results presented in this paper jeopardize the assumption of panmixia with respect to infection by Wolbachia bacteria, in populations of two-spotted spider mites. We predict that the infection will not be homogeneously distributed in space because spider mite females will either (i) start a patch of their own, thus promoting sib-mating (sibs will predominantly be of the same infection type); or (ii) allocate their eggs to patches of other females so that the majority of the egg clutches (80–90%) consist of eggs laid by females of the same infection type. Within-patch heterogeneity with respect to infection by Wolbachia may be predominantly due to cases of imperfect maternal transmission of the bacteria and/or of environmental curing. Furthermore, if given a choice, uninfected females will preferably mate to uninfected males, thus directly reducing opportunities for Wolbachia induced-CI to become expressed.
Perception of genetic relatedness, possibly through phenotypic similarity, is not uncommon in mites. Moreover, it has been shown to influence behavioural decisions. Perception of genetic relatedness may have evolved in the context of avoiding intra-specific competition. For example, females of the predatory-mite species *Iphiseius degenerans* prefer to oviposit near closely related conspecific eggs rather than near eggs of more distantly related females (Faraji et al., 2000). Also, when given a choice, spider mite males preferred to guard last-moult stages of females collected from the same host plant over females collected from another host plant (Gotoh et al., 1993). Finally, females of the predatory mite *Hypoaspis aculeifer* choose males based on their genotype and switch preference according to the type of food present (Lesna & Sabelis, 1999). These behavioural responses seem to be triggered by olfactory stimuli. Given the ability of mites to use cues in order to make inferences on genotype, and behave adaptively using that information, the ability to perceive presence of a symbiont may be a simple, albeit important, evolutionary step.

A behavioural trait that results in avoidance of CI clearly benefits uninfected females, because CI acts in the fertilized egg, and is thus unavoidable once an uninfected female is inseminated by an incompatible mate. In *T. urticae*, females may mate multiply (Cone, 1985) but there is currently no evidence that they can select sperm once inseminated, so they are likely to profit from being choosy. Therefore, we expect spider mite females to prefer uninfected mates and choose an uninfected patch for their offspring – our results meet this expectation. In *T. urticae*, males may mate multiply, but they invest time in guarding females, which restricts the number of possible mating opportunities. Therefore, infected males may also benefit from avoiding incompatible matings, and infected mothers may benefit by choosing an infected patch for their sons. For example, it may significantly pay males to choose when opportunities for multiple mating are scarce. Indeed, infected mothers do prefer infected patches, and infected males tended to prefer infected females (although not significant). Future studies should not exclude the possibility of male choosiness.

Empirical tests of the assumption of random mating between uninfected hosts and hosts infected with a CI-inducing Wolbachia have been performed in three instances: twice for infections in *D. simulans* (Hoffmann & Turelli, 1988; O’Neill, 1991) and once for an infection in *Tribolium confusum* (Wade & Chang, 1995). All three cases failed to find evidence for assortative mating. These experiments involved mating in large groups; individual male or female preference was not tested. Also, it is unclear whether assortative mating may be expected in *T. confusum*, as it is presently unknown if flour-beetle populations are polymorphic for CI-W infections. Hoffmann & Turelli (1988) used *D. simulans* flies collected from populations at an early stage of the CI-W epidemic in California – assortative mating with respect to Wolbachia may have evolved after this initial stage. Note that although assortative mating has not been found in fruitflies, genetic ‘host resistance’ to Wolbachia-induced CI has been described in *D. melanogaster* (Boyle et al., 1993; McGraw et al., 2001).

Wolbachia effects on mate preference have been found only in the isopod *Armadillidium vulgare* (Moreau et al., 2001). In this species Wolbachia infections are associated with feminization of genetic males, rather than CI. In feminization, infected genotypic males develop a female phenotype, and are fertile (Rigaud, 1997). It is reported that males prefer real females to neo-females (i.e. feminized males) (Moreau et al., 2001). In this case, however, a clear genotypic difference exists between the two types of female: neo-females (i.e. genotypic males) may lack the ability to produce female sexual pheromones and will therefore be less ‘attractive’. This genetic difference, rather than presence of the symbiont, may be the determinant factor leading to male preference. In our experiments we tested whether presence of Wolbachia could be implicated as the determinant factor in decision-making by two-spotted spider mite females and males. The possible role of nuclear genotype differences was reduced to a minimum by inbreeding prior to establishment of the uninfected sub-lines.

**Implications of host avoidance of CI**

Host behavioural traits that result in the avoidance of CI seem undisputedly adaptive – how these traits evolved, however, remains unknown. Further, if spider-mite populations are not panmictic with respect to Wolbachia, as our results suggest, this poses interesting new questions. When is CI maintained in the population if incompatible matings can be avoided through population structure and assortative mating? Our data show such strong population structure (Fig. 1, bottom) that we expect local extinction of CI (given the fitness cost of infection). If not for the benefit of the symbiont, why does CI persist? There are two possible explanations for this. The first is that CI indeed cannot persist. Because of the lack of data on CI frequencies in the field, the possibility that CI will go extinct cannot be excluded. In fact, one infected R-isofemale line has been isolated that does not express CI (Vala et al., 2002).

The second explanation is that CI persists because it is under positive selection. In the absence of an advantage to the symbiont, the simplest way to account for the latter is to argue that CI confers an advantage to the infected host. For example, in structured populations infected and uninfected patches can coexist through a migration-invasion trade-off (Egas et al., 2002). Local populations of infected hosts are related and CI will isolate them from unrelated (i.e. uninfected) intra-specific competitors. In this way, infected hosts stay in control of local resources. This is also advantageous to
Wolbachia because, being transmitted vertically, they benefit from the reproductive success of the hosts they occupy. In addition, there is an exclusive benefit to Wolbachia: CI may hamper reproductive success of related uninfected females that occasionally emerge by imperfect transmission.

**Conclusion**

Our results show that Wolbachia endosymbionts can play an important role in oviposition and mating behaviour in spider mites. Preference of uninfected females for uninfected males suggests that Wolbachia, like other symbionts, can be implicated in adaptive mate choice. Assortative aggregation of eggs and preference of uninfected females for uninfected males question the assumption of panmixia in polymorphic spider mite populations. Our data is in line with other results that show that suppression of CI can be host-dependent. The hypothesis that hosts of CI-Wolbachia are not necessarily ‘innocent bystanders’, i.e. that selection may favour mechanisms that avoid CI, is therefore pertinent. We have yet to unravel how such evolutionary steps proceed.

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