

***Wolbachia* infection reduces sperm competitive ability in an insect**

Fleur E. Champion de Crespigny* and Nina Wedell

Centre for Ecology and Conservation, The University of Exeter Cornwall Campus, Penryn TR10 9EZ, UK

The maternally inherited bacterium *Wolbachia pipientis* imposes significant fitness costs on its hosts. One such cost is decreased sperm production resulting in reduced fertility of male *Drosophila simulans* infected with cytoplasmic incompatibility (CI) inducing *Wolbachia*. We tested the hypothesis that *Wolbachia* infection affects sperm competitive ability and found that *Wolbachia* infection is indeed associated with reduced success in sperm competition in non-virgin males. In the second male role, infected males sired 71% of the offspring whereas uninfected males sired 82% of offspring. This is the first empirical evidence indicating that *Wolbachia* infection deleteriously affects sperm competition and raises the possibility that polyandrous females can utilize differential sperm competitive ability to bias the paternity of broods and avoid the selfish manipulations of *Wolbachia*. This suggests a relationship between *Wolbachia* infection and host reproductive strategies. These findings also have important consequences for *Wolbachia* population dynamics because the transmission advantage of *Wolbachia* is likely to be undermined by sperm competition.

Keywords: *Wolbachia*; sexual selection; *Drosophila*; sperm competition

1. INTRODUCTION

Selfish genetic elements (SGEs), such as the maternally inherited bacterium, *Wolbachia pipientis* are gaining notoriety both for their prevalence across taxa (Hurst & Werren 2001), and for the significant fitness costs they frequently impose on their hosts (O'Neill *et al.* 1997; Hatcher 2000). For instance, *Wolbachia* is thought to infect up to 75% of arthropods (Jeyaprasaksh & Hoy 2000), and is responsible for selfishly manipulating offspring sex ratios or host reproductive compatibilities in order to improve its own transmission. *Wolbachia* frequently induces cytoplasmic incompatibility (CI). CI occurs when the sperm of infected males fertilize the eggs of uninfected females and it results in embryo death. However, the reciprocal crosses are compatible (O'Neill *et al.* 1997). This has obvious negative consequences for the offspring production of both uninfected females and infected males, but promotes the spread of *Wolbachia* by decreasing the fitness of uninfected females relative to infected females not affected by CI. In addition to CI, *Wolbachia* has been shown to be associated with decreased fecundity in female (Hoffmann *et al.* 1990), and decreased fertility in male (Snook *et al.* 2000) *Drosophila simulans*. These fitness costs are thought to place selective pressure on hosts to avoid or reduce the effects of *Wolbachia*. Because *Wolbachia* affects the fertility of both males and females, it is likely that *Wolbachia* influence sexual selection and male and female reproductive strategies. However, despite the prevalence of *Wolbachia* and its impact on host fitness, this possibility is largely unexplored (Charlat *et al.* 2003).

Drosophila males produce sperm throughout their lives and *Wolbachia* has recently been shown to affect sperm production in male *D. simulans* (Snook *et al.* 2000).

Although sperm do not transmit *Wolbachia*, infected males produce approximately 40% fewer sperm than uninfected males (Snook *et al.* 2000). Sperm production could have a direct impact on male fitness as high sperm numbers are often important in obtaining paternity under sperm competition (Parker 1998). *D. simulans* females are highly polyandrous and it is therefore likely that multiple ejaculates compete for the fertilization of a given female's eggs. Paternity is typically biased towards the last male to mate. In double mating trials, the second male to mate sires on average 74% of the subsequent offspring (Price 1997).

Despite the consequences of *Wolbachia* infection for sperm production, a study of sperm competition in *D. simulans* manipulated by CI-inducing *Wolbachia* found no variation in sperm competitive ability between infected virgin and uninfected virgin males (Hoffmann *et al.* 1990). However, it is possible that differences in sperm competitive ability are best revealed when males have mated previously. Virgin males are likely to have a full complement of sperm and may therefore ejaculate similar quantities of sperm during their first mating, irrespective of infection status. Infected males, however, may suffer more pronounced sperm limitation under multiple mating. *D. simulans* males are highly polygynous, so sperm competitive ability as a non-virgin is likely to be important to male reproductive success.

