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Male-killing *Wolbachia* and male mate choice: a test with *Drosophila innubila*

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ABSTRACT

Hypothesis: Species infected with male-killing endosymbionts may exhibit reversal in sexual selection, with males exhibiting a preference for uninfected females.

Organism: *Drosophila innubila*, a species in which a substantial proportion of the females are infected with male-killing *Wolbachia*.

Methods: Laboratory experiments in which males were allowed to choose between infected and uninfected females within a population cage. Virgin or non-virgin males and females were tested separately in all four combinations, as prior mating history may affect motivation to mate and thus choosiness. In a separate experiment, males were allowed to mate repeatedly and offspring production was monitored to determine if multiple mating adversely affects male fertility.

Results: In the mate choice tests, the number of infected and uninfected females that mated was very similar in all tests, thus providing no evidence that males prefer uninfected females. Male fertility declined substantially in successive matings, indicating that they do not have unlimited capacity to sire offspring through repeated mating.

New hypothesis: If infected and uninfected females differ in some quantitative phenotypic trait, the probability that a female is infected depends not only on her phenotype but also on the prevalence of infection among females in the population. Hence, the phenotypic cues that might be used as the basis for adaptive male mate choice are likely to be ambiguous, thus impeding the evolution of male choosiness.

Keywords: *Drosophila innubila*, endosymbionts, male-killing, *Wolbachia*, sexual selection, sperm depletion.

INTRODUCTION

Innumerable species of arthropods are infected with maternally transmitted microbial endosymbionts, many of which enhance their transmission by manipulating host reproduction in a variety of ways (O'Neill *et al.*, 1997). Two of these mechanisms – feminization and male-killing – not only facilitate the spread of the endosymbionts, but also lead to female-biased sex ratios within populations of outbreeding, sexual species. Feminization entails the developmental conversion of endosymbiont-infected genetic males into functional females

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and has been documented in crustacea and mites (Rigaud, 1997; Weeks *et al.*, 2001). Male-killing, the death of male embryos that develop from eggs laid by infected females, has been found in diverse insects and is caused by a variety of microbial taxa (Hurst and Jiggins, 2000).

In species infected with male-killing endosymbionts, the infection status of the female of a mating pair determines offspring sex ratio. Consequently, males that mate with uninfected females will sire up to twice as many progeny as males that mate with infected females. Furthermore, because such symbionts cause the population-level sex ratio to be female biased, males are the more valuable sex (Fisher, 1930). Because sons have higher mean fitness than daughters in such a population, males that mate with uninfected females are expected to leave more than twice as many genes to future generations. In fact, because infected lineages persist by their females mating with uninfected males every generation, the nuclear genes present in such lineages are ultimately diluted out of existence as a result of this backcrossing scheme. In the long run, then, males that mate with infected females will leave no copies of their nuclear genes to posterity, except those that escape from infected lineages as a result of incomplete transmission of the endosymbiont or incomplete penetrance of the male-killing effect.

In a species infected with male-killing endosymbionts, the expected proportion of females within a population will range between $1/(2 - P)$ and $(1 + P)/2$, depending on the level of viability compensation by females for the male offspring that are killed by the infection, and where P is the prevalence of endosymbiont infection among females. The net effect on the population is a less male-biased operational sex ratio (Emlen and Oring, 1977). Thus, both the enhanced genetic payoff that comes from mating with uninfected females and the change in the operational sex ratio may favour the evolution of choosier males, in contrast to the situation in uninfected species of insects (Bateman, 1948). However, such choosiness would be favoured only if mating by a male entails a cost in terms of future reproductive success, such as increased susceptibility to predation during copulation or reduced fertility in subsequent matings. Furthermore, such choosiness requires that males be able to distinguish infected from uninfected females.

