

# Increased male mating rate in *Drosophila* is associated with *Wolbachia* infection

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## Abstract

The maternally inherited bacterium *Wolbachia pipientis* infects 25–75% of arthropods and manipulates host reproduction to improve its transmission. One way *Wolbachia* achieves this is by inducing cytoplasmic incompatibility (CI), where crosses between infected males and uninfected females are inviable. Infected males suffer reduced fertility through CI and reduced sperm production. However, *Wolbachia* induce lower levels of CI in nonvirgin males. We examined the impact of *Wolbachia* on mating behaviour in male *Drosophila melanogaster* and *D. simulans*, which display varying levels of CI, and show that infected males mate at a higher rate than uninfected males in both species. This may serve to increase the spread of *Wolbachia*, or alternatively, may be a behavioural adaptation employed by males to reduce the level of CI. Mating at high rate restores reproductive compatibility with uninfected females resulting in higher male reproductive success thus promoting male promiscuity. Increased male mating rates also have implications for the transmission of *Wolbachia*.

## Introduction

The maternally inherited bacterium *Wolbachia pipientis* is one of the most prevalent endosymbionts of arthropods. It is estimated to infect between 20% and 75% of terrestrial species (Werren *et al.*, 1995; Jeyaprakash & Hoy, 2000; Stevens *et al.*, 2001) and is renowned for manipulating host reproduction in order to improve its own transmission. *Wolbachia* achieves this in a variety of ways (Hoffmann & Turelli, 1997; Rigaud, 1997; Stouthamer, 1997; Hurst & Jiggins, 2000) and frequently inflicts physiological and fitness costs on its host. Because of its potential impact on host fitness, *Wolbachia* is thought to influence both sexual selection and host reproductive strategies (Zeh & Zeh, 1996, 1997; Hatcher, 2000; Charlat *et al.*, 2003). However, little is known about the consequences of *Wolbachia* infection on either host reproductive behaviour or the utilization of beha-

vioural adaptations that may enable infected individuals to avoid or reduce the detrimental effects of *Wolbachia*.

The flies *Drosophila simulans* and *D. melanogaster* are both infected with *Wolbachia* that induce cytoplasmic incompatibility (CI). When the sperm of an infected male fertilize the ova of an uninfected female the resulting embryos undergo abnormal mitosis and die (Lassy & Karr, 1996; Tram & Sullivan, 2002). All other crosses remain viable. Reducing uninfected female fitness indirectly increases the spread of *Wolbachia* because the relative fitness of infected females that transmit *Wolbachia* is higher. Theoretical models predict CI-inducing *Wolbachia* to spread rapidly to fixation within its host population (Caspari & Watson, 1959; Frank, 1997); however, this is rarely seen in natural *Drosophila* populations (Hoffmann *et al.*, 1990; Hoffmann & Turelli, 1997; Vala *et al.*, 2004). Intermediate infection frequencies may result from incomplete maternal transmission of the parasite, natural host curing events and incomplete CI induction. CI usually results in high levels of embryonic mortality but there is significant variation between species. The source of this variation is thought to be varying bacterial levels in the testes (Clark & Karr, 2002).

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In *D. simulans*, almost 100% of offspring resulting from incompatible crosses die (Hoffmann & Turelli, 1988) whereas in *D. melanogaster*, CI induction is generally relatively low, and is only evident when males are young (Reynolds & Hoffmann, 2002; Clark *et al.*, 2003). In addition to inducing CI, *Wolbachia* can impose physiological costs on hosts. Infected *D. simulans* males produce approximately 40% fewer sperm than uninfected males, reducing their fertility (Snook *et al.*, 2000). It is not yet clear whether infected *D. melanogaster* males experience similar fertility reductions but this seems likely, particularly when males are young and *Wolbachia* induce higher levels of CI.

