

Mate preferences in *Drosophila* infected with *Wolbachia*?

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Abstract The maternally inherited bacterium *Wolbachia pipientis* generates strong reproductive incompatibilities between uninfected females and infected males (cytoplasmic incompatibility), significantly reducing both female and male reproductive success. Such fitness costs are thought to place selective pressure on hosts to evolve pre-copulatory preferences for mating with compatible mates, thereby enabling them to avoid the reproductive incompatibilities associated with *Wolbachia*. Therefore, uninfected females are predicted to prefer mating with uninfected males, whereas infected males are predicted to prefer mating with infected females. Despite these predictions, previous investigations of pre-copulatory mate preferences in *Wolbachia*-manipulated *Drosophila* have not found evidence of female preference for uninfected or compatible males. However, none of these studies utilised a design where focal individuals are provided with a simple choice in a relatively non-competitive situation. We examined both female and male pre-copulatory mate preference based on mate infection status in *Drosophila simulans* and *D. melanogaster* using simple choice assays involving between 30–50 replicates per treatment. Although we found no evidence of female pre-copulatory mate preferences in either species, male *D. simulans* exhibited some preference for mating with females of the same infection status. However, this preference was not evident when we repeated the experiment to confirm this finding. Consequently, we conclude that neither male nor female *D. melanogaster* and

D. simulans exhibit significant *Wolbachia*-associated pre-copulatory mate preferences.

Keywords *Wolbachia* · Mate preferences · *Drosophila* · Sexual selection

Introduction

The maternally inherited bacterium, *Wolbachia pipientis*, is renowned for manipulating host reproduction to improve its own transmission (O'Neill et al. 1997). This is achieved in a variety of ways including feminising or killing male offspring or inducing parthenogenesis (O'Neill et al. 1997). However, it is probably best known for inducing cytoplasmic incompatibility (CI) and causing embryo death when the sperm of an infected male fertilises the ova of an uninfected female (Hoffmann and Turelli 1988). The reciprocal crosses are viable, and possessing *Wolbachia* is likely to confer a reproductive advantage to infected females because they are compatible with both infected and uninfected males. In contrast, when *Wolbachia* is not fixed in a population, CI is likely to dramatically reduce the reproductive success of both uninfected females and infected males, as the offspring production of singly mated uninfected females can be reduced by as much as 95% (Hoffmann and Turelli 1997; O'Neill et al. 1997). Such reductions/costs to both female and male fitness are thought to promote the evolution of mechanisms that enable hosts to override or avoid the detrimental effects of *Wolbachia* (Hoffmann et al. 1990; Zeh and Zeh 1996, 1997; Hatcher 2000; Vala et al. 2004; Champion de Crespigny et al. 2005, 2006; Champion de Crespigny and Wedell 2006). Pre-copulatory mate preference for compatible mates is one such mechanism that has been proposed as a potential

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strategy for overcoming the deleterious consequences of *Wolbachia* infection (Hoffmann et al. 1990; Vala et al. 2004). Female preference for uninfected mates may evolve because uninfected females that prefer compatible males avoid the reproductive costs of CI. Likewise, infected males can avoid CI if they mate with compatible (infected) females. In populations where the frequency of *Wolbachia* is not at fixation, genes or alleles encoding such mating preferences are expected to spread because the fitness costs of *Wolbachia* and CI are often severe (Champion de Crespigny et al. 2005). In some species, *Wolbachia* induces CI in almost 100% of fertilisations resulting from incompatible crosses. Hence, provided compatible mates exist within the population and costs associated with expressing preference are relatively minor, pre-copulatory mate preferences for compatible mates could have a significant selective advantage.