Randerson *et al.* (2000) recognized the potential importance of male choosiness in species infected with male-killing endosymbionts. They also showed that, in theory, an allele that confers upon males a mating preference for uninfected females can spread within species harbouring male-killing endosymbionts. While this notion is intuitively appealing, empirical tests of male mate choice in such species have yielded inconsistent results. For instance, males of the crustacean *Gammarus duebeni* show no preference for uninfected females over those infected with a feminizing microsporidian (Kelly *et al.*, 2001). In the terrestrial isopod *Armadillidium vulgare*, there are two types of females, those that are not infected with *Wolbachia* and which are heterogametic (WZ), and neo-females, which are homogametic genetic males (ZZ) that have developed as functional females due to infection with a feminizing *Wolbachia* (Rigaud, 1997). Matings involving infected neo-females yield solely neo-female offspring. When provided with a choice between these two types of females, males prefer the uninfected WZ females, a mating that results in the production of normal offspring sex ratios (Moreau *et al.*, 2001). However, when genetic WZ females were experimentally infected with *Wolbachia*, the males no longer exhibited a preference for the uninfected females (Moreau *et al.*, 2001). This suggests that a female's genotype, rather than infection status *per se*, is the basis for male mate choice. Perhaps neo-females, which are genetically male, do not have the normal complement of female behaviours or pheromones, thus making them less attractive to males.

Finally, Jiggins *et al.* (2002) examined male mating behaviour in the butterfly *Acraea encedon*, which is infected at high frequency by male-killing *Wolbachia*. The sex-ratio bias is so extreme in this species that there has been behavioural sex role reversal, with females forming lekking swarms to attract males for mating (Jiggins *et al.*, 2000). However, tests of mating behaviour in the field showed that males exhibited no discrimination in favour of uninfected females. One possible reason these butterflies fail to conform to theoretical expectations is that infection by *Wolbachia* is evolutionarily very recent (Jiggins, 2003), and thus there may have been insufficient time for the evolution of male mate choice.

The present study focuses on male mate choice in *Drosophila innubila*, a mycophagous species that inhabits mid- to high-elevation woodlands and forests of Arizona, New Mexico, Sonora and Chihuahua. This species is infected with a single strain of *Wolbachia* that causes embryonic male-killing. Male mate choice for uninfected females within *D. innubila* may be expected to have evolved for several reasons. First, patterns of diversity and geographic differentiation of mtDNA (which is co-transmitted with *Wolbachia*) indicate that the *Wolbachia* infection is evolutionarily old within this species (Dyer and Jaenike, 2004, 2005). Second, the observed prevalence of infection (up to 45% in the Chiricahua Mountains), the high rate of maternal transmission, and the nearly complete penetrance of male-killing in all populations studied (Dyer and Jaenike, 2004, 2005) indicate that *Wolbachia* is a potentially strong selective factor. Furthermore, general levels of nucleotide variation within *D. innubila* indicate that this species has a very large effective population size (Dyer and Jaenike, 2004), and thus may be well positioned to respond to selection for a change in male mating behaviour.

To determine if males of *D. innubila* preferred uninfected females as mates, we used population cages in which there were multiple males and females, as this mimics natural conditions better than small enclosures with one male and two females. In our experiments, the age, condition and mating history of the tested males were controlled, so that any differential mating based on female mate choice would be based on random differences among males. However, within each trial the females did differ systematically, with 50% being infected with *Wolbachia*. Thus, any difference between infected and uninfected females in their mating rate within trials would be due either to male mate choice or to differences among females in propensity to mate.

Our experiment expanded on previous tests of the Randerson *et al.* (2000) hypothesis in that we tested both virgin and non-virgin males and females in all combinations. This is because mating history may affect a fly's motivation to mate, which may in turn influence its mate preference. For instance, virgin females in many species of *Drosophila* are more willing to mate than are non-virgin females (Markow, 1996). If a male's probability of mating with a female is a function of her intrinsic suitability (e.g. infected or not) and her level of resistance to male courtship, then very low or very high levels of female resistance may hinder the expression of male choosiness. Finally, to determine whether there is a cost to mating for males of *D. innubila*, we tested whether male fertility declined as a consequence of previous matings.

METHODS

Drosophila strains and maintenance

The experiments utilized two uninfected strains of *D. innubila* that had been collected in the Chiricahua Mountains of southeast Arizona in 2000 (strain ST-1) and 2001 (strain ST-4).

Both strains produce 1 : 1 offspring sex ratios, and PCR reveals no evidence of *Wolbachia* infection. We also used two strains infected with *Wolbachia*, as determined by PCR, both of which consistently produce ~100% female offspring. These strains are designated *mk-1* and *mk-3*, and these had been maintained in the laboratory by mating them to males from strain ST-1. Cultures were maintained at 22°C and a light : dark cycle of 14 : 10 h on instant *Drosophila* medium (Carolina Biological Supply) plus a small piece of commercial *Agaricus bisporus* mushroom.