Alternatively, it has been suggested that hosts become 'adapted' to harbouring *Wolbachia*, resulting in a mutualistic relationship between the host and the bacterium. Models predict infections to become increasingly benign and levels of incompatibility to decrease over-time (Hoffmann & Turelli, 1997). This leads to the possibility that male fitness is improved by *Wolbachia* infection or alternatively, is reduced in the absence of *Wolbachia*. There is some evidence that *Wolbachia* increases female fitness in mosquitoes, although no effect on male fitness has been reported (Dobson *et al.*, 2002, 2004). Similarly, infected females of some strains of *D. melanogaster* have enhanced fecundity and survivorship in comparison with uninfected females. However, this does not appear to be consistent across the species and no positive effect on male fitness has been found (Fry & Wilkinson, 2004; Fry *et al.*, 2004).

Reduced sperm production has important implications for various aspects of host reproduction and behaviour. For example, *Wolbachia* infection reduces sperm competitive ability in *D. simulans* (Champion de Crespigny & Wedell *in press*), presumably because infected males are limited by sperm production. Similarly, male stalk-eyed flies carrying meiotic driving genes produce fewer sperm and suffer reduced sperm competitive ability because of smaller ejaculates and incapacitation of sperm by the seminal fluid of normal males (Wilkinson & Fry, 2001; Fry & Wilkinson, 2004). Sperm production may additionally limit the number of copulations achieved by males if copulation rate is regulated by the availability of sperm (Wedell *et al.*, 2002). Although this possibility has not been examined directly in *Drosophila* it is thought that accessory gland secretions may limit copulation frequency in *D. melanogaster* males (Lefevre & Jonsson, 1962).

Various aspects of male mating behaviour, such as mating rates, have been studied extensively in *D. melanogaster* (but less frequently in *D. simulans*). Most studies report a strong positive correlation between male mating success and fitness in *D. melanogaster* (Partridge & Farquhar, 1983; Partridge *et al.*, 1985, 1987a,b). In addition, there is significant heritable intra-specific variation in mating behaviour, such as courtship initiation and duration, which affect a male's mating ability or

success (Greenspan & Ferveur, 2000; Moehring & Mackay, 2004). Given the relationship between mating success and fitness, there should be strong selection on mating behaviours or adaptations that improve male mating success. Therefore, as *Wolbachia* affects host fitness, males may evolve mating strategies, such as variable mating rates, to counter the detrimental effects of *Wolbachia*.

Mating rates may be of particular importance to infected males. There is some evidence that male mating frequency affects the ability of *Wolbachia* to manipulate sperm. In both *D. simulans* and *D. melanogaster*, repeated mating leads to a decline in the level of CI induced by males (Karr *et al.*, 1998; Reynolds & Hoffmann, 2002). The level of CI expression is thought to be related to the rate of spermatogenesis, with faster spermatogenesis resulting in reduced CI (Karr *et al.*, 1998). Although CI induction declines with male age in *D. melanogaster*, the decline is more rapid under repeated mating (Reynolds & Hoffmann, 2002). Infected males whose sperm are not manipulated by *Wolbachia* do not suffer the fitness costs associated with CI, as crosses with uninfected females are compatible.

Here we investigate the possibility that *Wolbachia* infection affects male mating rate and copulatory behaviour in *D. melanogaster* and *D. simulans*. Decreased sperm production associated with *Wolbachia* infection may result in decreased mating rates in infected males compared with uninfected males because of more severe sperm limitation. Alternatively, infected males may mate at the same or higher frequency than uninfected males in order to purge *Wolbachia* manipulated sperm and restore reproductive compatibility with uninfected females. Male mating rates are examined in both *D. simulans* and *D. melanogaster* to allow comparison of the impact of *Wolbachia* infections that induce high or low levels of CI on male reproductive behaviour. High and low levels of CI are predicted to exert differing selective pressures on hosts to evolve mechanisms to avoid the manipulations of *Wolbachia*. Consequently, we expect any variation in mating frequency or copulatory behaviour to be greatest in *D. simulans* where *Wolbachia* induces high levels of CI and thus imposes larger fitness costs. We show that infected males of both species mate at higher rates than uninfected males. As predicted, we find the greatest difference in mating frequency in *D. simulans* where *Wolbachia* induces higher levels of CI.