Recent models of the evolution of female preference for uninfected mates under *Wolbachia*-induced CI have confirmed the theoretical potential for such strategies to evolve (Champion de Crespigny et al. 2005). In addition, the models demonstrated that alleles encoding preference for uninfected mates are likely to spread to infected females. This justifies studies of mate preferences in species or populations where all or most individuals are infected with *Wolbachia* and/or when uninfected individuals are obtained by curing a subset of the infected population because we expect these individuals to possess preference alleles despite their infected ancestry (Champion de Crespigny et al. 2005). Alleles encoding mate preferences may be maintained (against genetic drift) in such populations because infection frequencies are stochastic and subject to natural curing events (heat shock or naturally occurring antibiotics; Clancy and Hoffmann 1998) and imperfect maternal transmission of *Wolbachia* (Hoffmann and Turelli 1997). As many wild *Drosophila* populations exhibit long-term intermediate *Wolbachia* infection frequencies (Hoffmann and Turelli 1997), the conditions necessary for positive selection on mate preferences could be present for significant periods of time.

It is generally thought that females benefit from being 'choosy' more than males because their reproductive success depends to a lesser degree on the number of copulations they perform than on the 'quality' of their partner (Bateman 1948). In *Wolbachia*-manipulated populations, uninfected females obviously benefit from mating with a compatible, uninfected male because only clutches resulting from such matings are viable. Furthermore, infected females may benefit from mating with uninfected males because they produce more sperm than infected males (Snook et al. 2000). However, males may also benefit from being choosy in this scenario. Like females, they can improve their fertility by mating with compatible

(infected) mates and/or they can improve their reproductive success by mating with the most fertile mates. Field studies on *Drosophila simulans* have found that infected females are approximately 10% less fertile than uninfected females (Hoffmann et al. 1990), although infected females of some strains of *D. melanogaster* have enhanced fecundity and survivorship in comparison to uninfected females (Fry et al. 2004). Strategic ejaculation has been demonstrated in the amphipod *Gammarus duebeni* where males allocate more sperm to uninfected, high fecundity females than to females infected with the sex-ratio distorting microsporidian *Nosema granulosis* (Dunn et al. 2006).

Pre-copulatory mate preferences have been investigated in several species manipulated by *Wolbachia*. There is no evidence of male mate preferences in the butterfly *Acraea encedon* where *Wolbachia* kills male offspring (Jiggins et al. 2002), despite convincing theoretical predictions (and presumably strong selective pressure) that males should prefer uninfected females (Randerson et al. 2000). Likewise, there is no evidence of male preference for uninfected females in a recent study of *D. innubila*, which also is manipulated by male-killing *Wolbachia* (Sullivan and Jaenike 2006). However, male isopods (*Armadillidium vulgare*) interact more with uninfected females and attempt to mate with uninfected females more frequently than with feminised males (neo-females; Moreau et al. 2001). This is thought to occur as a result of behavioural and physiological differences between uninfected and feminised females rather than explicit evolution of preference for uninfected females in males. In addition, the spider mite, *Tetranychus urticae*, in which *Wolbachia* induces CI, exhibits plastic female mate choice. Uninfected females prefer to mate with uninfected males, whereas infected females exhibit no preference for either infected or uninfected males (Vala et al. 2004). Aside from the spider mite study, all other investigations of mate preferences in species manipulated by CI-inducing *Wolbachia* have found no support for infection related mate preferences (Hoffmann and Turelli 1988; Hoffmann et al. 1990; Wade and Chang 1995). However, none of these experiments utilised a basic choice design in which a single individual is provided with a choice between one infected and one uninfected mate. Instead, mating observations were made on large groups of (in some cases non-virgin) infected and uninfected individuals. Additionally, observations did not necessarily begin immediately after the males and females were combined, and assays were sometimes biased towards the fastest mating pairs. Hence, from these experiments, some or all of the following limitations apply: It is impossible to know whether individuals actually experienced a 'choice' between mates of opposing infection status, competitive interactions may have affected the outcome of courtship, and mating history may have affected mate preferences.

In this study, we investigate, by means of simple choice experiments, pre-copulatory mate preferences in both male and female *D. simulans* and *D. melanogaster*. Although pre-copulatory mate preferences have been previously examined in *D. simulans* (Hoffmann and Turelli 1988; Hoffmann et al. 1990), pre-copulatory mate preferences have not been investigated in *D. melanogaster* before this study. This experimental design minimises competitive interactions, controls for mating history and ensures focal individuals are exposed to both infected and uninfected mates for the same period of time.