Mate choice

To obtain flies for the mate choice experiments, the females of each strain (ST-1, ST-4, *mk-1* and *mk-3*) were crossed with males of an uninfected strain, ST-3, which had been collected in the Chiricahuas in 2001. These crosses were done to prevent inbreeding depression among the experimental flies. Uninfected cultures were set up using 3–4 females per vial, while cultures of infected flies were set up with 6–7 females per vial. For both types of strains, the females were 5 days old. Because the male offspring of infected females die as embryos, the two conditions yield similar densities of larvae within the cultures.

On the day of emergence, the flies to be used in the mate choice experiments were anaesthetized using CO_2 and then separated by sex. Three days after emergence, the females were again anaesthetized and a small piece was cut off the tip of the right wing for infected females and the left wing for uninfected females. This was done to allow subsequent identification during the mate choice experiments. Flies were then fed instant food plus *A. bisporus* mushroom. Flies that were to be tested as virgins were kept in vials in groups of males, infected females or uninfected females, with 10–12 flies per vial.

Flies that were to be tested as non-virgins were maintained as above, except that 3-day-old flies were placed with an equal number of uninfected *D. innubila* of the opposite sex carrying a *yellow* body mutation. Flies carrying this mutation mate readily, and the *yellow* phenotype is readily distinguishable from the wild-type. At 6 days post-emergence, the males were removed by aspiration and placed in vials with instant food plus mushroom.

Eight days after emergence, and 2 h after lights on, flies were placed in a $30 \times 23 \times 20$ cm population cage containing two slices of *A. bisporus* mushroom, as mating activity is enhanced in the vicinity of mushroom breeding sites. For the mate choice tests, all combinations of virgin and non-virgin males and females were examined. For each of the four trials of each combination, 100 infected and 100 uninfected females were first added to the cage and given 2 min to habituate, then 100 males were added. Copulating pairs were aspirated into individual vials and the females were identified as infected or uninfected by their wing clipping. The mating arenas were watched for 2 h or (more typically) until 50 pairs had copulated, which ever came first. Subsequently, all remaining unmated flies were aspirated from the cage and counted to ensure that none had escaped or died during the experiment.

An analysis of variance was used to assess whether the proportion of females that mated depended on female infection status, female mating history (virgin or non-virgin), male mating history (virgin or non-virgin), and the interaction between female mating history and male mating history. Each of the 16 trials provided one data point for infected females and one for uninfected females.

Multiple-mating and male fertility

To test sperm limitation resulting from multiple matings, we used the offspring of crosses between ST-1 females and ST-4 males. Examining the offspring of this cross reduces the effects of inbreeding depression. By using uninfected strains, we eliminate embryonic male-killing as a source of variation in offspring production. Flies to be tested were treated the same as those used in the mate choice experiment.

Females' wings were clipped 3 days after emergence. In total, 400 females were used in the experiment, 100 each of the following treatments: right wing clipped, left wing clipped, both wings clipped, neither wing clipped. Flies in all four categories were anaesthetized for a similar period (~45 s) for this procedure. After clipping, females were stored 10–12 per vial, and grouped according to wing-clipping treatment. They were transferred to fresh food at age 6 days.

From 3 to 6 days of age, males were kept in population cages either in the absence of females (to obtain virgin males) or in the presence of *yellow* females at a ratio of one male to two females. The latter treatment would allow males to have a normal rate of mating, since this is the sex ratio in the Chiricahuas (Dyer and Jaenike, 2005). Six days after emergence, the males were aspirated into vials of fresh instant *Drosophila* medium plus *A. bisporus*.

Nine days after emergence, one male and four females (each with a different wing-clipping pattern) were aspirated into a vial that was observed continuously for copulation. In total, 100 of these vials were set up. As mating occurred the wing type of each female was recorded, as this enabled us to determine which female mated first, which one was second, and so on. The flies were watched continuously for 9 h. Following the matings, the wing length of each male was measured from the anterior cross-vein to the tip of the third longitudinal vein, as this provides a measure of overall body size of a male. Each female that mated with a given male was placed individually in culture (instant *Drosophila* medium plus *A. bisporus*) and subsequently transferred to fresh food every 4 days for the next 20 days. All of the offspring of every female were counted.

To determine if the fertility of males is depressed by multiple mating, the number of offspring was examined as a function of male type (virgin or non-virgin), individual male within male type, and the order in which females mated with individual males.

