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The impact of *Wolbachia*, male age and mating history on cytoplasmic incompatibility and sperm transfer in *Drosophila simulans*

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Abstract

Most insects harbour a variety of maternally inherited endosymbionts, the most widespread being *Wolbachia pipientis* that commonly induce cytoplasmic incompatibility (CI) and reduced hatching success in crosses between infected males and uninfected females. High temperature and increasing male age are known to reduce the level of CI in a variety of insects. In *Drosophila simulans*, infected males have been shown to mate at a higher rate than uninfected males. By examining the impact of mating rate independent of age, this study investigates whether a high mating rate confers an advantage to infected males through restoring their compatibility with uninfected females over and above the effect of age. The impact of *Wolbachia* infection, male mating rate and age on the number of sperm transferred to females during copulation and how it relates to CI expression was also assessed. As predicted, we found that reproductive compatibility was restored faster in males that mate at higher rate than that of low mating and virgin males, and that the effect of mating history was over and above the effect of male age. Nonvirgin infected males transferred fewer sperm than uninfected males during copulation, and mating at a high rate resulted in the transfer of fewer sperm per mating irrespective of infection status. These results indicate that the advantage to infected males of mating at a high rate is through restoration of reproductive compatibility with uninfected females, whereas uninfected males appear to trade off the number of sperm transferred per mating with female encounter rate and success in sperm competition. This study highlights the importance *Wolbachia* may play in sexual selection by affecting male reproductive strategies.

Introduction

Most insects harbour a variety of maternally inherited endosymbionts, the most widespread being the α-proteobacterium *Wolbachia pipientis* that is estimated to infect between 20 and 75% of all insects (Werren *et al.*, 1995; Stouthamer *et al.*, 1999; Jeyaprakash & Hoy, 2000; Charlat *et al.*, 2003); a recent meta-analysis suggested that 66% of insect species harbour *Wolbachia* (see Hilgenboecker *et al.*, 2008). *Wolbachia* manipulate host reproductive systems to increase its transmission by inducing parthenogenesis, feminization, male killing or cytoplasmic incompatibility (CI) – the most common form of reproductive manipulation resulting in reduced hatching success. Unidirectional CI occurs when the sperm of infected males fertilize the eggs of uninfected females, whereas reciprocal crosses and matings between infected males and females are viable (Yen & Barr, 1971; Callaini *et al.*, 1996; Lassy & Karr, 1996; O'Neill *et al.*, 1997; Tram *et al.*, 2003). The presence of *Wolbachia* in the testes modifies sperm requiring a corresponding *Wolbachia*-induced rescue by the egg of the modified sperm at fertilization to ensure successful

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production of infected offspring (Werren, 1997). CI results in early embryo developmental failure and death due to disruption of the pronuclear chromatin condensation and misalignment of the chromosomes during mitosis (Riparbelli *et al.*, 2007), although the molecular mechanism is as yet unknown. CI may cause complete clutch failure although a range of CI expression (proportion of viable offspring) has been observed (O'Neill *et al.*, 1992; Giordano *et al.*, 1995; Sinkins *et al.*, 1995). CI is also induced when males and females are infected with different strains of *Wolbachia* (bidirectional incompatibility) (Yen & Barr, 1971; O'Neill & Karr, 1990; Turelli *et al.*, 1992; Rousset & Solignac, 1995; Charlat *et al.*, 2003).

The reproductive manipulations of *Wolbachia* causing CI can have a far-ranging impact on sexual selection and host reproductive strategies (Wedell, 2013). For example, male Mediterranean flour moths *(Ephestia kuehniella)* infected with CI-inducing *Wolbachia* produce fewer fertile sperm than uninfected males, whereas there is no impact of *Wolbachia* infection on nonfertile sperm production (Lewis *et al.*, 2011). Similarly, male *Drosophila simulans* infected with the *wRi* strain produce fewer sperm cysts than uninfected males (Snook *et al.*, 2000) and as a consequence are poor sperm competitors compared to uninfected males (Champion De Crespigny & Wedell, 2006). Furthermore, uninfected female *D. simulans* mating to both an infected and an uninfected male can recuperate their fitness loss due to CI by promoting sperm competition and reduce the probability of fertilizing their eggs with infected males' sperm (Champion de Crespigny *et al.*, 2008). In addition, infected male *D. simulans* and *D. melanogaster* have been shown to mate at a higher rate than uninfected males. It is suggested that this may be a male behavioural adaptation to reduce the level of CI and restore reproductive compatibility with uninfected females, assuming that the level of CI decreases with increasing male mating rate (Champion De Crespigny *et al.*, 2006). However, this is as yet unknown and is the focus of the current study.

