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A cost of *Wolbachia*-induced sex reversal and female-biased sex ratios: decrease in female fertility after sperm depletion in a terrestrial isopod

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A number of parasites are vertically transmitted to new host generations via female eggs. In such cases, host reproduction is an intimate component of parasite fitness and no cost of the infection on host reproduction is expected to evolve. A number of these parasites distort host sex ratios towards females, thereby increasing either parasite fitness or the proportion of the host that transmit the parasite. In terrestrial isopods (woodlice), *Wolbachia* bacteria are responsible for sex reversion and female-biased sex ratios, changing genetic males into functional neo-females. Although sex ratio distortion is a powerful means for parasites to increase in frequency in host populations, it also has potential consequences on host biology, which may, in turn, have consequences for parasite prevalence. We used the woodlouse *Armadillidium vulgare* to test whether the interaction between *Wolbachia* infection and the resulting excess of females would limit female fertility through the reduction in sperm number that they receive from males. We showed that multiple male mating induces sperm depletion, and that this sperm depletion affects fertility only in infected females. This decrease in fertility, associated with male mate choice, may limit the spread of *Wolbachia* infections in host populations.

Keywords: Oniscidea; parasite; feminization; fertility; sperm depletion

1. INTRODUCTION

When parasites gain transmission to a new host generation via female eggs (vertical transmission), host reproduction is an intimate component of parasite fitness, and no cost of the infection on host reproduction is expected to evolve (Fine 1975). Among vertically transmitted parasites, *Wolbachia* are intracytoplasmic bacteria that infect many arthropod and nematode species (Rousset *et al.* 1992; Werren *et al.* 1995; Bandi *et al.* 1998). In arthropods, the effects of *Wolbachia* infection on basic host fitness components (fecundity, survival) vary according to parasite variants and/or host genotype, but in most cases *Wolbachia* have no significant effect on host fitness (e.g. in *Drosophila*: see Hoffmann *et al.* 1994; Giordano *et al.* 1995; Poinsot & Mercot 1997), consistent with the general theory. In a few host–*Wolbachia* associations, the bacterial infection imposes a physiological cost to the host (Hoffmann *et al.* 1990; Fleury *et al.* 2000). This cost is nevertheless often counterbalanced by the fact that most *Wolbachia* strains manipulate the reproductive biology of their hosts, thus increasing their propagation within host populations (see the review in Stouthamer *et al.* 1999). For example, several of these parasites distort host sex ratio, increasing either their fitness or the proportion of their transmitting vector (the female sex) (Rigaud 1997; Hurst *et al.* 1997). In terrestrial isopods (woodlice), *Wolbachia* induce feminization in most host species, inducing reversion of genotypic males into functional females (e.g. Juchault *et al.* 1993; Bouchon *et al.* 1998; Rigaud *et al.* 1999). As a result of maternal transmission, *Wolbachia*-infected mothers produce highly female-biased progeny, and *Wolbachia* are associated with an overall female-biased sex ratio in their hosts (e.g. Juchault *et al.* 1993; Moreau & Rigaud 2000, 2003).

Sex-ratio distortion is a powerful means by which parasites increase their frequency in host populations, but it also has potential consequences for host biology that might, in turn, have consequences for parasite prevalence. Extinction of the host population (and consequently parasite population) is one of these potential consequences (Hamilton 1967; Hatcher *et al.* 1999). Evolutionary changes in host reproductive strategy and patterns of reproductive competition have also been suggested or shown in several cases: by distorting population sex ratio (and operational sex ratio), *Wolbachia* alter the intensity or the direction of competition for mates (Jiggins *et al.* 2000; Moreau *et al.* 2001; Dyson & Hurst 2004). An excess of females induces a new situation where females are the sex in competition for mating. For example, in woodlice, uninfected females mate more frequently than infected ones when there is male choice because of the 'imperfect female status' of infected females (Moreau *et al.* 2001). Such a selective male mate choice would, by itself, be a factor capable of limiting *Wolbachia* maintenance or invasion in populations (Hatcher 2000; Randerson *et al.* 2000). In addition, males always provide fewer sperm to females infected by *Wolbachia*, compared with uninfected females (Moreau *et al.* 2001). One could speculate that fewer sperm may reduce female fertility, which may induce an additional limitation for the increase of