RESULTS

Mate choice

In total, we conducted 16 trials in which *D. innubila* males could mate either with *Wolbachia*-infected or -uninfected females, 4 trials each of virgin males with virgin females, virgin males with non-virgin females, non-virgin males with virgin females, and non-virgin males with non-virgin females. In each of the 16 trials, the proportion of infected females that mated was very similar to the proportion of uninfected females that mated, and in no case was the difference significant (Fig. 1). Analysis of the entire data set revealed that female infection status had no effect on her probability of mating ($P = 0.16$; Table 1). However, virgin females were significantly more likely to mate within the designated observation period than were non-virgin females ($P < 0.0001$; Table 1), although prior mating history of males did not have such an effect.

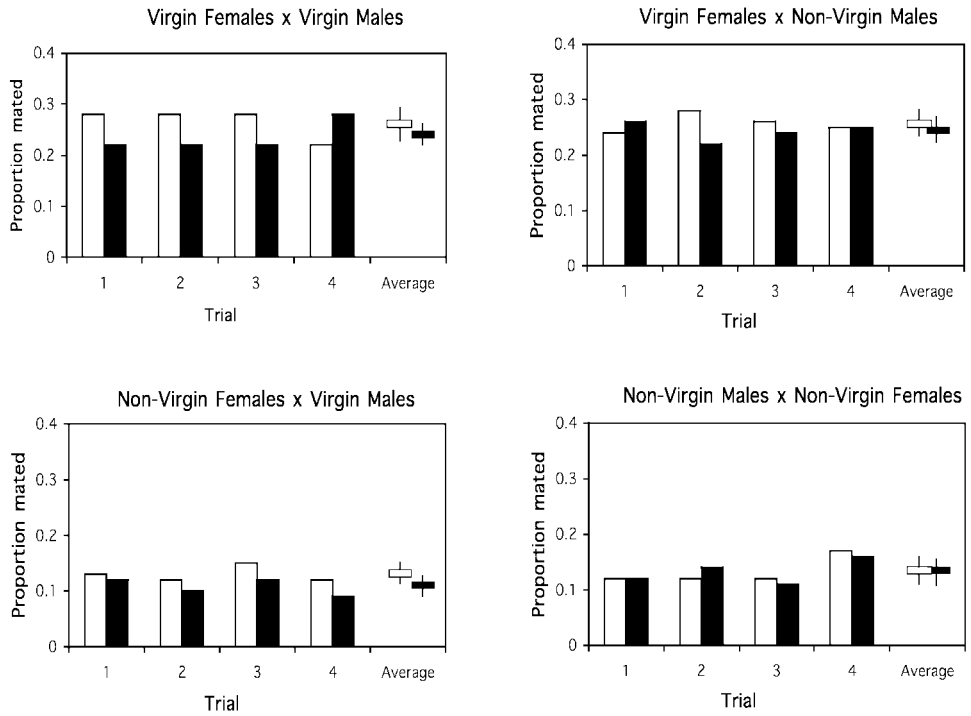

Fig. 1. Proportion of infected and uninfected females ($n = 100$ for each type) that mated in the four combinations of virgin or non-virgin males and virgin or non-virgin females. Four trials of each combination were carried out. In no individual trial was there a significant difference in the proportion of infected (solid bars) and uninfected (open bars) females that mated. Also plotted is the mean (± standard error) proportion of females that mated across the four trials.

Table 1. Results of ANOVA of proportion of females mated in male mate choice trials

Source of variation	d.f.	*F*	*P*
Female infection status [male type, female type]	4	1.82	0.16
Female type (virgin vs. non-virgin)	1	259.6	0.0001
Male type (virgin vs. non-virgin)	1	1.32	0.26
Female type × male type	1	1.32	0.26
Error	24		

Multiple-mating and male fertility

The number of offspring produced by females was strongly affected by the order in which they mated with a male ($P < 0.0001$; Table 2), with the mean number of offspring declining from 96 for the first female to 16 for the fourth female with which a male mated (Fig. 2). Non-virgin males sired slightly more offspring in total (mean = 80 ± 4) than did virgin males (mean = 75 ± 4), a difference of borderline significance ($P = 0.06$, Table 2). However, there was a highly significant difference among individual males in their fertility ($P < 0.0001$); the

