Sexual selection in an isopod with *Wolbachia*-induced sex reversal: males prefer real females

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Abstract

A variety of genetic elements encode traits beneficial to their own transmission. Despite their 'selfish' behaviour, most of these elements are often found at relatively low frequencies in host populations. This is the case of intracytoplasmic *Wolbachia* bacteria hosted by the isopod *Armadillidium vulgare* that distort the host sex ratio towards females by feminizing the genetic males they infect. Here we tested the hypothesis that sexual selection against *Wolbachia*-infected females could maintain a polymorphism of the infection in populations. The infected neo-females (feminized males) have lower mating rates and received less sperm relative to uninfected females. Males exhibited an active choice: they interacted more with uninfected females and made more mating attempts. A female behavioural difference was also observed in response to male mating attempts: infected neo-females more often exhibited behaviours that stop the mating sequence. The difference in mating rate was significant only when males could choose between the two female types. This process could maintain a polymorphism of the infection in populations. Genetic females experimentally infected with *Wolbachia* are not exposed to the same sexual selection pressure, so the infection alone cannot explain these differences.

Introduction

Intracytoplasmic symbionts frequently use a vertical mode of transmission. Their survival and reproduction is dependant upon the survival and reproduction of their hosts (Fine, 1975). However, in the context of anisogamy, such symbionts are only maternally transmitted via the cytoplasm of the eggs. Consequently, if these micro-organisms are able to cause an excess of females in their host, they will increase their transmission, even in the absence of advantageous effects and even if a cost is associated with the infection (Cosmides & Tooby, 1981;

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Werren & O'Neill, 1997). The condition that must be satisfied is that the eventual cost of the infection is outweighed by the excess of infected daughters produced by infected females (Taylor, 1990).

One of the most spectacular types of cytoplasmic sex ratio distorters (CSRD) are those that convert males into functional females (Rigaud, 1997). Such an alteration of sex determination is induced by several micro-organisms in several species of crustaceans, and particularly by *Wolbachia* bacteria in terrestrial isopods (Martin *et al.*, 1973; Bouchon *et al.*, 1998). In isopods, *Wolbachia* transform genotypic males (homogametic sex, ZZ) into phenotypic females (ZZ + *Wo*, called neo-females). Basic population dynamic models predict that such sex ratio distorters should increase in frequency in host populations previously uninfected (Hatcher & Dunn, 1995), and that uninfected genetic females should be replaced by infected neo-females in a few generations (Taylor, 1990). All infected females found so far in wild populations of the woodlouse *Armadillidium vulgare* are of the ZZ + *Wo*

type, supporting the models; however, their frequency is often lower than those predicted by the models (Juchault *et al.*, 1993; Rigaud *et al.*, 1999a). Recent research suggests that polymorphism for the infection by CSRD may be explained by several processes, e.g. population structure of the host, but may also be explained by the insufficiency of knowledge of some biological traits (Hatcher, 2000). In particular, infected females may suffer a fitness disadvantage relative to uninfected females. In terrestrial isopods, evidence exists that *Wolbachia* induce various costs (Rigaud & Juchault, 1998; Rigaud *et al.*, 1999b). However, such costs are generally low or limited to stringent conditions, such that they can hardly explain the general low prevalence of feminizing *Wolbachia* in natural populations.

Recently, it has been suggested that the role of mate choice is a promising area in explaining CSRD polymorphism in populations (Hatcher, 2000; Randerson *et al.*, 2000). Indeed, in populations infected by CSRD, choosiness may have evolved because of the rarity of males (Emlen & Oring, 1977). In a butterfly harbouring a male-killing *Wolbachia*, infected females have a lower probability of being mated than uninfected ones when the symbionts reach near-fixation, i.e. when the sex ratio reaches extreme female-biased values (Jiggins *et al.*, 2000). Similar findings were obtained in terrestrial isopods infected by feminizing *Wolbachia*, even if biases in sex ratios were less severe (Moreau & Rigaud, 2000). One hypothesis is that males are able to discriminate between females on the basis of infection and prefer to mate with uninfected females. However, mate choice has not been clearly tested in these systems. We therefore tested this hypothesis using experiments designed to test whether sexual selection against *Wolbachia* infection exists within the terrestrial isopod *Armadillidium vulgare*. We discuss the consequences of our results with respect to host population stability and the evolution of parasite prevalence.