## Materials and methods

### Experimental stock

The *D. simulans* stock arose from an iso-female line of flies infected with *Wolbachia* that was originally collected from Riverside, California and maintained in laboratory populations for at least 2 years. Uninfected flies were obtained by antibiotic treatment approximately

9 months prior to use in the experiment: two consecutive generations were raised on food containing 0.03% tetracycline hydrochloride. Approximately eighty infected females (enclosed with eighty males) laid eggs on the tetracycline medium. Similar numbers of first generation offspring interbred and laid eggs on new tetracycline medium. More than 150 female (and >150 male) generation two offspring founded the uninfected population. The *D. melanogaster* derived from an iso-female line established from an OregonR (OreR) laboratory strain infected with *Wolbachia*. Similar to *D. simulans*, uninfected flies were obtained by antibiotic treatment, however in *D. melanogaster*, three generations were raised on tetracycline medium and the curing process was undertaken approximately 18 months prior to the experiment. Between 50 and 100 flies of each sex contributed offspring to each generation. Given the lengths of time between tetracycline treatment and experiment for both species, it is unlikely that uninfected flies suffer from reduced gut flora or other side effects of antibiotic treatment. Stock populations, consisting of thousands of infected and uninfected flies, were maintained in the same way with a constant supply of food/medium on which to lay eggs. This, in combination with low genetic variation caused by inbred iso-female lines, reduces the likelihood that populations experienced different selection regimes and/or have diverged as a result of genetic drift. Therefore, any difference between flies of opposing infection status can be attributable to the presence/absence of *Wolbachia*. Flies were maintained on a standard low yeast *Drosophila* medium. One litre of medium consists of 10 g of Agar, 85 g of sugar, 60 g of oats, 20 g of yeast, 1 g of Nipagin and 1 L of water.

In our flies, offspring mortality in incompatible *D. simulans* crosses is typically >95% when males are young and virgin. However, infected males taken at random from the stock population usually produce some viable offspring when mated to uninfected (incompatible) females. This indicates that CI induction declines in nonvirgin, older males. Offspring mortality is similarly high in incompatible *D. melanogaster* crosses when males are 1- to 2-day-old virgins. However, CI declines rapidly and does not appear to be induced by older nonvirgin males.

The infection status of flies of both species was confirmed by polymerase chain reaction (PCR). DNA was extracted from whole flies with a salt extraction. *Wolbachia* was detected using universal *Wolbachia* specific primers (Wsp 81F and Wsp 691R) that amplify the surface protein gene (Zhou *et al.*, 1998). To confirm that the PCR correctly amplified *Wolbachia* DNA, the PCR product of three infected *D. simulans* and three infected *D. melanogaster* samples was purified and the DNA sequenced. The PCR product was purified using a Qiagen purification kit (Quiquick PCR purification) and sent to Lark Technologies (Houston, TX, USA) for sequencing. The sequences were compared using Blast and the

sequences were confirmed to be homologous with the expected *Wolbachia* strain in each species: *w*Ri in *D. simulans* (Braig *et al.*, 1998) and *w*Mel in *D. melanogaster* (Zhou *et al.*, 1998). All individuals screened for *Wolbachia* showed the correct infection status after PCR.

### Collection of virgins

Eggs were collected from stock populations and the larvae reared at 25°C with a 12:12-h light-dark cycle in density-controlled vials (25 larvae per vial containing 6 mL of food medium). Approximately 700 larvae of each infection status were collected daily. Vials were inspected every 6 h (three times during the light cycle) for newly eclosed flies. All vials were emptied of newly emerged flies, combined within infection groups (i.e. infected and uninfected combined separately) and the new adults were chilled on ice, sexed and the sexes placed in separate, density-controlled vials (≤40 adults per vial) containing food. This process maximizes randomization of adult flies and eliminates any effect of larval rearing vial from the experimental design. Before being used in experiments, all vials containing females were inspected for larvae as an additional confirmation of virginity status.