We tested the hypothesis that *Wolbachia* infection is associated with decreased sperm competitive ability using male *D. simulans* that had mated twice prior to mating with the focal female. This study provides the first empirical evidence for reduced success in sperm competition associated with *Wolbachia* infection in *Drosophila*. These findings may have important consequences for the population dynamics of *Wolbachia* and for the evolution of host reproductive strategies (e.g. polyandry) that may mediate the fitness costs of *Wolbachia* infection.

* Author for correspondence (f.decrepigny@exeter.ac.uk).

Table 1. Experimental design: experiment treatment and final sample sizes.

treatment	first male		second male		sample size
	infection status	phenotype	infection status	phenotype	
1	infected	ebony	uninfected	wild-type	18
2	uninfected	ebony	infected	wild-type	21
3	uninfected	wild-type	infected	ebony	23
4	infected	wild-type	uninfected	ebony	23

2. MATERIAL AND METHODS

(a) *Experimental stock*

The flies were derived from an iso-female line infected with *Wolbachia* that was originally collected in Riverside, California and has been maintained in laboratory populations for at least 2 years. This line was introgressed, over several generations of backcrossing, with *D. simulans* homozygous for a recessive ebony phenotype marker to enable paternity assignment in the double mating trials. Uninfected flies were obtained by antibiotic treatment six months prior to the experiment: two consecutive generations were raised on food containing 0.03% tetracycline hydrochloride (Hoffmann *et al.* 1986). Approximately 80 infected females (enclosed with 80 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 more than 150 male) offspring from the second generation founded the uninfected population.

The curing process generated four groups of varying phenotype and infection status: both infected and uninfected wild-type and ebony groups. The infection status of each group was confirmed by PCR (See Snook *et al.* 2000 for general methodology).

(b) *Collection of virgins*

Eggs were collected from stock populations and the larvae were reared at 25 °C with a 12/12 h light–dark cycle in density-controlled vials. Vials were inspected every 6 h (three times during light cycle) for newly eclosed flies. New adults were chilled on ice, sexed and the sexes were placed in separate, density-controlled vials containing food.

(c) *Experiment design*

Infected ebony females were assigned to one of four experimental treatments (table 1) in which they mated with both an infected non-virgin and an uninfected non-virgin male. Males were mated twice prior to mating with the focal female. Infected females were used to avoid CI since they are reproductively compatible with both infected and uninfected males. In all treatments one male had the ebony phenotype. Infection status and order of mating were controlled, so approximately equal numbers of ebony males were infected or uninfected and in the first or second male role. Infected ebony females were also assigned to one of four control treatments in which they mated to two non-virgin males of the same phenotype and infection status. This allowed us to account for any differential mortality of the marker or infection by comparing offspring production of the different male types. However, we found no difference in offspring production between the control groups (ANOVA: $F_{3,23}=1.476$, $p=0.251$). We also compared the offspring production of

females mated once only to non-virgin, twice mated males. We found no difference in offspring production between infected and uninfected males (ANOVA: $F_{1,30}=1.610$, $p=0.215$).

(d) *Experimental procedure*

The experiments were performed at 25 °C under controlled lighting conditions. All males and focal females were 3 days old. Males and focal females were placed in individual vials 12 h before the experiment commenced. Two virgin females were introduced to each male's vial and copulations were observed. Both females were removed within 15 min of the completion of the male's second copulation. Males were then transferred to the focal female's vial after both male and female were lightly anaesthetized using CO₂. Typically, the flies recovered within 5 s of transfer. Copulations were observed and the durations recorded. Males were removed within 15 min of completing copulation and were frozen for measurements. Body size was estimated by measuring the length between the intersection of the anterior cross vein with the longitudinal vein 3 (L3) and the intersection of L3 with the distal wing margin (Partridge *et al.* 1987). Females were mated to the second male approximately 24 h later following the same procedure as above. After copulating for the second time, females were transferred to fresh vials. Fresh vials were provided to each female at 48 h intervals for a total of 6 days. Offspring were collected from vials, counted and paternity was assigned by visual inspection under a dissecting microscope. Females that produced fewer than 10 offspring were discarded from the analysis.