Increased mating rates by infected males should generally be favoured if it restores reproductive compatibility with uninfected females or, in the case of bidirectional incompatibility, with infected females that harbour other *Wolbachia* strains. CI is known to decline with increasing male age in several species including *D. simulans* (Turelli & Hoffmann, 1995; Karr *et al.*, 1998; Reynolds & Hoffmann, 2002; Weeks *et al.*, 2007), but so far the mechanism by which male ageing decreases CI expression is not clear. It is suggested that *Wolbachia* density in the testes may play a role; weaker CI in older males is associated with lower *Wolbachia* density in testes (Binnington & Hoffmann, 1989; Bressac & Rousset, 1993; Clark *et al.*, 2002; Veneti *et al.*, 2003). In addition, the length of time that sperm spend in the males' testes directly impacts upon CI expression. Karr *et al.* (1998) demonstrated that the sperm of previously mated males spent less time in contact with *Wolbachia* in the testes resulting in weaker CI expression. In contrast, sperm of young virgin males that had spent longer time in contact with *Wolbachia* during development was associated with stronger CI expression (Karr *et al.*, 1998). Therefore, high turnover of sperm through repeated mating could potentially reduce CI induction by limiting the exposure to *Wolbachia* in the testes.

Alternatively, high mating rate among infected males may be advantageous to *Wolbachia*. If infected males mate with more females than uninfected males, the overall level of CI in the population will initially increase due to incompatible pairings with uninfected females and *Wolbachia* will spread. This is because infected females will have greater reproductive success relative to uninfected females than if infected and uninfected males mated at the same rate. However, the transmission advantage for *Wolbachia* depends on high levels of CI being induced in all copulations irrespective of male mating history, which is currently not known. Karr *et al.* (1998) have shown that mated *D. simulans* males induced lower CI expression than virgin males. However, they did not disassociate mating history and male age, and thus, the relative impact on CI of mating at high rate was not assessed.

The aim of this study was to determine the consequences of varying mating rates in *Wolbachia*-infected male *D. simulans* on the level of CI induced. We examined the hypothesis that high mating rates may accelerate the rate of compatibility restoration (i.e. decline in CI induction) over and above the documented effect of male age in *D. simulans*. We quantified the number of sperm transferred to females during copulation by uninfected and infected males of varying ages and mating rates, and the level of CI induced in crosses between infected males and uninfected females. This allowed us to independently examine the impact of male infection status, male mating history and male age on the number of sperm transferred to virgin females during copulation and the resulting hatching success. We show that, as predicted, reproductive compatibility was restored faster in infected males that mate at higher rate and that this effect was over and above the effect of male age on CI induction.

Materials and methods

Study species

The impact of male age and mating rate on CI induction and sperm transfer was investigated in *D. simulans*. Flies infected with the *Wolbachia Riverside* strain *(wRi)* were originally collected from Riverside, California (USA), and maintained in large laboratory populations since 2003 (obtained from G. Hurst, UCL, that originate from the Riverside '89 strain as described in Hoffmann

et al., 1990). Uninfected flies were obtained by antibiotic treatment (tetracycline hydrochloride) of large numbers of infected flies at least 2 years before the experiment. The large numbers of individuals involved in the infection elimination process mean that it is unlikely that the uninfected population suffers from inbreeding depression or differs in genetic background from the infected population. Thus, any differences between the infected and uninfected populations are likely due to the presence/absence of *Wolbachia*. Both infected and uninfected populations were maintained under the same conditions at 25 °C on a 12:12-h light/dark cycle with a constant supply of food and medium on which to lay eggs.

The infection status of flies from both infected and uninfected populations was confirmed by polymerase chain reaction (PCR). Universal *Wolbachia*-specific primers (Wsp 81F and Wsp 691R) were used. The infection status was confirmed before and after the experiment using the procedure as described in Champion De Crespigny *et al.* (2006). The expected infection status was confirmed in all cases.

Egg and larvae collection

Eggs were collected from stock populations by inserting small petri dishes containing a standard egg laying medium (12.5 g agarose, 275 mL deionized water, 150 mL grape juice and 0.5 g Nipagin) and 0.5 g of yeast paste into the population cages. The amount of yeast was controlled in case this affected male spermatogenesis. Eggs were collected every 24 h for 8 successive days. Larvae were collected in the morning with a metal pointer and placed into vials containing 15 mL of *Drosophila* medium. They were reared on standard low yeast *Drosophila* medium (10 g agarose, 20 g yeast, 85 g granulated sugar, 60 g maize, 1000 mL deionized water and 1 g Nipagin). Only 25 individual larvae were put into each vial in order to control the density of larvae and ensure that larvae had *ad libitum* food during the rearing process. The flies were reared on a 12:12-h light/dark cycle. Approximately 500 larvae of each infection status were collected daily.