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Wolbachia in host populations. Indeed, a growing number of studies suggest that sperm quality or quantity may be limiting in arthropods, i.e. females inseminated with too few sperm may have a decreased fertility (e.g. Dewsbury 1982; MacDiarmid & Butler 1999; Baker *et al.* 2001; Schaus & Sakaluk 2001; Dyson & Hurst 2004; but see Andrade & Banta 2002). Moreau *et al.* (2001) demonstrated that the quantity of sperm received by isopod females is much higher than required to fertilize the number of eggs females that can lay. However, in the context of a female-biased sex ratio, the opportunities for males to mate multiply are much increased (male woodlice are able to mate with a large number of females, at least in species infected by feminizing *Wolbachia*; see Moreau & Rigaud (2003)), and sperm depletion could occur, which may alter female fertility.

We tested whether *Wolbachia* infection would limit fertility of female woodlice, relative to uninfected females, through the reduction in sperm numbers that they receive from males. We examined whether multiple male mating induces sperm depletion, whether this sperm depletion affects female fertility and, finally, whether differences in sperm numbers provided to females that are infected or uninfected by *Wolbachia* alter their fertility.

2. MATERIAL AND METHODS

(a) *Population origin, strain maintenance and general methods*

The woodlice used in this study belong to the cosmopolitan species *Armadillidium vulgare* (Crustacea, Isopoda, Oniscidea). All individuals were maintained under identical laboratory conditions for many years, on moistened soil, at 20 °C and at the natural photoperiod of Poitiers (latitude 46°40′ N). For each generation, males and females from the same brood were sorted by sex before they reached sexual maturity. They were then reared separately, thus ensuring that all females were virgin (see Moreau *et al.* (2001) for the routine procedure of strain maintenance). Two strains were used. Uninfected genetic females (heterogametic, WZ) and males (homogametic, ZZ) came from a strain collected in Nice (France) in 1961. Infected neo-females (genetic males reversed by the bacterium, ZZ + *Wolbachia*) came from a strain collected in Celles Sur belle (France) in 1991. A series of backcrosses was made to limit the genotypic differences between the two strains (see Moreau *et al.* 2001).

All experimental females and males were 1 year old. To obtain females receptive to mating, virgin females were reared under a 18 L : 6 D photoperiod, which stimulates the onset of reproduction. Female receptivity is limited to the end of a preparturial moult, when oocyte maturation is nearly over (Lefebvre & Caubet 1999). This stage was assessed by checking the shape of white plates of calcium carbonate on the ventral face, which differentiate a few days before moulting. Receptive females show incomplete plates compared with the complete plates of non-receptive individuals (Moreau & Rigaud 2002). Only females receptive to mating were used in the experiments.

(b) *General experimental procedure*

The following experiments took place in cylindrical boxes (diameter of 8 cm) with moistened soil, a piece of dead leaf and a slice of fresh carrot, at 20 °C for 12 h (Moreau *et al.* 2001). All experiments were initiated with virgin males and females. In each trial, five receptive females were presented to a single male. This was repeated over 4 days, the five females being changed every day. Each male therefore had 20 females available for mating in 4 days (figure 1). Mass (in mg ± 1 mg) was recorded for each individual using a Sartorius precision balance, and males were placed with females of approximately equal size (asymmetry random) to avoid any possibility of physical incompatibility for reproduction. Insemination is internal and, because isopod females possess two genital apertures each independently linked to one ovary, males must perform two successive inseminations to inseminate the female completely.

In terrestrial isopods, it is impossible to assess both the number of sperm received in one ejaculate and the fertility associated with this ejaculate in the same female (dissection of the female is needed to count the sperm within the oviduct). Therefore, the experimental series were assigned randomly to two different sets of analyses, to assess: (i) the quantity of sperm that a given female had received from a male (eight series of each category); and (ii) the fertility of each female (eight series of each category; figure 1). We first controlled the number of females inseminated. After dissection, mated females were characterized by the presence of large white balls of sperm in their two genital ducts (oviducts of unmated females are thin and transparent). In cases where eggs were laid, clutches showing no development in any egg were assigned to unfertilized females (eggs are visible through the transparent cuticular structure forming the marsupium). At the end of the experiments, males were dissected to control for the presence of macro-parasites. Males with parasites (e.g. nematodes) were excluded from the analysis to avoid confounding results owing to the usage of unhealthy males. Furthermore, to have a set of comparable experiments for the sperm depletion, the series where males mated with fewer than three females during the first day were also excluded from the analysis. Owing to these restrictions, data from only 20 males were available (10 males with infected females and 10 males with uninfected females; figure 1). In the series kept for analyses, all females had homogeneous weight (mean ± s.e.m.: females without *Wolbachia*: 83.47 ± 2.87 mg for the evaluation of female fertility and 86.37 ± 3.52 mg for the evaluation of sperm density in ejaculates, ANOVA (data log-transformed) $F_{1,101} = 0.47$, $p = 0.49$; females infected with *Wolbachia*: 73.367 ± 2.43 for the evaluation of female fertility and 80.23 ± 4.01 for the evaluation of sperm density in ejaculates, ANOVA (data log-transformed) $F_{1,80} = 2.16$, $p = 0.14$).

