

Wolbachia infection lowers fertile sperm transfer in a moth

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The endosymbiotic bacterium *Wolbachia pipientis* manipulates host reproduction by rendering infected males reproductively incompatible with uninfected females (cytoplasmic incompatibility; CI). CI is believed to occur as a result of *Wolbachia*-induced modifications to sperm during maturation, which prevent infected sperm from initiating successful zygote development when fertilizing uninfected females' eggs. However, the mechanism by which CI occurs has been little studied outside the genus *Drosophila*. Here, we show that in the sperm heteromorphic Mediterranean flour moth, *Ephesia kuehniella*, infected males transfer fewer fertile sperm at mating than uninfected males. In contrast, non-fertile apyrene sperm are not affected. This indicates that *Wolbachia* may only affect fertile sperm production and highlights the potential of the Lepidoptera as a model for examining the mechanism by which *Wolbachia* induces CI in insects.

Keywords: endosymbiont; sperm heteromorphism; Lepidoptera; spermatogenesis

1. INTRODUCTION

The endosymbiotic bacterium *Wolbachia pipientis* is potentially the most common symbiont on Earth [1]. The key to its success is its ability to manipulate host reproduction to increase its transmission. Commonly *Wolbachia* induces cytoplasmic incompatibility (CI), which causes embryo death when sperm from an infected male fertilizes the ova of an uninfected female; all other crosses are viable. These manipulations lead to an increase in the number of infected females, which is beneficial to *Wolbachia* as it is maternally transmitted.

CI is believed to occur as a result of *Wolbachia*-induced modifications to maturing sperm [2]. These modifications must occur in the early stages of spermatogenesis, as the bacteria are shed from mature sperm cells. The mechanism by which CI occurs is unknown, although it may occur via *Wolbachia*-induced modifications of cytoskeletal activity [2]. Sperm modifications by *Wolbachia* also affect host fertility—in *Drosophila simulans*, infected males produce fewer

sperm than uninfected males [3] and have reduced paternity in sperm competition [4]. However, it is unknown whether similar effects are seen in other non-drosophilid–*Wolbachia* host systems.

Here, we examine whether *Wolbachia* is associated with reductions in sperm transfer in the Mediterranean flour moth, *Ephesia kuehniella*. The effect of CI-inducing *Wolbachia* on sperm production is particularly interesting in the Lepidoptera, as males are sperm heteromorphic, producing both fertile and non-fertile sperm [5]. Both are transferred during copulation, and non-fertile sperm can constitute up to 95 per cent of an ejaculate [6]. Non-fertile sperm appear to suppress the female receptivity by filling their sperm storage organ [7]. It is unknown whether *Wolbachia* modifies both sperm types, or if it selectively modifies fertile sperm, the only type relevant to CI induction. Examining the effect of *Wolbachia* on lepidopteran heteromorphic sperm may increase our understanding of how *Wolbachia* induces CI in insects. In Lepidoptera, male mating capacity is directly related to sperm production (e.g. [8]). If *Wolbachia* infection is detrimental to male fertility, then we predict that infected males will transfer fewer sperm than uninfected males and, as a consequence, achieve fewer matings in their lifetime.

2. MATERIAL AND METHODS

Moths were obtained from a laboratory population, singly infected with group A *Wolbachia* inducing high levels of CI (greater than 80%), collected from the wild in Yokohama, Japan [9]. Both infected and uninfected individuals were maintained in a large interbreeding laboratory population (derived from approx. 100 matriline; electronic supplementary material, S1). By combining the offspring of approximately 60 infected and approximately 40 uninfected matrilines, we established separate infected and uninfected populations prior to the onset of the experiment. All moths were reared following a standard protocol [9]; see electronic supplementary material, S1 for further details on the populations used. Following eclosion, 50 virgin males from each population were individually coupled with virgin *Wolbachia*-infected females. For every subsequent day of his life, each male was allowed access to a fresh virgin female for 6 h. The first two females to mate with each male were removed immediately following copulation, and used to estimate sperm transfer. Males that failed to mate were isolated, and presented with a fresh female the following day. This continued until death and adult longevity noted. Lifetime mating success was measured as the total number of matings. Male body size was estimated by measuring the length of the forewing [10]. The total number of fertile and non-fertile sperm present in the male's first and second spermatophores was measured following a standard protocol [6]. Infection status was determined by PCR for the universal *Wolbachia*-specific primers *wsp81F* and *wsp691R* [11].