(e) *Statistics*

Male success in achieving paternity was estimated by calculating the logit (Snedecor & Cochran 1978) of the number of offspring sired by the second male to mate relative to the total number of offspring produced by the female. This method accounts for variation in the number of offspring produced by each female and provides a more accurate estimation of paternity than simple proportions. The data were analysed in a general linear model (GLM; table 1) and the residuals were tested for normality. The independent variables in the GLM were the infection status and the phenotype (wild-type/ebony) of the second male to mate. The size of the first and second male and the durations of the first and second copulation were included as covariates. All two-way interactions were modelled and terms were removed stepwise. This resulted in the removal of the covariate 'size of male 2' and the inclusion of the interaction between size of male 1 and the duration of the second copulation. Copulation duration data were log transformed to improve normality. All data presented in the text are mean \pm s.e. unless otherwise stated.

Table 2. General linear model of paternity achieved by second male to mate.

	d.f.	mean square	F	P
<i>independent variables</i>				
<i>Wolbachia</i>	1	33.504	6.814	0.011
ebony marker	1	105.353	21.427	0.000
<i>covariates</i>				
size of male 1	1	44.621	9.075	0.003
duration of 1st copulation	1	21.361	4.344	0.04
duration of 2nd copulation	1	44.057	8.554	0.005
interaction: size of male 1/duration of 2nd copulation	1	43.532	8.854	0.004
<i>corrected model</i>	6	33.710	4.571	0.000
<i>intercept</i>	1	44.834	0.000	0.003
<i>error</i>	78	4.917		
r^2	0.345			
adjusted r^2	0.295			

3. RESULTS

Males infected with *Wolbachia* sired significantly fewer offspring than uninfected males (table 2 and figure 1). In the second male role, infected males sired $71.5 \pm 0.05\%$ of offspring whereas uninfected males sired $82.3 \pm 0.04\%$. A previous study of sperm competition in *D. simulans* found that the second male to mate sires approximately 74% of the offspring (Price 1997). The duration of both the first and second copulation had a significant effect on paternity in the present study. In both cases, longer copulations increased the paternity achieved by each male. This is consistent with many studies of sperm competition in both dipterans (Parker *et al.* 1999), and other taxa (Simmons 2001). Although there was no difference between the size of the first and second males (Paired *t*-test: $t_{95} = -1.74$, $p = 0.085$), the size of the first male to mate had a significant association with the outcome of sperm competition, with larger males achieving higher paternity. In addition, there was an interaction between the size of the first male to mate and the length of the second copulation. However, we were unable to determine the precise nature of this interaction. The phenotypic marker (ebony) used to assign paternity also had a significant association with the outcome of sperm competition. For unknown reasons, ebony males obtained more paternity than wild-type males. However, this effect was controlled for in our experimental design and there was no interaction between the phenotypic marker and infection status in their effects on paternity.

4. DISCUSSION

These results provide the first empirical evidence that the cytoplasmically inherited micro-organism *Wolbachia* can disadvantage its host in sperm competition. The reduction in male fertility caused by decreased sperm production that is associated with *Wolbachia* infection in *D. simulans* provides a potential mechanism for the observed paternity disadvantage of infected males. Under multiple mating scenarios, infected males are likely to be sperm limited (Snook *et al.* 2000), and may transfer fewer sperm than

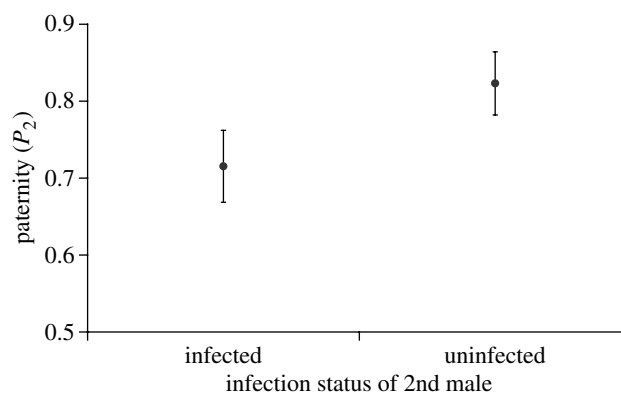


Figure 1. The mean \pm s.e. paternity achieved by infected ($n = 44$) and uninfected ($n = 41$) males. Paternity is presented as P_2 , the proportion of offspring sired by the second male to mate.

uninfected males, indicating that high sperm numbers may be important to fertilization success. Another possibility is that infected males have sperm of inferior quality. However, this is unlikely to fully explain the observed variation in paternity, as we found no difference in offspring production by females singly mated to infected and uninfected non-virgin males. Finally, females may be able to exercise choice of sperm. It is possible that *Wolbachia* leaves an imprint on the sperm providing a cue to females during the fertilization process. These hypotheses are not mutually exclusive and, irrespective of the exact mechanism(s) of biasing paternity, these results may have important consequences for female ability to avoid CI and for the population dynamics of *Wolbachia*.