Table 2. Results of ANOVA of offspring number as a function of previous male type (virgin vs. non-virgin), individual male within male type, and female mating order

Source of variation	d.f.	*F*	*P*
Female mating order	3	24.98	0.0001
Male type (virgin vs. non-virgin)	1	3.78	0.06
Male [male type]	50	3.43	0.0001
Error	96		

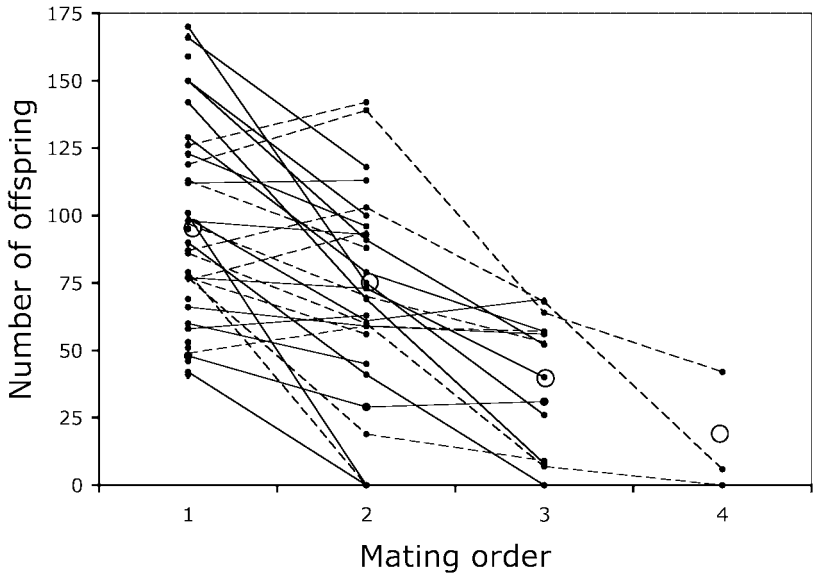

Fig. 2. Offspring production by females as a function of the order in which they mated with individual males. Lines connect different females mated to the same male. Points plotted as 0 indicate that a female did mate with a male, but produced no offspring. The number of points per male indicates the number of females with which he mated. Solid lines indicate males that were virgin at the start of the experiment, whereas dashed lines indicate non-virgins. The large open circles are means for a given mating order category. Note the decline in fertility with mating order and similar rank order fertility among individual males from one mating to the next.

experimental males had the same approximate rank order fertility in the successive matings, as is evident in Fig. 2. Males sired significantly fewer offspring in mating 2 than in mating 1 ($P < 0.0001$) and significantly fewer in mating 3 than in mating 2 ($P < 0.0001$). The decline from mating 3 to mating 4 was not significant, but this may be related to the very small sample size for mating 4.

The difference among males was not related to size, as there was no correlation between male wing length and the number of offspring produced in either the first mating ($r = -0.05$, $P = 0.72$) or summed across all matings ($r = -0.07$, $P = 0.63$).

DISCUSSION

Males within a species that is polymorphic for infection with a male-killing endosymbiont are expected to pass on far more of their genes to future generations if they mate with uninfected females. The use in the present experiment of flies that were controlled for age, nutritional condition and mating history allowed us to examine the role of infection *per se* on mate choice. Despite the more than two-fold benefit of male mating with uninfected females, we found no evidence that males of *D. innubila* prefer to mate with such females. This lack of discrimination was found consistently across multiple trials in all combinations of virgin and non-virgin males and females.

There are several possible reasons for the lack of mate choice by *D. innubila* males. If the potential fertility of males is unaffected by mating, there may be only a small penalty to be paid for mating with an infected female. However, our results showed that multiple mating by males dramatically lowered male fertility, as measured by the number of offspring produced. Thus, mating with an infected female adversely affects a male's subsequent fertility. As a member of the quinaria species group, it is likely that females of *D. innubila* re-mate fairly frequently (Markow, 2002). Moreover, natural populations of *D. innubila* are female-biased (Dyer and Jaenike, 2005). Therefore, males may experience high rates of mating in the wild.