Adult collection and sexing

Approximately 8–9 days after the eggs were laid, the adults began to eclose. The vials were inspected every 6 h (three times during the light cycle) for newly eclosed flies. New adults were chilled on ice, sexed, and the sexes were placed in separate vials containing *Drosophila* medium with 40 individuals per vial. This process ensured that all adult flies were virgins at the beginning of the experiment. Both male and female flies were kept at 25 °C on a 12:12-h light/dark cycle.

CI Induction experiment

In order to examine the impact of mating history and male age on CI induction, male mating rate and mating history were carefully manipulated. To investigate the impact of mating rate on CI and to distinguish the impact of mating rate from the impact of ageing, three different treatments were used. First, to control for the effect of male age on CI induction, virgin infected males aged 5, 6, 7, 8 or 11 days old were mated to virgin uninfected females and CI/hatching success scored (see below for procedure and also Table 1) (one mating only for each male at a set age). This treatment is referred to as 'senescence control – SC'. Under the second treatment – low mating rate (LMR), males mated with a single virgin uninfected female when they were 4, 5, 6, 7, 8 and 11 days old, respectively (6 matings in total for each individual male), and CI/hatching success scored for each of these matings (see below). In the final treatment – high mating rate (HMR), males were mated to three virgin uninfected females when they were 4, 5, 6, 7, 8 and one mating when they were 11 days old (16 matings in total for each individual male). Only the first female that an HMR male mated with on each day was used to score CI/hatching success.

At the beginning of the experiment, 3-day-old male flies were put into 7.5 × 2.5 cm mating vials (one male/vial). They were divided into SC, LMR and HMR treatments randomly with a sample size of 15, 20 and 25 flies, respectively. For the SC treatment, the sample size was 15 males of each individual age. Single virgin uninfected female flies (4–5 days old) were added to each vial at the beginning of each day's matings (according to the treatment) and were removed after copulation had completed. We recorded the time males started mating (TSM) and the time males finished mating (TFM). As soon as mating finished, female flies were transferred to a coloured low yeast *Drosophila* medium to oviposit. Eggs were incubated for 24 h at 25 °C on a 12:12-h light/dark cycle. 24 h after, TFM females were removed, frozen and the infection status

Table 1 Experimental design of sperm transfer to uninfected females at mating. Males were mated when they were 4, 5, 6, 7, 8 and 11 days old, respectively, in each cross. In all cases, male flies were mated with uninfected females.

Treatments	Males	Sample size	Mating history of males
Senescence males	Infected	15 per age	Virgin male and a single mating per day
	Uninfected	15 per age	Virgin male and a single mating per day
Low mating rate	Infected	20	6 matings in total per male
	Uninfected	20	6 matings in total per male
High mating rate	Infected	25	16 matings in total per male
	Uninfected	25	16 matings in total per male

of the females was later confirmed by PCR. The eggs laid by the females in the 24 h after mating were counted twice: 48 and 72 h after TFM. However, at 72 h, only the unhatched eggs were counted.

The percentage of all the eggs laid by each individual female during the 24-h period after mating that subsequently hatched is termed hatching success. Hatching success percentage was used as a measurement of CI expression. Full CI expression would result in 0% hatching success. An increase in the percentage of eggs that hatched means a decline in CI expression and a restoration of reproductive compatibility. Thus, CI is negatively related to hatching success.

Sperm transfer experiment

Treatments and mating procedure

Three infected and three uninfected treatments (Table 1) were used to investigate the effects of infection status, male age and mating rate on the number of sperm transferred to a female at copulation. In all cases, the females were uninfected with *Wolbachia*. Infected and uninfected males were randomly allocated to SC, LMR and HMR treatments (as per the CI induction experiment described previously) (Table 1) with sample sizes of 15, 20 and 25 flies for each treatment, respectively. Similar to the CI induction experiment, males of 4, 5, 6, 7, 8 and 11 days old and uninfected females of 4–5 days were used. At the beginning of each day of the experiment, single female flies were added to each male's vial. TSM and TFM were recorded. As soon as mating finished, the female flies were transferred to Eppendorf tubes and frozen in liquid nitrogen 40 min after TFM. The females were then transferred to a −80 °C freezer for later dissection.