### Experiment design and procedure

To determine male mating rate, 40 infected and uninfected *D. melanogaster* males and 35 infected and uninfected *D. simulans* males were placed in individual vials containing a standard amount of fly medium and maintained at 25°C over night. All males were 1 day old on the first day they were exposed to females. The following day, three virgin (3–6 days old) females were placed, using a pipette, into each vial. Both infected and uninfected females were randomly allocated to males after being mixed prior to the experiment. Both personal observation and previous studies of mating preferences have found no evidence of assortative mating by males based on female infection status (Hoffmann & Turelli, 1988; Hoffmann *et al.*, 1990). The females were chilled on ice to prevent their escape and facilitate transfer to the vials. They typically became active within a minute of their introduction. The time females were present in the vials was recorded and the vials were observed constantly for 6 h (*D. melanogaster*) or 4 h (*D. simulans*) each day for three sequential days. *Drosophila simulans* were observed for a shorter period of time owing to their rapidity of mating and there being a limited supply of females. The experiment was started at the same time each day (within 2 h of 'lights on'). When males commenced their third, sixth or ninth copulation, three additional virgin females were added to the vial via pipette. These females usually recovered from the chilled transfer process before the male had finished copulating. Therefore, males had constant access to virgin females with

which to mate. We recorded the times at which males commenced each mating and, in *D. simulans*, the time copulations were completed. Copulation duration was not recorded in *D. melanogaster* for logistical reasons. At the end of the observation period, the females were removed from each of the vials and the time of removal recorded. The experiments were performed in a constant temperature room (25°C) with one artificial light source from above.

After the experiment was completed all males were frozen for size measurement. Both wings were dissected and male size was measured as wing length: the distance between the intersection of the anterior cross vein and longitudinal vein 3 (L3) and the intersection of the L3 with the distal wing margin (Partridge *et al.*, 1987b). Wings were mounted on microscope slides and measured using the biometrics program, MicroMeasure (version 3). The average size of both wings was calculated where possible. However, some wings were damaged during the dissection process and these were discarded from the analysis.

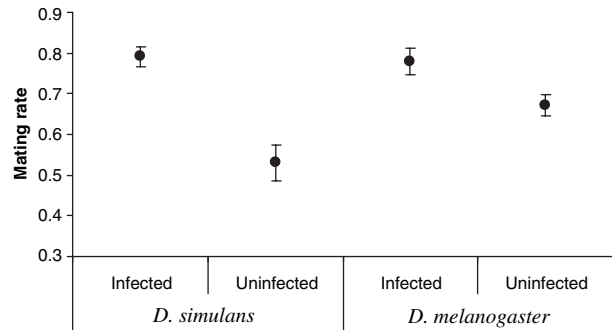
### Statistics

In order to take in to account variation in exposure time to females, male mating rates (the number of copulations per hour of female exposure) were calculated for each male as an estimate of his mating capacity. Mating rates over the 3 days of the experiment were analysed in REML variance components analyses with male size as a covariate and the residuals of the models were inspected for normality. Average mating rates were analysed in general linear models with male size as a covariate. Data are presented as mean  $\pm$  SE unless otherwise specified. Males that did not copulate at all during the 3 days of the experiment were excluded from the analyses. This resulted in the exclusion of seven uninfected but no infected *D. simulans* males. All *D. melanogaster* males copulated on at least 1 day of the experiment. The data were analysed using SPSS 11.5 and GENSTAT (7th edition).

## Results

### *Drosophila simulans*

*Wolbachia* had a significant effect on male mating rates (REML VCA: Wald<sub>1,61</sub> = 24.73,  $P < 0.001$ ). Infected males mated significantly more frequently than uninfected males on each day of the experiment and when mating rates were averaged for the duration of the experiment (GLM:  $F_{1,61} = 29.55$ ,  $P < 0.001$ ; Fig. 1). On average, infected males mated 49.01% more frequently than uninfected males. In addition, mating rates increased significantly over the three experimental days (REML VCA: Wald<sub>2,61</sub> = 16.14,  $P < 0.001$ ). Although there was no interaction between *Wolbachia* infection and experimental day, increasing mating rates were only



**Fig. 1** Mean  $\pm$  SE mating rates (number of copulations per hour) of infected and uninfected *Drosophila simulans* and *D. melanogaster*. The mating rates presented are the average for each male across the 3 days of the experiment.