(c) *Assessment of sperm density in ejaculates*

Each oviduct was dissected in a watchglass containing 15 ml of Ringer solution. After gentle homogenization, six drops of 10 µl of this solution were deposited on a microscope slide. Sperm were visualized using DAPI (Sigma, concentration of 0.04 mg ml^{-1}) (Moreau *et al.* 2001). Observations were made under epifluorescence on a Zeiss Axioplan microscope. The estimated density of sperm received by each female was based on the total count of the 12 drops after the homogeneity between drops had been controlled for (results not shown).

(d) *Assessment of female fecundity and fertility*

After 12 h with one male, each female was isolated. The eggs were allowed to develop for at least three weeks. Embryos were then flushed out from the marsupium using a water flow. The numbers of fertilized and unfertilized eggs were recorded by checking the development status of each egg. Unfertilized eggs remain brown and no cell division can be observed, whereas developing eggs contain a visible larva. Some females laid clutches containing only unfertilized eggs. These were determined to be free of sperm by dissection, and were counted as unfertilized. Fecundity

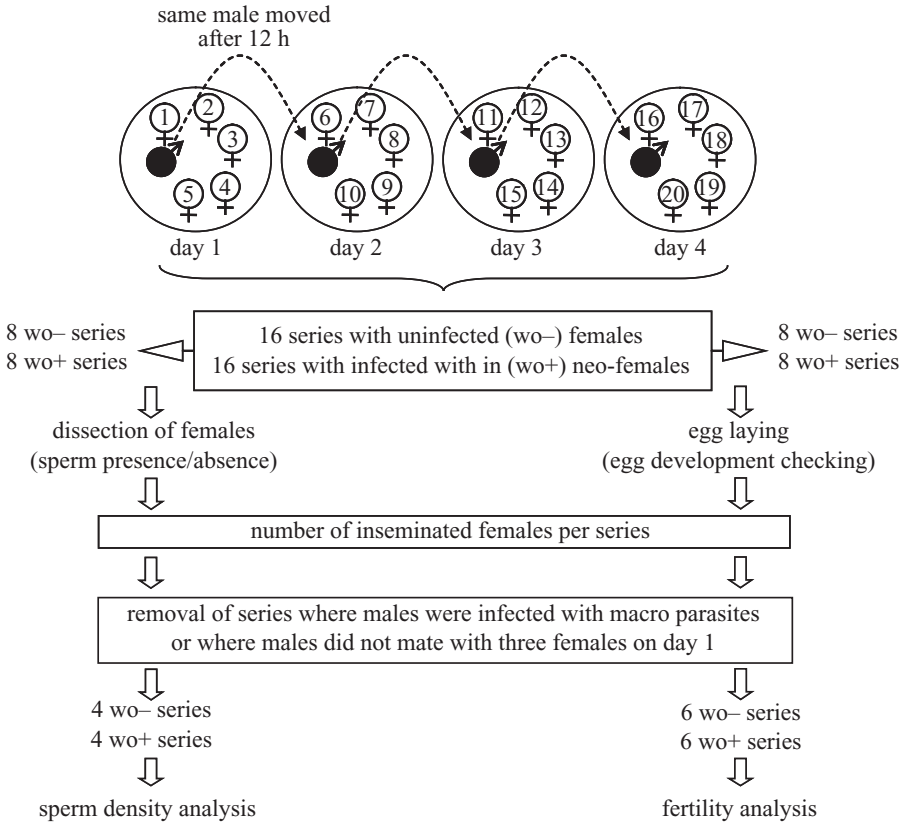

Figure 1. Experimental protocol (see § 2b for details).

was measured as the total number of eggs laid (fertilized + unfertilized). Fertility was estimated as the ratio of fertilized eggs out of the total number of eggs laid in the marsupium.