We examine the hypothesis that uninfected females and infected males prefer to mate with compatible mates. Specifically, we expect uninfected females to prefer uninfected males and infected males to prefer infected females. Infected females and uninfected males are expected either to exhibit no pre-copulatory mate preferences or to prefer the most fertile mates (uninfected females and males). In *D. simulans*, *Wolbachia* induces high levels of CI in incompatible crosses between uninfected females and infected males (Hoffmann and Turelli 1988). However, in *D. melanogaster*, CI induction is relatively low and generally only evident when young infected virgin males mate with uninfected females (Reynolds and Hoffmann 2002; Clark et al. 2003). Such variation in fitness costs should create different selective pressures on the evolution of pre-copulatory mate preferences. Consequently, we expect to see stronger patterns of mate preference in *D. simulans* as a result of the high levels of CI induced in this species.

Materials and methods

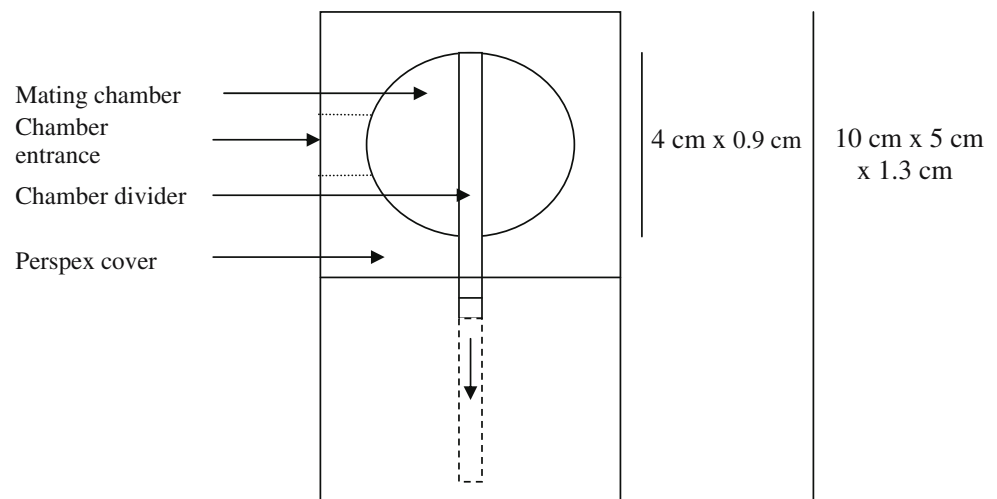
Study species The *D. simulans* used in this experiment derived from infected flies originally collected from Riverside, California (USA) and subsequently maintained in large laboratory populations. The *D. melanogaster* derived from an OregonR (OreR) laboratory strain. Both infected lines descend from iso-female lines established before obtaining them for this study. Uninfected flies of both species were obtained by tetracycline treatment of a large (>80 females and >80 males) random sample of the infected population approximately 1 year before the experiment: Two consecutive generations were raised on food containing 0.03% tetracycline hydrochloride. More than 150 female and 150 male offspring from the second generation founded the uninfected populations. Note that the uninfected *D. simulans* used to repeat the mate preference experiment were from a separate and later cured population due to the time elapsed between the initial and repeat experiment. However, the curing process and subsequent maintenance of the populations was identical.

Both infected and uninfected flies were maintained in stock populations consisting of 1,000s of flies. The populations were treated in the same way, and flies were provided with a constant supply of food/medium on which to lay eggs. The *Wolbachia* infection status of the populations was confirmed by PCR using *Wolbachia*-specific primers before and after the experiment using the methodology described in Champion de Crespigny et al. (2006). The expected infection status was confirmed in all cases.

Experimental design Mate preference in *D. simulans* and *D. melanogaster* was assessed via simple mate choice trials in which virgin flies were provided with both virgin infected and uninfected mates. Virgin flies were obtained by collecting eggs from stock populations and raising the larvae in density controlled vials (five larvae per millilitre agar) at 25°C on a 12/12-h light/dark cycle. When adult flies began to eclose, the vials were inspected three times during the light cycle for emerging flies. Any flies present in the vials were chilled on ice and sexed, and the sexes were separated to vials containing not more than 40 adult flies. Before experimentation, all vials were inspected for larvae to ensure virginity.