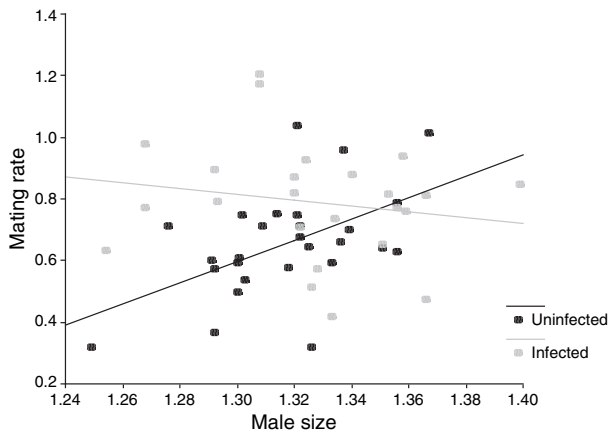
apparent in infected males (Friedman test:  $\chi^2_2 = 17.15$ ,  $P < 0.001$ ) as the mating rate of uninfected males remained similar throughout the experiment (Friedman test:  $\chi^2_2 = 0.50$ ,  $P = 0.779$ ). There was a significant effect of male size on mating rate (REML VCA: Wald<sub>1,61</sub> = 6.05,  $P = 0.014$ ) but there was no interaction between *Wolbachia* and male size (REML VCA: Wald<sub>1,61</sub> = 0.06,  $P = 0.805$ ), and no difference in size between infected and uninfected males (Independent samples  $t$ -test:  $t_{59} = 1.90$ ,  $P = 0.850$ ). Interestingly, there is no effect of male size on mating rate when mating rate is averaged over the three experimental days.

In addition to mating at higher rate, on average, infected males copulated for greater lengths of time (Independent samples  $t$ -test:  $t_{61} = 3.315$ ,  $P = 0.002$ ) than uninfected males. The mean copulation duration for infected males was  $26 \pm 0.5$  min compared with  $22 \pm 1.0$  min for uninfected males. Furthermore, the average time interval between mating (the time between the completion of one copulation and the start of the next) throughout the experiment was significantly shorter for infected males than uninfected males (Independent samples  $t$ -test:  $t_{59} = -2.342$ ,  $P = 0.025$ ). Typically, infected males began mating with a new female  $42 \pm 2.0$  min after they finished mating with the previous one, whereas the interval between mating for an uninfected male was  $59 \pm 7.0$  min.

In summary, *Wolbachia*-infected male *D. simulans* (i) mate for longer periods of time and (ii) have a shorter interval between mating, than uninfected males. As a result, infected males mate more frequently than uninfected males in a given time period. In contrast to uninfected males, infected males also increase their mating rate over the 3 days of the experiment.

### *Drosophila melanogaster*

Similar to *D. simulans*, *Wolbachia* infection in *D. melanogaster* had an effect on mating rate with infected males



**Fig. 2** The relationship between male body size [wing length (mm)] and average (over 3 days of experiment) male mating rate (number of copulations per hour) in infected and uninfected *Drosophila melanogaster*.

mating at a higher rate than uninfected males on each day of the experiment (REML VCA: Wald<sub>1,50</sub> = 7.21,  $P = 0.007$ ) and when mating rates were averaged for the experiment as a whole (GLM:  $F_{1,75} = 6.667$ ,  $P = 0.012$ ; Fig. 1). On average, infected males mated 15.9% more frequently than uninfected males. In contrast to *D. simulans*, there was no effect of experiment day on mating rates. Although there is no difference in body size between infected and uninfected males (Independent samples  $t$ -test:  $t_{49} = 1.132$ ,  $P = 0.263$ ), and no effect of male size on mating rates (REML VCA: Wald<sub>1,50</sub> = 0.60,  $P = 0.440$ ), there was a significant interaction between *Wolbachia* infection and male size on mating rate (REML VCA: Wald<sub>1,50</sub> = 6.49,  $P = 0.011$ ). The mating rate of uninfected males increases with male size (Pearson:  $r = 0.519$ ,  $n = 27$ ,  $P = 0.006$ ; Fig. 2), but there is no relationship between male size and mating rate in infected *D. melanogaster* males (Pearson:  $r = -0.174$ ,  $n = 24$ ,  $P = 0.417$ ).