(e) *Statistical analysis*

Continuous data were neperian log(ln)-transformed and percentage estimates were arcsine-square-root transformed to meet homogeneity of variances and normality. The average proportions of females that a male can inseminate per day were compared with an ANOVA for repeated measures (data were not independent since the mating pattern of one given day depends on the mating pattern of the preceding day). The following analyses compared ejaculate density in females that had been mated. In these cases, it was not possible to use an ANOVA for repeated measures because the number of females inseminated by a given male during the 12 h was not the same between the sub-series. To take into account the male effect for the different female categories, a nested ANOVA was conducted, where the 'male' factor was nested in the 'female infection status' factor. The differences in female fertility according to their infection status were analysed day-by-day, using an ANOVA. In all cases, the male and female weights were taken as covariables, but they never significantly influenced the results (data not shown). They were therefore removed from the final analysis, as were the non-significant interactions between the factors under test. The fecundities were nevertheless compared using an analysis of covariance, with the female weight as covariable, since it is known that fecundity is linked to female size in terrestrial isopods (e.g. Moreau *et al.* 2002).

All tests were performed using the JMP statistical software (v. 5.0, SAS Institute Inc.).

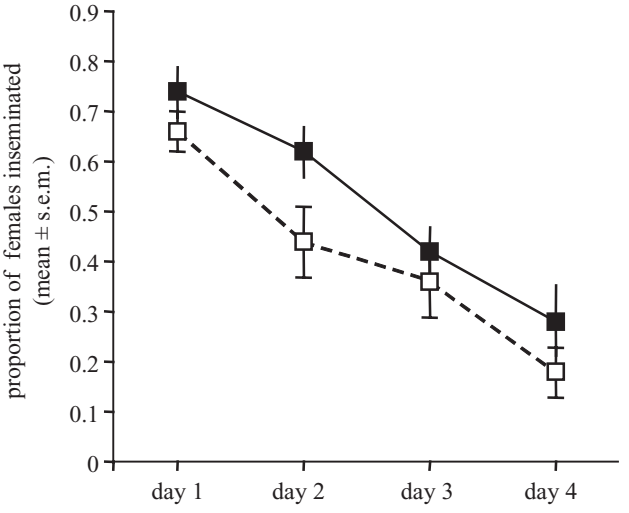

Figure 2. Proportion of females inseminated per male in *Armadillidium vulgare*, according to day of the experiment and infection status of females. Each day, five females were presented to each male for mating. Sample size was 10 males per series: infected, open squares; uninfected, filled squares.

3. RESULTS

Females possess two genital tracts, but males may occasionally inseminate only one tract. In our experiments, this happened in 18 females out of 118, with an increase over time (from 4.6% in day one to 28.6% in day four, $\chi^2_3 = 9.75$; $p = 0.02$). This nevertheless did not occur more frequently in infected than in uninfected females (8/56 versus 10/62, respectively, $\chi^2 = 0.08$; $p = 0.78$). These 'half-inseminated' females were counted as inseminated. The

Table 1. ANOVA tables for ejaculate density received by inseminated females (log-transformed data) testing the effects of day, infection by *Wolbachia* and individual males.
(The 'male effect' was nested within the 'infection' effect.)

source	d.f.	sum of squares	*F*-ratio	*p*
day	3	26.21	19.13	< 0.001
infection	1	2.30	5.04	0.028
male(infection)	6	4.91	1.79	0.117
error	56	25.57		
total	66	61.09		

proportion of females inseminated each day (among the five available per male) decreased with time (figure 2). At the beginning of the experiment, *ca*. 70% of females were inseminated, whereas on the fourth day, only *ca*. 20–30% were inseminated, demonstrating that male mating capacity decreases after several copulations. Slightly fewer infected females were inseminated than uninfected ones (ANOVA for repeated measures made on arcsine-square-root transformed data: between-groups effect (*Wolbachia* infection): $F_{1,18} = 5.05$; $p = 0.037$; within-group: effect of the day: $F_{3,16} = 23.28$; $p < 0.0001$; interaction day × infection: $F_{3,16} = 0.52$; $p = 0.671$).

The density of the ejaculate provided to females also decreases with time (table 1; figure 3*a*), i.e. males provide fewer sperm after multiple copulations. Females infected by *Wolbachia* received less sperm than their uninfected counterparts (table 1).