In order to further test our hypothesis that *Wolbachia* is detrimental to male fertility, we also repeated the first part of our experiment using a population of *E. kuehniella* produced via antibiotic curing (see electronic supplementary material, S2). Comparison with our *Wolbachia*-infected population allowed us to examine the impact of *Wolbachia* on male fertility while excluding potential genetic differences between the two populations initially used.

Analyses were performed in R (v. 2.0.1). The impact of *Wolbachia* infection on sperm transfer, and the total number of matings, was analysed using generalized linear models [12], specifying Poisson error distributions (data corrected for over-dispersion) with male size as a covariate. The effect on longevity was analysed using Cox's proportional hazards analyses on uncensored data, again with male size as a covariate. Model simplification was carried out with interactions removed before main effects.

3. RESULTS

There was no difference with respect to infection status in the number of sperm transferred on a male's first mating, for either fertile (uninfected (*U*): mean

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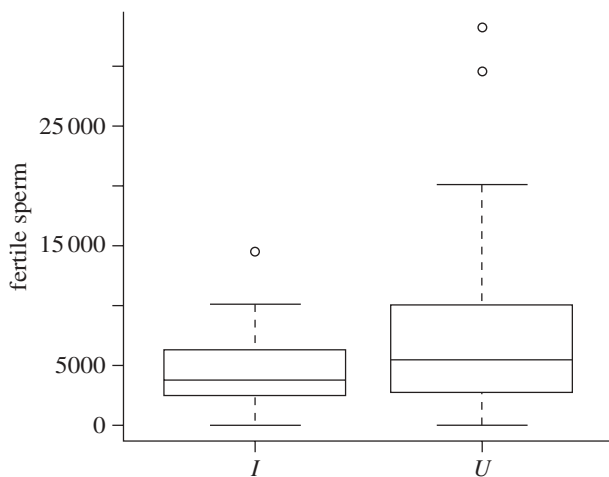


Figure 1. The number of fertile eupyrene sperm transferred by *Wolbachia*-infected (*I*, $n = 24$) and uninfected (*U*, $n = 38$) males to females on their second mating. Median \pm upper and lower quartile.

7873 ± 961 s.e.; infected (*I*): 6641 ± 939 , $F = 0.097$, $p > 0.7$, d.f. = 53), or non-fertile sperm (*U*: $25\,751 \pm 2200$; *I*: $26\,178 \pm 3386$, $F = 0.002$, $p > 0.9$, d.f. = 47). *Wolbachia* infection did, however, affect fertile sperm transfer on males' second mating; infected males transferred approximately 38 per cent fewer fertile sperm compared with uninfected males ($F = 5.80$, $p = 0.0199$, d.f. = 49, figure 1; fertile sperm transfer was also reduced on a male's third mating) ($F = 5.25$, $p = 0.026$, d.f. = 46). In contrast, infection status did not affect the number of non-fertile sperm transferred on a male's second mating (*U*: $27\,042 \pm 3633$; *I*: $27\,756 \pm 4858$, $F = 0.065$, $p > 0.8$, d.f. = 49). This result was confirmed using antibiotically cured males (see electronic supplementary material, S2).

There was no effect of *Wolbachia* infection on lifetime number of matings: infected and uninfected males mated on average five times (range 1–7; s.e. ± 0.345 and 0.266 , respectively; $F = 0.772$, $p > 0.3$, d.f. = 59). In addition, there was no difference in size between uninfected and infected males (mean \pm s.e. mm, *U*: 6.44 ± 0.035 ; *I*: 6.46 ± 0.054 , $F = 0.038$, $p > 0.8$, d.f. = 79). There was also no effect of *Wolbachia* infection on adult male longevity ($\chi^2 = 2.5$, $p > 0.1$), although uninfected males tended to live longer (10.7 ± 0.285 days) than infected males (9.4 ± 0.707 days).