There was no difference in the average time interval between consecutive mating (measured here as the difference between the starting times of the mating) throughout the experiment (Mann–Whitney  $U$ :  $Z = -0.085$ ,  $n = 75$ ,  $P = 0.932$ ) between infected and uninfected *D. melanogaster* males.

In summary, infected *D. melanogaster* males copulate at a higher rate than uninfected males. Because there is no difference between infected and uninfected males in the time interval between consecutive mating, it appears that uninfected males become reluctant to copulate with females earlier than infected males.

## Discussion

These results are the first demonstration of an association between *Wolbachia* that induce CI and male mating

behaviour. *Wolbachia*-infected males of both *D. simulans* and *D. melanogaster* mate at a higher rate than uninfected males. Furthermore, infected male *D. simulans* copulate for longer periods of time and the interval between mating is shorter than for uninfected males. Based on these results it seems unlikely that male mating rate is limited by sperm production: despite producing fewer sperm (Snook *et al.*, 2000), infected male *D. simulans* mate at higher rate than uninfected males.

There are two possible explanations for these findings: either *Wolbachia* or the male host benefit from increased male mating rate. Parasite mediated manipulation of host behaviour in order to improve transmission is well documented (Moore & Gotelli, 1996). *Wolbachia* would benefit from elevated male mating rate providing it is linked to an increased rate of CI induction, which causes *Wolbachia* to spread more rapidly through populations. However, this depends on the level of CI induced at each mating. If high levels of CI are induced in crosses between infected males and uninfected females irrespective of male mating history, then offspring of uninfected females will be killed, promoting the spread of *Wolbachia*. If CI instead declines rapidly to zero through repeated male mating, then reduced CI under high male mating rate will undermine the transmission advantage to *Wolbachia*. Empirical data from both *D. melanogaster* and *D. simulans*, suggest the decline in CI associated with mating history is rapid and dramatic (Karr *et al.*, 1998; Reynolds & Hoffmann, 2002). Therefore, repeated male mating could reduce the transmission advantage of *Wolbachia*. However, there is likely to be an optimal rate at which the spread of *Wolbachia* through high mating rate is balanced by the reduction of CI as a consequence of male mating rate *per se*. This will depend on the precise relationship between male mating rate and the level of CI induced; *Wolbachia* will be favoured by high male mating rate if it overall results in higher rate of CI in crosses with uninfected females, whereas *Wolbachia* will lose its transmission advantage if repeated male mating is associated with additional reductions in CI. Further documentation of the consequences of repeated mating by infected males for CI induction in uninfected females is required, and ideally the relationship between the rate of CI induction in relation to male mating rate and mating history modelled.

The decrease in CI induction associated with increased male mating frequency could instead benefit the male host. Nonvirgin *D. simulans* males express approximately 50% less CI than virgin males (Karr *et al.*, 1998) whereas nonvirgin *D. melanogaster* males express approximately 60% less CI than virgin males (Reynolds & Hoffmann, 2002). Reduction in CI induction of this magnitude dramatically improves the fitness of infected males because they regain reproductive compatibility with the uninfected females in the population. This creates a strong selective advantage on males to mate at high rate

when infected with *Wolbachia*, promoting higher levels of male promiscuity.

The question remains why uninfected males mate at lower rate? Mating is known to be costly in *Drosophila*. For instance, multiple mating in *D. melanogaster* reduces male fertility (Lefevre & Jonsson, 1962; Markow *et al.*, 1978), suppresses immune function (McKean & Nunney, 2001) and reduces longevity (Partridge & Farquhar, 1983). Infected males may trade off the costs associated with high mating frequency against the benefits of reducing CI. Because uninfected males are compatible with both infected and uninfected females, there is no additional fitness gain from mating at higher frequency through restoring compatibility. Therefore, mating at higher frequency may generate greater costs than benefits in uninfected males. Many studies suggest that *Drosophila* males are incapable of fertilising eggs beyond three or four consecutive mating within a short period of time (Lefevre & Jonsson, 1962; Markow *et al.*, 1978). Infected males in our study mated as many as 11 times within a 4- to 6-h period. Hence increased mating rates could be disadvantageous for uninfected males, but may be a response triggered by the presence of *Wolbachia* in infected males that ultimately increases their fitness. The fitness consequences for infected and uninfected males of varying mating history are currently being investigated.