The fertility was significantly different between the two female categories from only the third day of the experiment (figure 3*b*), i.e. only after sperm depletion in males was important (figure 3*a*). This difference was caused by a decrease in the fertility of infected females (ANOVA on transformed data: $F_{3,55} = 5.93$; $p < 0.001$), but not in uninfected females (ANOVA: $F_{3,58} = 1.10$; $p = 0.36$; figure 3*b*).

Female weight was found to affect fecundity ($F_{1,114} = 150.17$, $p < 0.0001$), regardless of infection status ($F_{1,114} = 1.01$, $p = 0.32$; interaction weight × infection: $F_{1,114} = 0.02$, $p = 0.87$; figure 4). The fecundity was therefore the same between the two categories of females.

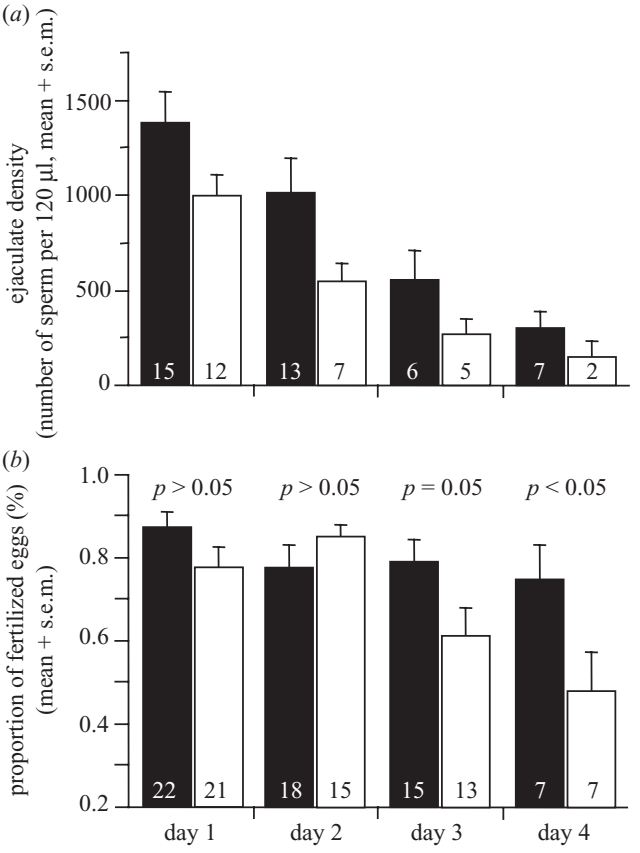

Figure 3. (*a*) Average ejaculate density and (*b*) fertility in *Armadillidium vulgare* females, according to day of the experiment and infection status of females. Numbers in the bars are the numbers of females investigated. *p*-Values in (*b*) refer to ANOVA analysis comparing the fertility of the two female types each day: infected, open bars; uninfected, filled bars.

4. DISCUSSION

Our results highlight that sperm depletion occurs after multiple mating by *A. vulgare* males, and that this sperm depletion affects the fertility of females differently according to their infection status by the *Wolbachia* bacteria.

The sperm depletion might be owing to the fact that males do not have enough time to refresh their sperm reserve between the serial copulations. The pattern of this sperm depletion fits well with the pattern of mating capacity: both regularly decrease with time. Owing to the ejaculate expenditure (sperm are provided in large numbers, at least during the first copulations), this suggests that it might be costly to produce sperm rapidly (see Dewsbury (1982) for a discussion on sperm cost).

Confirming previous results (Moreau *et al.* 2001), males tend to inseminate fewer females infected by *Wolbachia* bacteria relative to uninfected females, and fewer sperm were provided to infected females. The difference in sperm density provided to infected versus uninfected females nevertheless tends to be less obvious in cases of sperm depletion.

The fertility differed between females infected by *Wolbachia* and uninfected females, but only after males had experienced several copulations and provided fewer sperm to females. However, since sperm depletion occurred in uninfected females (figure 3*a*) with no significant changes in female fertility (figure 3*b*), there was no direct link between sperm depletion in males and fertility in females in this experimental series. Conversely, in infected females, fertility was affected by a low number of sperm in the ejaculate, consistent with several observations in other invertebrates (e.g. MacDiarmid & Butler 1999; Baker *et al.* 2001; Schaus & Sakaluk 2001). However, from the comparison between figures 3*a* and 3*b*, the relationship between sperm