The internal anatomy of *D. innubila* also suggests that male fertility can be a limiting factor in offspring production. The seminal receptacles of *D. innubila* females are 12–14 mm long (J. Jaenike, unpublished), similar to other members of the quinaria group and considerably longer than those of most *Drosophila* (Pitnick *et al.*, 1999). Across *Drosophila* species, there is a positive correlation between sperm length and seminal receptacle length, but a strong negative correlation between sperm length and number of sperm transferred per copulation (Pitnick, 1996; Pitnick *et al.*, 1999). Our data on male fertility after multiple matings, together with the long seminal receptacles, are consistent with the possibility that sperm or accessory gland products (Wolfner, 1997) can limit male fertility in *D. innubila*. Thus, unlimited male fertility does not explain why males fail to prefer uninfected females.

Multiple mating by females, which is common in *Drosophila* (Markow, 2002) and many other insects (Eberhard, 1996), could also affect the evolution of male preference. If males did prefer to mate with uninfected females, sperm competition would be reduced within infected females, thus reducing the penalty of mating with such females. Consequently, selection for male choosiness could conceivably act in a negative frequency-dependent manner.

Perhaps most intractably from an evolutionary perspective, males may be unable to reliably distinguish infected from uninfected females. Suppose infected and uninfected females differ in some quantitative trait, x, such as size or wing beat frequency during courtship interactions. For insect species infected with male-killing endosymbionts, female size may differ between infected and uninfected females. We have previously shown that the male-killing *Wolbachia* that infects *D. innubila* spreads as a result of reduced larval competition experienced by infected females and that female body size declines as a function of larval density within a breeding site (Jaenike *et al.*, 2003). Thus, infected females may, on average, be somewhat larger than uninfected females during or following years when conditions result in significant competition among sibling larvae.

The question for a male then becomes, what is the probability that a female is infected, given that she has a certain phenotype x_i? The question can be addressed using the general approach of signal detection theory (Wiley, 1994). In the present case, the probability that a

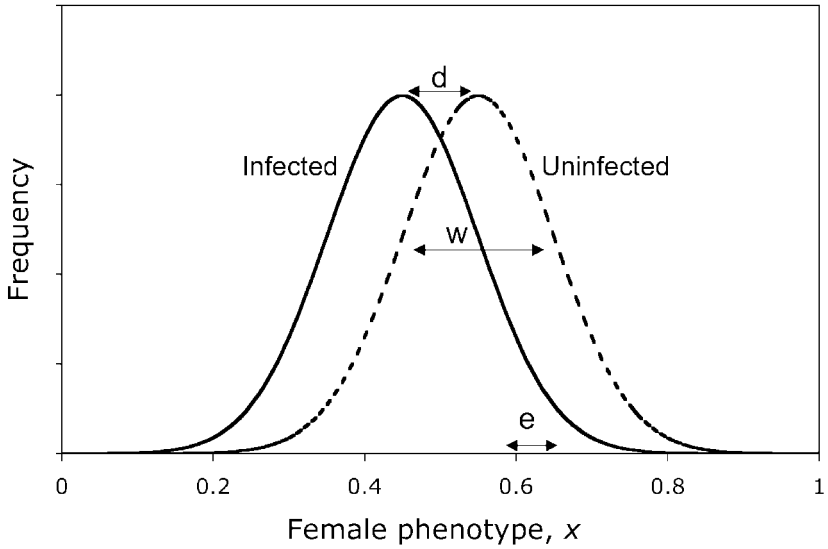

Fig. 3. Hypothetical frequency distribution of a quantitative variable (x) for infected and uninfected females. d = difference between the means of the two classes; w = standard deviation within classes; e = error associated with male estimates of female trait value.

female is infected depends on several factors (see Fig. 3), including: (1) the difference in mean phenotype between infected and uninfected females (d); (2) the phenotypic variation within infection classes (w); (3) the error (e) associated with a male's estimate of the female phenotype, where e can be considered an inverse measure of the accuracy of the male's assessment; and (4) the prevalence of *Wolbachia* infection within the insect population (P). For simplicity, assume that there is no error ($e = 0$) associated with a male's estimation of a female's phenotype. In this case, the probability that a female of phenotype x_i is infected can be expressed according to Bayes's theorem as:

$$\Pr(I|x_i) = \frac{\Pr(x_i|I)P}{\Pr(x_i|I)P + \Pr(x_i|U)(1-P)} \quad (1)$$

where $\Pr(I \mid x_i)$ is the probability that a female is infected given she has a phenotype x_i, $\Pr(x_i|I)$ and $\Pr(x_i|U)$ are the probabilities that a female has phenotype x_i, given that she is either infected (I) or uninfected (U) (Bradbury and Vehrencamp, 1998).