Sperm is stored in the female's seminal receptacle (SR) and her paired spermathecae (Gillott, 2005). The exact time at which sperm storage commences was not known for *D. simulans*. In a separate study prior to this experiment, we determined the optimum time to count sperm in the females' uterus prior to storage in females' SR and spermathecae (data not presented). Counting sperm in the uterus is preferable because of the difficulties associated with dissecting the SR and spermathecae. The uterus contained the greatest numbers of sperm 40 min after the end of mating. Hence, females were frozen in liquid nitrogen 40 min after TFM in this experiment.

Dissection and sperm counting procedure

Frozen female flies were thawed at room temperature for at least 10 min before dissection as rehydration helped to better disassociate sperm bundles into individual sperm. The female reproductive system is located at the ventral posterior of the abdomen and the uterus at the posterior end of reproductive system (Wilt & Hake, 2003). A phosphate buffer solution (PBS) was used as a basic medium to preserve sperm. 50 μL of PBS was pipetted onto a microscopic slide. The female fly was placed next to the PBS under a dissecting microscope. The body was gently squeezed with tweezers to protrude the ovipositor duct that was pulled out with fine tweezers. The uterus was immediately placed in PBS. Fine dissection needles were used to cut off the circular organs at the anterior end of the uterus. A needle was used to hold the opposite end of the cut, and with another needle, the sperm were squeezed out. The female parts were removed and the sperm were mixed with PBS. An extra 50 μL aliquot of PBS was added and mixed. The slides were left for 24 h to dry before being submerged into deionized water twice for 10 s. The slides were left covered at approximately a 45° angle for a further 24 h. The sperm were counted under dark field at 100× magnification. Both sperm bundles and individual sperm were counted. Each bundle contains 64 sperm (Snook *et al.*, 2000).

Statistics

Repeated-measures ANOVA was used to investigate the effects of male age and mating history on CI induction and the effects of infection status, male age and mating history on the number of sperm transferred during copulation (see Table 1). Bonferroni corrections were applied to all repeated-measures ANOVA to control for multiple comparisons. In addition, an adjustment was made to the numbers of degrees of freedom of the ages in the CI induction experiment by Greenhouse–Giesser due to violated sphericity (Greenhouse & Geisser, 1959). Homogeneity of slopes between mating history treatments was compared by ANCOVA in the CI induction experiment. The data are presented as mean ± SE. SPSS version 20 was used to analyse the data.

Results

CI Induction

Both male age and mating rate affected the level of CI expressed in crosses between infected male and uninfected female *D. simulans*. CI induction decreased (i.e. hatching success increased) with increasing male age ($F_{3.7,141.4} = 97.821$, $P < 0.001$; Fig. 1) and with previous mating history ($F_{2,120} = 44.7$, $P < 0.001$, Fig. 1). In addition, there was a significant interaction between male age and mating history ($F_{17,303} = 35.746$, $P < 0.001$). Virgin males (SC males) conferred a hatching success that ranged between 3% for 4-day-old males and 27% for 11-day-old males (Fig. 1). In contrast, the hatching success of males that mated once and three times per day was close to 58% and 70% at 11 days old, respectively (Fig. 1). This shows that although male age influences CI induction, mating

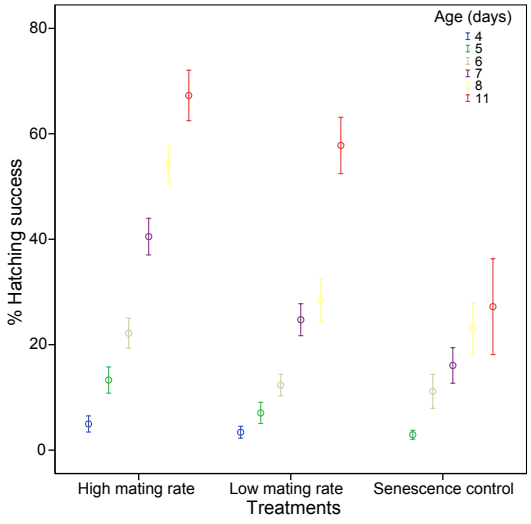

Fig. 1 Mean ± SE hatching success (percentage of viable offspring) of *Drosophila simulans* in crosses between *wRi*-infected males and uninfected females. The hatching success percentages are the average for each male of the 4 to 8 and 11 days old in each mating history treatment. The treatments are senescence control (SC, one mating only for each male and at a set age), low mating rate (LMR), males mated with a single virgin uninfected female when they were 4, 5, 6, 7, 8 and 11 days old (6 matings in total for each individual male) and high mating rate (HMR), males were mated to three virgin uninfected females when they were 4, 5, 6, 7, 8 and one mating when they were 11 days old (16 matings in total for each individual male).