How males might recognize their infection status and adjust their mating behaviour remains unknown. However, behavioural plasticity in terms of mating strategies such as copulation duration, ejaculate size, nuptial gift size and mating rates is well-documented in many taxa and attributed to a variety of reasons including the risk of sperm competition (Cook & Gage, 1995; Wedell & Cook, 1999; del Barco-Trillo & Ferkin, 2004; Garcia-Gonzalez & Gomendio, 2004), variation in female quality (Gage, 1998; Engqvist & Sauer, 2001) and in response to parasitic infections (Polak & Starmer, 1998). Plastic behavioural responses to *Wolbachia* by *Drosophila* seem likely when one considers that host populations typically evolve under intermediate infection frequencies and incomplete maternal transmission of *Wolbachia* (Hoffmann & Turelli, 1997). Because infected females frequently produce both infected and uninfected offspring, plastic behavioural responses to *Wolbachia* infection are likely to have a selective advantage to males. It is unknown whether this might apply to other species infected with CI-inducing *Wolbachia*, however plastic behavioural adaptations, e.g. mate choice have been demonstrated in female spider mites infected with CI-inducing *Wolbachia* (Vala *et al.*, 2004).

Despite finding increased mating rates in *Wolbachia*-infected males in two species of *Drosophila*, without further survey in different taxa it is not possible to evaluate the generality of our results. Our findings may be specific to our particular *Drosophila* strains. This could arise by chance or because, at least in the case of *D. melanogaster*, the consequences of *Wolbachia* infection

for host fitness may be dependent on interactions between host and *Wolbachia* genotypes (Olsen *et al.*, 2001; Fry *et al.*, 2004). If arising by chance, it is interesting that we find increased male mating rates in two species of fly where there is no *a priori* reason to expect mutations to manifest in the same behavioural response. Although it seems unlikely that male mating rates are dependent on host/*Wolbachia* genome interactions, extrapolation of our findings to other species/strains infected with *Wolbachia* must be treated with caution.

It is possible that the association between *Wolbachia* and male mating rates arises from a benefit of infection. *Wolbachia* infections are predicted to become increasingly benign and potentially confer benefits to the host over-time (Hoffmann & Turelli, 1997). However, this seems unlikely because, although fitness benefits of infection have been demonstrated in females of some species (Dobson *et al.*, 2002, 2004; Fry *et al.*, 2004), fitness advantages to males have so far not been found. On the contrary, there is substantial evidence of significant fitness costs of *Wolbachia* infection to male *Drosophila* (O'Neill *et al.*, 1997; Snook *et al.*, 2000). Similarly, it is unlikely that these results arise from differences in maturation time between infected and uninfected males in either species. There is no apparent difference in development time or onset of reproduction between infected and uninfected males (F.E. Champion de Crespigny, personal observations). Infected and uninfected males are both capable of fertilising eggs at 1 day. Additionally, no consistent effect of infection status on male longevity has been found in either *D. melanogaster* (Fry *et al.*, 2004) or *D. simulans* (Champion de Crespigny & Wedell, unpublished data). Hence it is unlikely that increased mating rates by infected males represent a terminal investment in reproduction. A final possibility is that the lower mating rate of uninfected males could be a consequence of infection loss, if mating rate is a co-adaptation to the presence of *Wolbachia*. More simply, mating rate may be an unselected consequence of adaptation to the presence of *Wolbachia*, and curing flies of their infection could result in a simultaneous loss of mating capacity. However, this premise relies on a strong linkage between mating rate and infection, i.e. consistent across species.

Mating success in *D. melanogaster* is frequently positively associated with male body size (Partridge & Farquhar, 1983; Partridge *et al.*, 1987a,b). There was no difference in size between infected and uninfected males in this study so male size cannot explain the higher mating rates associated with *Wolbachia* infection. Although no relationship was found between body size and mating rate in infected *D. melanogaster*, mating rates were positively correlated with male size in uninfected flies. This supports previous findings (Partridge & Farquhar, 1983; Partridge *et al.*, 1987a,b), although it is evident that *Wolbachia* infection overrides any effect of male size as infected males copulate at consistently high frequency in *D. melanogaster*.