4. DISCUSSION

We found no effect of *Wolbachia* infection status on the number of fertile or non-fertile sperm transferred on a male's first mating. Infected males may eclose with sufficient sperm supplies to produce a 'standard'-sized ejaculate on their first mating. In contrast, *Wolbachia*-infected males transferred fewer fertile sperm on their second mating. The difference in sperm numbers could be the result of different genetic backgrounds of the infected and uninfected females used to establish the matrilines. However, this is unlikely as moths were maintained in a large interbreeding population, and the infected and uninfected lines were maintained

separately for only one generation prior to the start of the experiment (electronic supplementary material, S1). In addition, this finding was subsequently corroborated in a sample of 15 infected and 15 tetracycline-cured males derived from the same population (electronic supplementary material, S2). Similar results have been found in *D. simulans*, where infected males produce 40 per cent fewer spermatozoa and have slower sperm maturation than uninfected males, but this was revealed only in non-virgin males [3]. Our finding thus mirrors that of *Drosophila* and is the first evidence that *Wolbachia* affects sperm production in a non-drosophilid species.

The mechanism by which *Wolbachia* affects sperm is largely unknown. In *D. simulans*, *Wolbachia* is present in developing sperm cysts but removed during spermatid elongation [2]. The removal of the bacteria may be energetically costly, and place constraints on sperm production. The same could be the case in the Lepidoptera. The presence of *Wolbachia* may also directly damage the sperm. In the moth *Philudoria potatoaria*, fertile spermatocytes harbouring large numbers of intracellular bacteria—likely to have been *Wolbachia*—were altered during the advanced stages of spermatogenesis, causing nuclear degeneration [13]. It is possible that a similar mechanism is responsible for the observed reduction in fertile sperm number in *E. kuehniella*.

In contrast to our results on fertile sperm, we found no effect of *Wolbachia* infection on non-fertile sperm transfer. To date, there has been no examination of the impact of *Wolbachia* infection on non-fertile sperm, although there are several possibilities why *Wolbachia* may exert a differential effect on fertile and non-fertile sperm. *Wolbachia* may specifically target fertile sperm because only they are involved in CI induction, or may affect both, but have only a detrimental effect on fertile sperm. There may be differences between fertile and non-fertile sperm in sensitivity to *Wolbachia* manipulation; fertile spermatogenesis is known to be more sensitive to both genetic and experimental manipulation (e.g. [14]). It could also be due to differences in the timing and duration of *Wolbachia* exposure during spermatogenesis. In the Lepidoptera, fertile spermatogenesis begins in the larvae and stops in the pupae, but non-fertile spermatogenesis begins close to pupation and continues in the adult [14]. In addition, the duration of meiosis differs markedly between non-fertile and fertile sperm, with non-fertile meiosis lasting a considerably shorter time [15]. Finally, the differential effect of *Wolbachia* may arise as a consequence of their dichotomous spermatogenesis. CI modification of sperm involves changes in sperm nuclear function and in *Drosophila melanogaster* at least, *Wolbachia* does not appear to disrupt any extra-nuclear sperm factors [16]. Therefore in the Lepidoptera, it is possible that *Wolbachia* cannot adversely affect apyrene sperm as they lack nuclei. These explanations are not necessarily mutually exclusive, and some or all of them may contribute to the observed differences.

We found no difference between infected and uninfected males in lifetime number of matings. In many insects including Lepidoptera, mating capacity is

directly related to males' ability to produce ejaculates (e.g. [8]). Contrary to our findings, we predicted that *Wolbachia*-infected males would mate less frequently as a result of reduced fertile sperm production. Sperm production in the Lepidoptera is often positively associated with male body size (e.g. [17]). However, there was no difference in body size between infected and uninfected males and body size did not explain differences in sperm transfer between males. Body size, therefore, cannot explain the observed association between *Wolbachia* infection and lowered fertile sperm transfer by non-virgins. We also found no effect of *Wolbachia* infection on longevity. Reported effects of *Wolbachia* on male longevity in *Drosophila* are inconsistent (e.g. [18,19]), although in *D. melanogaster* the virulent *Wolbachia* popcorn strain causes prolific tissue degeneration, which significantly shortens lifespan [20].

This study is the first to show that *Wolbachia* affects sperm transfer in a non-drosophilid host; we have shown that male *E. kuehniella* moths infected with *Wolbachia* transfer fewer fertile sperm when remating than uninfected males. Intriguingly, no such effect was observed for non-fertile apyrene sperm. As a result of their sperm heteromorphism, the Lepidoptera potentially represent an ideal model system to unravel the mechanisms underlying *Wolbachia*-induced CI.

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