Both male and female mate preference was assessed. Flies were randomly allocated to one of two experimental trials (female choice or male choice). In both cases, the focal fly, a virgin of one sex (*D. simulans*: female choice: infected $n=32$, uninfected $n=33$; male choice: infected $n=33$, uninfected=32; *D. melanogaster*: female choice: infected $n=48$, uninfected $n=50$; male choice: infected $n=30$, uninfected=35) was enclosed in a small mating chamber with a virgin infected and a virgin uninfected fly of the opposite sex. Five-day-old females and 2-day-old males were used in all trials. Potential infected and uninfected mates were marked with either fluorescent pink or fluorescent blue paint dust 24 h before the experiment. The flies usually cleaned themselves and removed most of the visible dust before the experiment. However, small paint particles remained adhered to the cuticle and hairs in various places, and these were clearly visible when viewed under a dissection microscope. Both flies were marked, and the marking was randomised with respect to infection status. This ensured that both infected and uninfected males were marked with both colours in equal numbers, but that each trial consisted of only one pink and one blue mate. This enabled identification of the potential mates but controlled for any preference for paint dust or colour. Neither males nor females of either species exhibited preference for a particular coloured mate during the trials (female preference: *D. simulans*—Pearson: $\chi^2=0.019$, $df=1$, $p=0.890$; *D. melanogaster*—Pearson: $\chi^2=2.613$, $df=1$, $p=0.106$; male preference: *D. simulans*—Pearson: $\chi^2=2.585$, $df=1$, $p=0.108$; *D. melanogaster*—Pearson: $\chi^2=1.643$, $df=1$, $p=0.2$).

Fig. 1 Diagram of the mating chambers used in the mate choice experiments



The experiments were performed in small, circular mating chambers (Fig. 1) that could be divided in half. This enabled the sexes to remain separated until the mating trial was started. The flies were chilled on ice to facilitate their transfer to the chambers, and they were given at least 20 min to recover before the experiment commenced. When all the flies were active, the divider within the mating chamber was withdrawn, and the flies were able to interact. The flies were observed until copulation occurred whereupon the copulating pair was separated from the third individual using the chamber divider. The identity of the first mating partner was determined by inspecting both the mating partner and its isolated competitor under a dissecting microscope for fluorescent dust particles. The colour and thereby the infection status of the first mate was recorded. The experimental trial lasted for 2.5 h, and flies not copulating within this time were discarded. Typically, more than 80% of the pairs copulated during each trial. The experiments were performed at room temperature, and both natural light and a desk lamp illuminated the vials.

Results

Female preference

Neither infected nor uninfected female *D. simulans* exhibited preference for mating with either infected or uninfected males (Pearson: $\chi^2=0.14$, $df=1$, $p=0.708$). When the data for the females were pooled, binomial tests revealed no preference for either infected or uninfected males (Binomial test, $n=65$, uninfected=0.49, infected=0.51, $P>0.99$). Thirty-three of 65 females mated with infected males.

Similarly, we found no evidence that female *D. melanogaster* prefer to mate with either infected or uninfected males (Pearson: $\chi^2=2.591$, $df=1$, $p=0.107$). Again, pooling the female data did not reveal any bias towards infected or uninfected males (Binomial test, $n=98$, uninfected=0.48, infected=0.52, $P=0.762$). Fifty-one of 98 females mated with infected males.