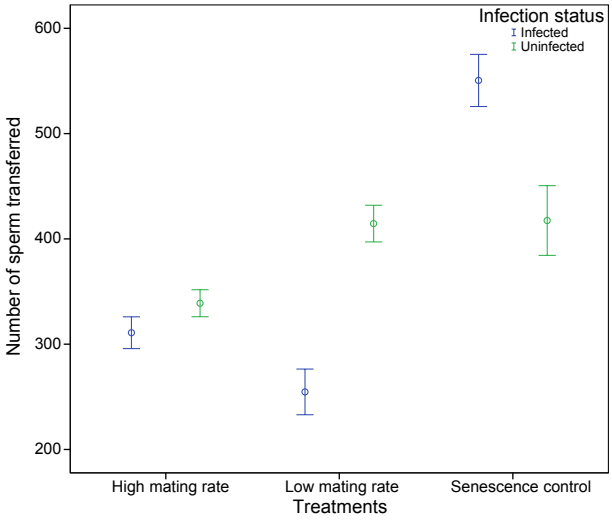

Fig. 2 Number of sperm (mean ± SE) transferred by males at mating with three different mating history treatments (senescence control, low mating rate and high mating rate) for *wRi*-infected and uninfected *D. simulans* males.

history affects hatching success over and above the impact of male age.

Sperm transfer

There was a significant difference between the number of sperm transferred by infected and uninfected males on their first mating ($F_{1,131} = 7.266$, $P = 0.008$; Fig. 2). Infected virgin males transferred on average more sperm (549 ± 30) than uninfected virgin males (433 ± 31) on their first mating. However, infected males that had mated previously under the LMR treatment transferred fewer sperm than uninfected males with the same mating history ($F_{1,220} = 33.985$, $P < 0.001$; Fig. 2). On average, infected LMR males transferred 249 ± 20 sperm, whereas uninfected LMR males transferred 411 ± 20 sperm per mating. Under the HMR treatment, there was no difference in the number of sperm transferred to females by infected and uninfected males ($F_{1,219} = 2.603$, $P = 0.108$; Fig. 2). Infected and uninfected HMR males transferred 295 ± 16 and 331 ± 16 sperm per mating, respectively, to females (Fig. 2).

Male age also affected the number of sperm transferred during copulation ($F_{4,141} = 5.205$, $P < 0.01$). Irrespective of infection status, younger males transferred more sperm than older males, but when the males were given a 'rest' for three days, the number of sperm transferred increased again for both infected and uninfected males. The number of sperm transferred was not associated with the duration of copulation ($F_{1,453} = 2.35$, $P = 0.126$, $r^2 = 0.005$). There was also no effect of infection status ($F_{1,622} = 1.772$, $P = 0.184$) or mating history on copulation duration ($F_{2,621} = 1.78$, $P = 0.17$). The average copulation lasted 20 min for SC, 19 min for LMR and 18 min for HMR males. There was also no difference between infected and uninfected males in terms of body size, as estimated by wing length ($F_{1,226} = 1.111$, $P = 0.293$). These measurements were highly repeatable with no difference between the wing length measurements at first and second measure ($F_{1,443} = 0.079$, $P = 0.779$, repeatability of 0.857, Lessells & Boag, 1987). The number of sperm transferred was also not significantly influenced by male body size ($F_{1,548} = 3.517$, $P = 0.061$).

A repeated-measures ANOVA was performed to examine the combined effect of mating history (SC, LMR and HMR), male age, copulation duration and body size on number of sperm transferred per mating. Male age, copulation duration and body size were included as covariates in the model, and mating history and infection status were fixed effects. The number of sperm transferred to females during copulation was affected by male mating history ($F_{2,618} = 4.888$, $P = 0.008$). The number of sperm transferred decreased with increasing male mating rate. Regardless of infection status, SC males transferred more sperm per mating overall than LMR and HMR (SC = 486 ± 22, LMR = 336 ± 15 and

HMR = 314 ± 11). However, there was a significant interaction between male infection status and mating history ($F_{2,618} = 4.322$, $P = 0.014$). In the SC treatment, infected males transferred more sperm than uninfected males ($F_{1,131} = 7.266$, $P = 0.008$); in the LMR treatment, uninfected males transferred more sperm than infected males ($F_{1,220} = 33.985$, $P < 0.001$); and in the HMR treatment, uninfected males transferred slightly more sperm than infected males, but the difference was not statistically significant ($F_{1,219} = 2.603$, $P = 0.108$). There was no 3-way interaction between mating history, male age and infection status ($F_{8,560} = 1.376$, $P = 0.204$).