The incorporation of infection prevalence substantially complicates the situation for a choosy male. Figure 4A shows the probability of female infection when the mean phenotypic difference between infected and uninfected females (d) is equal to the standard deviation (w) within each class, as illustrated in Fig. 3. There are several important consequences of casting the female identification problem in this manner. First, if the prevalence of infection is either very high (as in some butterflies) or low [as in some other insects (see Hurst and Jiggins, 2000)], then almost all females encountered will be either infected or not infected, *regardless of their phenotypes*. Second, the prevalence of infection may vary through time or among populations, as is known to occur in *D. innubila* (Dyer and Jaenike, 2004, 2005). Consequently, the probability that females of a given phenotype x_i are infected would

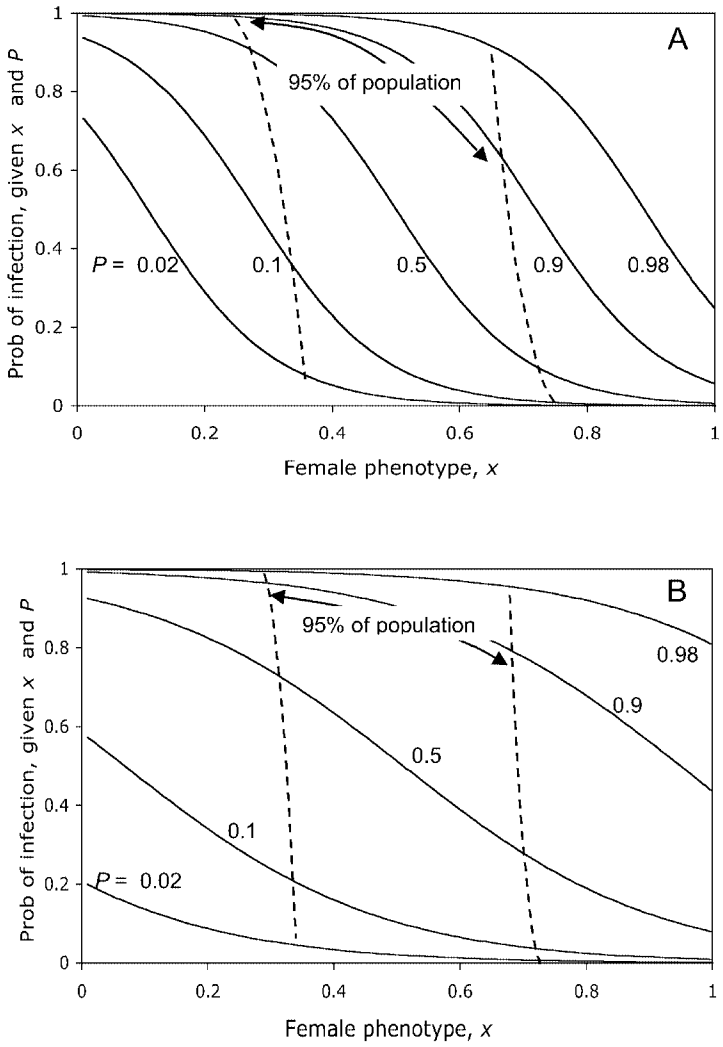

Fig. 4. Probability of a female being infected as a function of her phenotype x (see Fig. 3) and the prevalence of *Wolbachia* infection (P) among females in the population. (A) Mean (d) and standard deviations (w) as shown in Fig. 3. (B) As in (A), but the difference between means (d) is only half as great. The dashed lines encompass 95% of the phenotypic variation within a population, which varies as a function of P.

itself be variable. Under this scenario, males should prefer such females at some times, but discriminate against them at others. However, because fluctuations in infection prevalence probably occur much more rapidly than evolutionary changes in male preference, this may further impede the evolution of adaptive mate choice by males in species infected with male-killing endosymbionts.

In summary, in species infected with male-killing endosymbionts, males that mate with uninfected females pass on far more genes to future generations than those that mate

with infected females. Although this could favour the evolution of male preference for uninfected females, our laboratory studies of *D. innubila* revealed no evidence for such male discrimination. We suggest that the uncertainty associated with the assessment of a female's infection status may impede the evolution of such discrimination.

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