Male mating rates in natural populations are unlikely to be as extreme as we, and others find in the laboratory (Bateman, 1948; Lefevre & Jonsson, 1962). This is primarily because of access to receptive females. Because we were specifically interested in male mating behaviour we ensured male mating rate was not limited by female availability in this study. Although little is known about male mating rates in the field it seems unlikely that males have a constant supply of receptive females. Males probably wait by food sources/oviposition sites and court all females that arrive at the site (Markow, 1988). Female availability will depend on population size and willingness to mate. It is likely that males will court previously mated females more frequently than virgin females (Partridge *et al.*, 1987b; Gromko & Markow, 1993). Our *D. simulans* females remate readily after 24 h (although this could also be a laboratory phenomenon) so it is possible that many of the nonvirgin females arriving at a site will be willing to mate (but see Gromko & Markow, 1993). However, even if males do not copulate frequently, this may not alter the selective advantage of mating at high rate. Previous work by Karr *et al.* (1998) and Reynolds & Hoffmann (2002) indicates that only relatively few mating (compared with the number our males performed) are required to substantially decrease CI induction.

This study provides an opportunity to compare the impact of fitness variation resulting from high and low CI-inducing *Wolbachia* infections on the strength of selection on host behavioural adaptations. Because CI induction in *D. simulans* is higher than in *D. melanogaster*, a stronger response is expected. This prediction is corroborated by the finding that a greater difference in mating rate between uninfected and infected males is present in *D. simulans* (~50%) compared with *D. melanogaster* (~16%). However, despite the typically low levels of CI in *D. melanogaster*, males still display a behavioural response associated with *Wolbachia* infection.

The findings of this study have important implications for *Wolbachia* population dynamics. Reducing CI induction may undermine the transmission advantage of *Wolbachia* and consequently decrease its rate of spread, although this will depend on the precise relationship between male mating rate and mating history and the overall level of CI induced. Furthermore, this process could extend the length of time *Wolbachia* remains at low frequency in populations when it may be vulnerable to stochastic events, such as natural curing, which may cause its loss from populations (Champion de Crespigny *et al.*, 2005). In contrast to theoretical predictions, host populations of *Drosophila* sometimes display intermediate levels of infection in the wild (Hoffmann *et al.*, 1990; Turelli & Hoffmann, 1995; Vala *et al.*, 2004). Loss of CI induction through increased male mating rates may contribute to the maintenance of such infection polymorphisms in the wild as the presence of uninfected females maintains the selective advantage to infected

males of mating at high rates. The frequency of uninfected females in a population manipulated by *Wolbachia* will affect the selective advantage of males mating at high frequency. When the infection invades a population and the majority of females are uninfected, the selective advantage to infected males is greater than when *Wolbachia* is near fixation. However, maternal transmission failure of *Wolbachia*, and natural curing are thought to provide a constant source of uninfected individuals and this may be sufficient to maintain the selective advantage of high mating rates in infected males. Although surveys of levels of CI-inducing *Wolbachia* infections within wild populations of other species are generally lacking, a significant number reveal intermediate infection frequencies (see Vala *et al.*, 2004 for a review). This is in contrast to male-killing *Wolbachia* in butterflies for example, where it can reach almost 100% prevalence in some populations (Dyson & Hurst, 2004). Consequently, it would be interesting to investigate male mating rates in other species manipulated by CI-inducing *Wolbachia*.

In conclusion, this is the first study to identify a potential male behavioural adaptation that may have evolved in response to the selfish manipulations of CI-inducing *Wolbachia*. We suggest that *Wolbachia* infected males mate at high frequency in order to reduce the induction of CI. This restores reproductive compatibility with uninfected females, increasing male fitness. The selective advantage of this strategy depends critically on the availability of receptive, uninfected females and further work is required to elucidate the exact mechanisms involved and to confirm CI as the driving force behind increased male mating rates. This study highlights the potential importance of *Wolbachia* infection in sexual selection.

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