Sperm numbers and CI induction

There was an association between the number of sperm transferred at mating and the level of CI induced in relation to male mating history. For *Wolbachia*-infected HMR males, the mean number of sperm transferred per mating was negatively correlated with the average hatching success ($r = -0.939$, $P = 0.005$, $N = 6$). As the number of sperm transferred decreased (with number of previous matings), the hatching success increased. However, when controlling for the impact of male age on the number of sperm transferred and level of CI, the association no longer remains ($r = -0.423$, $P = 0.403$, $N = 6$). Similarly, there was no relationship between number of sperm transferred and CI induction for LMR males when controlling for male age ($r = -0.025$, $P = 0.962$, $N = 6$). There was a tendency for a negative relationship for the senescence control (SC) males, but this was not significant ($r = -0.710$, $P = 0.179$, $N = 6$). As the number of sperm transferred is influenced by male age, we also examined the impact of male mating rate on CI expression per sperm. That is to say, we looked at variation in hatching success that is not related to variation in number of sperm transferred (Fig. 3). This clearly shows that HMR and LHR males show increased hatching success with age compared to SC (treatment: $F_{2,14} = 8.963$, $P = 0.003$), highlighting the impact of mating rate on CI expression over and above the effect of male age (mean hatching success per sperm regardless of age for SC is 0.0280, LMR 0.090 and HMR 0.126).

Discussion

The results of this study provide important insights into the impact of *Wolbachia* on host sexual selection and the potential role of *Wolbachia* in shaping male reproductive strategies. Mating at high rate confers an advantage to infected males because it enables them to restore reproductive compatibility with uninfected females over and above the rate at which compatibility is increased as males age. Four-day-old virgin infected males had a hatching success of <5%, whereas the hatching success of 11-day-old infected males was 27% for virgin males (SC), 58% for LMR and 70% for HMR males, respectively. For some HMR males, hatching success was even 100%. These data clearly show that mating at a high rate restores compatibility faster than mating at low rate. It is not known whether a higher mating rate directly decreases reproductive incompatibility of infected males, or whether it is due to other impacts of mating at a high rate. For example, high mating rate may result in increased sperm viability or mobility *per se* as a consequence of being stored for a shorter duration in the testes. This possibility cannot be ruled out, as we did not quantify hatching success in relation to mating rate in uninfected males. However, previous work does not support this suggestion. For example, Snook *et al.* (2000) found no difference in hatching success of uninfected *D. simulans* Riverside males allowed 4 consecutive copulations (Snook *et al.*, 2000). Other studies have reported similar findings with multiply mated uninfected males having a hatching success that is vastly greater than the hatching suc-

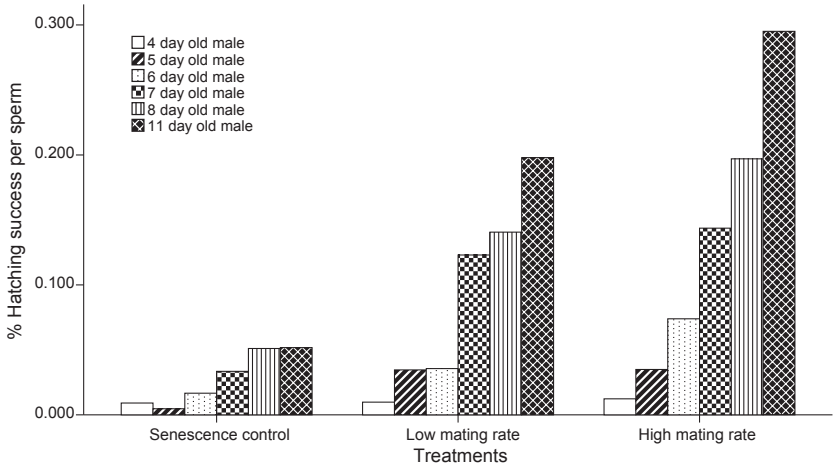

Fig. 3 The hatching success per sperm (%) *of wRi*-infected *D. simulans* males at different ages experiencing varying mating rates. Low mating rate (1 copulation per day), high mating rate (3 copulations per day).

cess of incompatible crosses due to CI in *wRi*-infected *D. simulans* males (e.g. Turelli & Hoffmann, 1995). Therefore, in populations with a high frequency of uninfected females (as is the case when *Wolbachia* invades), or where females harbours a different strain of *Wolbachia* to the male, infected males mating at a high rate will be favoured as this ensures reproductive compatibility with all the females in the population. This in turn means that high mating males are likely to enjoy greater reproductive success over their lifetime than infected males that mate at a lower rate.