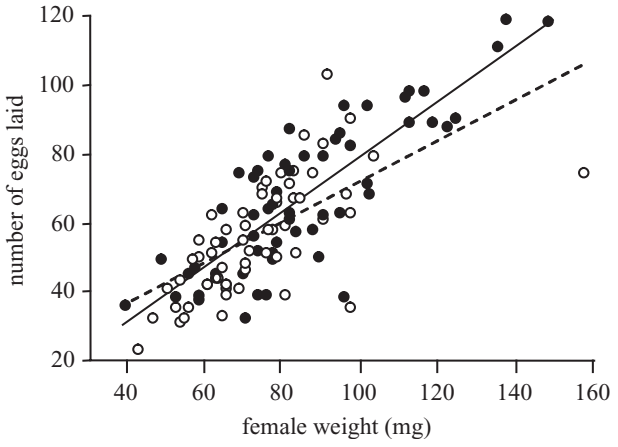

Figure 4. Fecundity of *Armadillidium vulgare* females according to their weight and infection status: infected, open circles; uninfected, filled circles.

depletion and reduced fertility is not linear and we can reasonably propose that a threshold exists above which fertility is affected by a (relatively) low sperm number. This threshold exists only in females infected by *Wolbachia*. From this observation, we therefore propose that infected females do not use sperm as efficiently as uninfected females.

We cannot exclude the possibility that the difference in fertility is directly owing to *Wolbachia* infection. However, previous results obtained after experimental infections have shown that the infection was not the direct cause of differences in the amount of sperm that males provide to the two types of female (Moreau *et al.* 2001). In fact, the difference should be more owing to the fact that naturally infected females are not genetic females (heterogametic for sex chromosomes, WZ), but are genetic males (homogametic ZZ) reversed into females by *Wolbachia* (called neo-females; see Rigaud 1997). The lack of the W female chromosome in these individuals has consequences for their behaviour during sexual courtship, the behavioural sequence normally leading to copulation being abnormal in neo-females (Moreau *et al.* 2001). The incomplete ability to use male sperm in the case of sperm depletion is another case of dysfunction of a female trait in these sex-reversed individuals.

The decrease in fertility after sperm depletion in infected neo-females, associated with female-biased sex ratios and male mate choice (Moreau *et al.* 2001), may have consequences for the maintenance of *Wolbachia* infection in host populations. With no fitness cost associated with *Wolbachia* and a strong symbiont transmission rate, the infection should spread deterministically in host populations (Taylor 1990). However, during *Wolbachia* spread, the population sex ratio becomes more and more female-biased (Taylor 1990; Rigaud *et al.* 1992), inducing: (i) the establishment of male mate choice, the males preferring uninfected females as mating partners (Moreau *et al.* 2001); and (ii) increased opportunities for multiple copulations in males because of female-biased operational sex ratios (Moreau & Rigaud 2003). The uninfected females would therefore be more likely to receive the first mating, and the larger amount of sperm. The infected neo-females would mate later and therefore receive inadequate numbers of sperm. Since lower sperm density in ejaculate induces decreased fertility, it is likely that infected neo-females would suffer a decrease in fitness. By these means, frequency-dependent selection for *Wolbachia* infection would occur in host populations: the more frequent the infection, the more female-biased the sex ratio and consequently the higher the probability of a fitness cost. This phenomenon will, of course, depend on encounter rates between mating partners in the field. However, since *A. vulgare* is a highly gregarious species, with local densities reaching more than 1000 individuals m^{-2} (e.g. Rigaud & Juchault 1995), this seems to describe a realistic situation.

The opportunity for female multiple mating to increase the number of sperm that they receive (as observed in some insect species; see, for example, Baker *et al.* (2001)) has not been investigated in this study. However, most *A. vulgare* females reject a second mating within the same receptivity period (Moreau *et al.* 2002) and infected neo-females reject copulations more frequently than uninfected females (Moreau *et al.* 2001), making neo-female multiple mating unlikely. The possibility should nevertheless be tested that neo-female multiple mating can be a strategy to avoid reduced fertility only in cases of sperm depletion.

Despite this restriction, the frequency-dependent phenomenon described above could explain why *Wolbachia* infections are rarely at fixation in host woodlice populations, and would therefore explain the maintenance of an infection polymorphism for this sex-ratio distorter (Randerson *et al.* 2000). This study suggests that *Wolbachia*-induced manipulation of high-level biological traits, such as sex ratio, may induce side-effect costs for the infection.

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