Male preference

In contrast to females, infected and uninfected male *D. simulans* seemed to exhibit some preference for mating with infected or uninfected females depending on their own infection status (Pearson: $\chi^2=4.481$, $df=1$, $p=0.034$; Fig. 2). Infected males mated more frequently with infected females (61% of matings), whereas uninfected males mated more frequently with uninfected females (66% of matings). However, these patterns were not significant when compared within either infected males (Pearson: $\chi^2=1.485$, $df=1$, $p=0.223$) or uninfected males (Pearson: $\chi^2=3.125$, $df=1$, $p=0.077$). To clarify these findings, we repeated the male preference experiment and increased the number of replicates (infected males $n=48$ and uninfected males $n=50$). However, males did not exhibit the same pattern of mating with females of the same infection status, and we, therefore, found no evidence of male mate preferences based on female infection status in the new data set (Pearson: $\chi^2=0.187$, $df=1$, $p=0.666$; Fig. 2).

D. melanogaster males exhibited no preference for either infected or uninfected females (Pearson: $\chi^2=0.332$, $df=1$, $p=0.565$). When the data for the males were pooled, binomial tests revealed no preference for either infected or uninfected females (Binomial test, $n=65$, uninfected=0.54, infected=0.46, $P=0.620$). Thirty of 65 males mated with infected females.

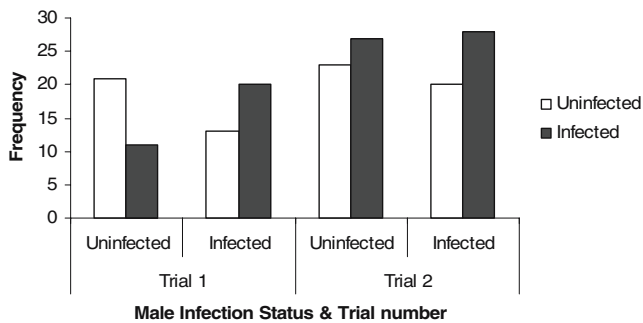


Fig. 2 Male mate preferences in *D. simulans*; the frequency with which uninfected and infected males mated with uninfected (white) and infected (black) females in trial 1 and in trial 2 when the experiment was repeated to confirm the initial results

Discussion

Pre-copulatory mate preference is a widespread feature of reproductive behaviour (Kokko et al. 2003); however, it has rarely been demonstrated in species manipulated by *Wolbachia* despite the often severe fitness costs associated with infection. Consistent with other studies of mate preferences in *Wolbachia*-manipulated *Drosophila* (Hoffmann and Turelli 1988; Hoffmann et al. 1990), females did not exhibit preference for males of particular infection status. Similarly, male *D. melanogaster* did not prefer to mate with either infected or uninfected females. In contrast, *D. simulans* males in the initial experiment seemed to prefer to mate with females of the same infection status as their own. However, this pattern was not repeatable, and it seems unlikely that either *D. simulans* or *D. melanogaster* males and females utilise pre-copulatory mate preferences associated with the infection status of potential mates.

The lack of evidence for female mate preferences in our study may not be surprising. Females may not be able to distinguish between males of different infection status before copulation. To the human observer, there are no obvious differences in courtship behaviour between infected and uninfected males in terms of latency to courtship initiation and the commencement of copulation (F. Champion de Crespigny, unpublished data). However, infected males (of both species) mate more frequently than uninfected males (Champion de Crespigny et al. 2006). Male body size is an important predictor of male mating success in *D. melanogaster*, with females mating more frequently with larger males (Partridge and Farquhar 1983; Partridge et al. 1985, 1987a,b). Although body size was not measured in this study, it is probably unlikely that infected and uninfected flies differed in body size because they were reared at the same density with ad lib food. Furthermore, no difference in body size between infected and uninfected flies has been found in other studies utilising the same

rearing methodology and flies from the same populations (Champion de Crespigny et al. 2006; Champion de Crespigny and Wedell 2006).