Previous research has provided mixed results with respect to the impact of male age on the level of CI expression. In *D. simulans* and *D. melanogaster* for example, CI decreases with increasing male age (Karr *et al.*, 1998; Reynolds & Hoffmann, 2002). However, this pattern is not universal. In *Aedes albopictus* mosquitoes, there is no effect of male age on the level of CI, which remains high also in older males (Moretti & Calvitti, 2012), and in *Culex fatigans* mosquitoes, CI is only slightly lower in older males (Singh *et al.*, 1976). Similarly in the plant hopper *Sogatella furcifera, Wolbachia*-infected males only show partial CI and lose their ability to induce CI as they age, whereas in *Laodelphax striatellus* plant hoppers the level of CI is much higher overall and is unaffected by male age (Noda *et al.*, 2001). It is suggested that the differences in the level of CI decline with age are due to the density of *Wolbachia,* particularly in the sperm cysts in the testes, with higher densities resulting in higher severity of CI expression (Boyle *et al.*, 1993; Breeuwer & Werren, 1993; Giordano *et al.*, 1995; Mercot *et al.*, 1995; Bourtzis *et al.*, 1996, 1998; Poinsot *et al.*, 1998; Rousset *et al.*, 1999; Veneti *et al.*, 2003). This suggestion is corroborated by the observation that *Wolbachia* density decreases within male germ cells with the onset of male age in *D. simulans* (Riparbelli *et al.*, 2007).

Why do infected males that mate at a higher rate induce lower CI independent of age? It has been suggested that the exposure time of sperm to *Wolbachia* in the testes directly influences the level of CI (e.g. Karr *et al.*, 1998). This could explain why male *D. simulans* that mate at a higher rate more rapidly restore reproductive compatibility than infected males mating at a lower rate. Spermiogenesis in *Drosophila* begins immediately after meiosis and continues throughout larval, pupal and adult stages (Fuller, 1993). Thus, the first ejaculate is likely to contain sperm that have spent much of their 'preadult life' in contact with *Wolbachia* and most spermatozoa will be affected as nearly all sperm cysts in young males are *Wolbachia* infected (Bressac & Rousset, 1993; Karr *et al.*, 1998; Clark *et al.*, 2002, 2003; Veneti *et al.*, 2003). In contrast, sperm in subsequent ejaculates will have had less residence time in the testes and therefore have had less exposure to *Wolbachia* manipulation. The suggestion that the level of CI is a function of residence time of sperm in testes is by no means universal. In *Wolbachia*-infected *D. melanogaster* for example, the severity of CI is largely related to male development time, with faster developing males expressing the strongest CI levels. The mechanism is not known, but is independent of *Wolbachia* density, maternal effects and male body size (Yamada *et al.*, 2007). However in *D. simulans*, variation in male development does not influence the severity of CI induction (Yamada *et al.*, 2007), indicating that exposure time of sperm to *Wolbachia* in the testes may indeed be important. *Wolbachia* appears to be depleted from the testes over time in *D. simulans* with the loss of *Wolbachia*-infected cysts occurring around day 3 post-eclosion (Clark *et al.*, 2002). Early developing sperm may therefore contain *Wolbachia* that induce CI, whereas sperm produced later may lack *Wolbachia* and hence are not modified (Clark *et al.*, 2003, 2008). Repeated mating by infected males may deplete the stocks of older *Wolbachia*-modified sperm and subsequent ejaculates instead containing an increasing proportion of new and unmodified sperm compared to males mating at a lower rate of a similar age. The net result is that males that mate at a higher rate will induce lower levels of CI as observed.

Previous work in *Drosophila* has shown that there is a positive correlation between CI levels and proportion of sperm infected by *Wolbachia* (Clark *et al.*, 2003). However, it is worth noting that direct contact with *Wolbachia* during sperm development is not necessarily a prerequisite for *Wolbachia*-induced sperm modification across insects. In *Nasonia vitripennis* wasps and *Chelymorpha alternans* beetles for example, sperm modification resulting in CI induction was present even if *Wolbachia* was not found in individual spermatocytes or spermatids, providing it was present in the testes (Clark *et al.*, 2008). Furthermore, no effect of the number of previous matings on CI induction was found in *N. vitripennis* males (Clark *et al.*, 2008). It is not clear why the impact of *Wolbachia* on sperm and CI differs between *Nasonia* and *Drosophila*. In *Nasonia* wasps, *Wolbachia*-induced CI causes paternal chromosome loss and production of haploid male offspring, rather than embryo death and hatching failure as in *Drosophila*. Importantly, male *Nasonia* eclose with the majority of their lifetime sperm supply, meaning that sperm used in consecutive matings will be derived from the same sperm population (Pennypacker, 1958). This is in contrast to *Drosophila* where spermatogenesis continues also in the adults (Fuller, 1993). An additional possibility is that the age of *Wolbachia* infection may also play a role. *wRi* in *D. simulans* has rapidly spread throughout California since it was first observed in southern California in 1984 (Hoffmann *et al.* 1986) indicating a recent association with its host, whereas the *Wolbachia* association with *Nasonia* is believed to be ancient (Werren, 1997). Differences in the co-evolutionary histories could therefore in part be responsible. This is corroborated by the