A previous study of sperm competition in *D. simulans* infected with CI-inducing *Wolbachia* found no difference in sperm competitive ability between infected and uninfected males (Hoffmann *et al.* 1990). However, virgin males were competed and there was a 4 day interval between each mating. The impact of decreased sperm production on sperm competitive ability of infected males may only be apparent under multiple mating since virgin males (either infected or uninfected) may have a full compliment of sperm and therefore deliver similar ejaculate quantities during their first mating. Increasing the number of prior copulations by infected males is likely to affect the competitiveness of their ejaculates as they may suffer sperm depletion. Importantly, since this species is highly polygynous, the probability that the ejaculates of two virgin males compete for fertilizations in nature is likely to be low. Instead, the majority of sperm competition events will involve the ejaculates of non-virgin males. The time interval between sequential matings could also affect the outcome of sperm competition via sperm loss during oviposition and sperm ageing and death (Snook & Hosken 2004). In this study, intervals between matings were short and constant, providing a more sensitive estimate of sperm competitive abilities since it ensures that both ejaculates compete with minimal sperm loss due to egg laying and sperm death.

Our findings also contrast with a study of the beetle *Tribolium confusum* (Wade & Chang 1995), where males infected by *Wolbachia* appear to enjoy a fertility advantage. However, this study did not utilize an experimental design typical of studies of sperm competition. Females were provided with a variable number of infected and uninfected

males for a minimum of 18 days and neither the number of matings nor the order of matings was recorded. Both mating order and frequency are known to affect the outcome of sperm competition (Simmons 2001). Greater paternity can be achieved by being the last male to mate and/or by providing the most sperm. *Wolbachia* is associated with increased male mating rates in both *D. simulans* and *D. melanogaster* (Champion de Crespigny *et al.* submitted), although it is not known whether this is the case in *T. confusum*. The effects of *Wolbachia* infection on sperm production are also unknown in this species.

Reduced sperm competitive ability in *D. simulans* males infected by CI-inducing *Wolbachia* may provide females with the means to bias the paternity of their broods. Polyandrous *D. simulans* females could reduce the paternity of infected males by promoting sperm competition. This has the potential to improve the fitness of both infected and uninfected females: Infected females of the related *D. melanogaster* have greater fecundity when mated to uninfected males (Fry *et al.* 2004), and uninfected females avoid the fitness costs associated with CI. This could lead to selection for polyandry in species manipulated by CI-inducing *Wolbachia*. This theory is also applicable to other SGEs that affect sperm production, such as sex-linked meiotic driver genes (Wilkinson & Fry 2001; Atlan *et al.* 2004).

Reduced sperm competitive ability in males infected by *Wolbachia* may have direct and critical consequences for the population dynamics of *Wolbachia* in nature. Infection frequencies of *Wolbachia* in wild populations of *D. simulans* are often significantly less than 100% (Turelli & Hoffmann 1991). Consequently, the ejaculates of infected and uninfected males are likely to compete in the wild and could decrease the rate of CI induction. Decreasing the rate of CI induction as a consequence of sperm competition, undermines the advantage to infected females and therefore slows the spread of *Wolbachia* (Champion de Crespigny *et al.* 2005). The rate of spread of *Wolbachia* may be strongly linked to the successful maintenance of the infection, since *Wolbachia* is thought to be vulnerable at low frequency due to stochastic environmental conditions (Champion de Crespigny *et al.* 2005).

We have shown that *Wolbachia* infection is associated with reduced sperm competitive ability in *D. simulans*. Given their widespread occurrence, SGEs, including *Wolbachia* may play an important and hitherto overlooked role in sexual selection and as a selective force driving the evolution of female mating patterns. In addition, sperm competition is likely to have an impact on *Wolbachia* population dynamics by undermining its transmission advantage.

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