In addition to potential problems associated with identifying *Wolbachia* infection in mates before copulation, female post-copulatory processes may more reliable in identifying mate compatibility or biasing fertilisations towards compatible males than pre-copulatory mate preferences. This may occur because interactions at the molecular and cellular level may be better equipped to identify abnormalities or incompatibilities (Zeh and Zeh 1997), or simply because there are inherent differences in the sperm competitive ability of infected and uninfected males. For instance, in *D. simulans*, *Wolbachia*-infected males produce fewer sperm (Snook et al. 2000) and have less competitive ejaculates in sperm competition than uninfected males (Champion de Crespigny and Wedell 2006). Hence, it is possible that uninfected polyandrous females can reduce the cost of CI induction by remating and therefore promoting sperm competition. Females may also reduce or avoid CI if they can assess the infection status of sperm in their reproductive tract. *Wolbachia* is known to cause protein composition changes in infected tissues. For example, a 135-kDa protein that generates an immunogenic response has been demonstrated in *Wolbachia*-infected alfalfa weevils (*Hypera postica*; Leu et al. 1989). If these proteins affect sperm phenotype in the same way in *Drosophila*, then it is possible that post-copulatory mechanisms could identify *Wolbachia*-modified sperm, allowing females to bias fertilisation towards the most suitable genotype.

In contrast to females, the only opportunity for males to ensure mate compatibility is before sperm transfer. Although males are typically considered the less choosy sex, with male reproductive success depending on number, rather than quality, of copulations (Bateman 1948), evidence is accumulating suggesting that male *Drosophila*, at least, are limited in terms of their fertility by sperm production and accessory gland secretions (Lefevre and Jonsson 1962; Champion de Crespigny 2005; Sullivan and Jaenike 2006). Consequently, preference for compatible females may be more advantageous than traditionally thought because the first matings in particular may be crucial in determining male reproductive success. In this study, we initially found weak evidence of male mating preferences in *D. simulans*. Infected and uninfected males preferred, to mate with females of the same infection status, but these patterns of mating were not supported statistically when compared within either infected males or uninfected males. Nor were the mating patterns replicated in a follow-up experiment. It is not clear why this was the case, but it could be accounted for by differences in the stock

populations in the initial and subsequent experiment. The uninfected flies used in the repeat experiment were cured separately to the initial uninfected population. However, the time between curing and experiment was the same for both replicates, as were the stock maintenance conditions. Unfortunately, this situation was impossible to avoid due to the amount of time elapsed between the initial and subsequent experiment. However, there is no a priori reason to expect the populations to differ, especially as they originate from the same infected stock population. Furthermore, strong divergence is unlikely due to the large population sizes and the high level of inbreeding associated with iso-female lines. Therefore, if we assume that our initial finding was a type 1 error, it seems unlikely that strong male mating preferences exist in this species, although they cannot be ruled out. The possibility remains that males assess mate compatibility while in copula and transfer different quantities of sperm to infected and uninfected females with respect to mate compatibility.

What is of real interest is that, with the possible exception of the spider mite study by Vala et al. (2004), there is an almost total lack of evidence of mate preferences in species manipulated by *Wolbachia* that bias sex ratios or cause profound reproductive incompatibilities, despite strong and convincing theoretical predictions. Perhaps the rate of spread of *Wolbachia* within populations is too rapid for the evolution of mate preferences. However, where *Wolbachia* causes CI at least, many populations exhibit intermediate infection levels (Vala et al. 2004), presumably placing continuous strong selection on behavioural adaptations such as mate preferences. Nevertheless, neither species with both recent (Jiggins 2003) nor long-term (Sullivan and Jaenike 2006) (including the species studied here) evolutionary associations with *Wolbachia* exhibit mate preferences with respect to *Wolbachia* infection status. It seems apparent that without physiological changes associated with infection (e.g. feminised woodlice; Moreau et al. 2001) or existing mate discrimination systems based on genotype (e.g. spider mites; Vala et al. 2004), it is difficult for individuals to avoid incompatible or less desirable mates. In both the previous examples, avoidance of *Wolbachia* is likely to be a side effect of another process [preference for female pheromones (woodlice) or sibs (spider mites)] rather than direct preference with respect to absence of infection. Future investigations of mate preferences associated with *Wolbachia* should concentrate on species where *Wolbachia* changes important aspects of the host's reproductive behaviour or physiology and/or where assessing mate genotype is intrinsic to host reproductive strategies. Additionally, pre-copulatory mate preferences may be more important in species where females and/or males are essentially monandrous, and there is little or no scope for post-copulatory processes to bias paternity.

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