finding that *wRi* has rapidly evolved from conferring a fecundity cost to infected females 20 years ago to currently be associated with a 10% fecundity advantage (Weeks *et al.*, 2007). It remains to be seen if there has been a similar shift in the effect of *wRi* on sperm number and CI induction in males. Our current study was conducted using males originating from flies collected in the wild in the late 1980s, and so repeating the experiment of recently collected flies is required to evaluate this possibility. However, it is not clear whether a similar 'co-evolution' from parasitic to mutualistic is to be expected also in males as they represent a dead end for *Wolbachia* because it is only transmitted in females and hence there may have been no ameliorative selection in males.

The number of sperm transferred at mating appears to influence the level of CI induced, but this pattern was only present in males mating at a high rate where transfer of fewer sperm in later copulations resulted in higher hatch rates (i.e. lower CI, see Fig. 3). There was a tendency for the same pattern to be the case also in control males mating only once at varying ages, but not for males mating at a lower rate. It is not clear whether this pattern is due to the number of sperm transferred *per se*, or the number of *Wolbachia*-modified sperm reflecting the impact of *Wolbachia* modification of sperm in the testes. It is likely to be the latter as male mating rate will affect the residence time of sperm in the testes and may translate into reduced number of *Wolbachia*-modified sperm transferred.

Why do infected and uninfected males differ in the number of sperm transferred to females at mating? It may be due to different reproductive strategies adopted by infected and uninfected males. Infected males may mate at a high rate in order to purge themselves of *Wolbachia*-manipulated sperm and restore reproductive compatibility with uninfected females. We have previously shown that *Wolbachia*-infected *Drosophila* males mate at a higher rate than uninfected males and that the difference in mating rate is greater in *D. simulans* (50% higher) that have a high level of CI (>95%) than in *D. melanogaster* (16% higher) that express a lower level of CI (<30%) (Champion De Crespigny *et al.*, 2006). Consequently, an increased mating rate by infected males may be a behavioural adaptation to increase their reproductive success. It is unlikely that *Wolbachia* infection is beneficial to males as it is shown to reduce both sperm production (Snook *et al.*, 2000) and sperm competitive ability (Champion De Crespigny & Wedell, 2006). While *wRi* can confer antiviral protection to *D. simulans*, this benefit is enjoyed by both sexes (Osborne *et al.*, 2009) and cannot explain why infected males should mate at a higher rate than uninfected males. It is therefore likely that the increased mating rate by infected males is an adaptive behaviour. This in turn will affect the overall level of multiple mating in *Wolbachia*-infected fly populations increasing the scope for sexual selection, but also sexual conflict over female remating rate (Wedell, 2013). Uninfected males, on the other hand, may trade off the number of sperm provided at mating in relation to their expected fertilization returns given the risk of sperm competition and female encounter rate (Wedell *et al.*, 2002). It is therefore possible that infected and uninfected males will adopt different reproductive strategies in *Wolbachia*-infected populations to maximize their respective fertilization returns. Infected males enjoy higher reproductive success by maintaining high mating rate to restore reproductive compatibility, whereas uninfected males instead balance the trade-off between the number of sperm transferred in relation to female encounter rate and success in sperm competition. Previous work has shown that *Wolbachia* infection also influences female mating decisions and hence has the potential to affect insect mating systems (Wedell, 2013).

In conclusion, this is the first study to quantify the number of sperm transferred in relation to CI expression and male age and mating rate in *Wolbachia*-infected *D. simulans*, showing that a high mating rate results in reduced CI expression. Infected males may therefore mate more frequently in order to restore their reproductive compatibility with uninfected females to a greater extent than males mating at lower rate. This may also explain why infected males transfer more sperm on their first mating than uninfected males, if this is a mechanism to 'rid' themselves of *Wolbachia*-modified sperm that induce CI reducing fertilization success. These results indicate that *Wolbachia* infections may force infected males to trade off restoring their reproductive compatibility against poor sperm competitive ability to increase their lifetime reproductive success, and demonstrate the potency of *Wolbachia* to influence insect mating patterns.

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