Materials and methods

Population origin, strain maintenance and general methods

Woodlice used for this study belong to the species *Armadillidium vulgare* (Crustacea, Isopoda, Oniscidea). They were all maintained in the same laboratory conditions, on moistened soil, at 20 °C and at the natural photoperiod of Poitiers (latitude 46°40′N). Food consisted of dead leaves and slices of fresh carrots provided *ad libitum*. Couples were bred individually in small boxes (diameter 8 cm). Offspring were separated from parents immediately after their birth, and reared in wider boxes (26 × 13 cm) in order to avoid competition effects. Four months after their birth, when individuals can be sexed, prereproductive males and females were reared in isolation to avoid sib-mating. All individuals were 14 months old at the time of the experiments. Two strains were used (20 lines in each strain). Uninfected genetic females (WZ) and males (ZZ) originally came from a strain collected in Nice (France) in 1961. To avoid inbreeding depression in this strain, females were serially crossed with males from various uninfected populations of different geographical origins (Sao-Paulo [Brazil], Rabat [Morocco], Sète, Moulis [France], Marbella [Spain], and Heraklion [Crete]). Males from a mass culture from Heraklion (original population consisted of 54 males and 84 females) were used during the nine last generations. Infected neo-females (ZZ + *Wo*) came from a strain collected in Celles sur Belle (France) in 1991. In order to limit the genotypic differences between the two strains, the infected strain was introgressed with the uninfected genome, by crossing males from the uninfected strain with females from the infected strain for six generations (six years) prior to the experiments.

To obtain virgin females receptive to mating, females from both strains were reared under a stimulatory photoperiod of LD 18:6, at 20 °C. Female receptivity is limited to the end of a preparturial intermoult, when oocyte maturation is nearly over (Lefebvre & Caubet, 1999), and can be estimated by the appearance of incomplete white plates of calcium carbonate on the ventral face, a few days before moulting (Steel, 1982; Moreau, personal observation).

Evaluation of mating success

To examine male mating inclination, encounters were performed by pairs, one male with either one infected neo-female (ZZ + *Wo*) or one uninfected female (WZ) ('no-choice' experiment). Twenty-one replicates were done with uninfected females and 27 with infected females. Pairs were placed in a cylindrical box (diameter 8 cm) with moistened soil and a piece of dead leaf, at 20 °C for 12 h (time sufficient for mating, see Mead, 1973). Generally, males were bigger than females, or approximately of equal weight, to avoid any physical mating incompatibility (the difference between males and females ranged between −10 mg and +70 mg; mean ± SEM = +14.7 ± 2.9; there was no difference between infected and uninfected females, $t = 0.61$, $P = 0.55$).

To determine the role of male choice and/or female–female competition, 24 triads were made, with one male encountering both an infected neo-female (ZZ + *Wo*) and an uninfected WZ female ('Choice 1' experiment), in the conditions described above. To distinguish the females, both were marked at different places on the cuticle. Differences in weights of infected and uninfected females never exceeded 7 mg (never more than 5.5% of body mass), and differences between male and female body sizes were in the same range as those in the previous experiment (ranging between −10 mg and +80 mg; mean ± SEM = + 19.7 ± 3.2; no difference

between infected and uninfected females, $t = 0.25$, $P = 0.80$).

To investigate the role of the *Wolbachia* infection *per se*, 24 triads were made with one male encountering two females from the uninfected strain (WZ females), one of which was previously infected experimentally with *Wolbachia* and the other injected with Ringer solution only ('Choice 2' experiment). The method for experimental infection, by inoculation, has previously been described (e.g. Rigaud & Juchault, 1995). The delay between inoculation and the choice test was 90 days, a delay large enough for the bacteria to infect all tissues of transinfected individuals, including oocytes (Rigaud & Juchault, 1995). The procedure described before was used, with the same care taken for differences in male and female sizes (difference ranging from + 4 mg to +56 mg, mean ± SEM = 26.1 ± 2.1; no difference between infected and uninfected females, $t = 1.66$; $P = 0.11$).

After each experiment, females were dissected in order to determine whether they had been mated. Insemination is internal, and, as the isopod females possess two genital apertures each independently linked to one ovary, males have to introduce sperm into both of them to inseminate the female completely. Inseminated females were characterized by the presence of large white balls of sperm in their genital ducts, while oviducts of nonmated females were thin and transparent. To investigate the ejaculate number, inseminated oviducts were dissected in a watch glass containing 15 mL of Ringer solution. After gentle homogenization, six drops of 10 μL per sample were deposited on a microscope slide. Sperm were revealed using DAPI (Sigma, concentration of 0.044 mg mL^{-1}). Observations were made under epifluorescence on a Zeiss Axioplan microscope. For each oviduct, the estimated number of sperm was based on the total counting of the six drops, after the homogeneity between drops had been controlled for (results not shown).

Analysis of mating behaviour

Both male and female behaviours were studied for 1 h using direct observation, under 'choice test' conditions. All behaviour took place in a Petri dish lined with moistened paper (diameter = 9.5 cm), topped with a transparent glass slide in order to limit air disturbance, at 20 °C, light = 100 lux, relative humidity = 90%. In experiment 1, one infected neo-female and one uninfected female were introduced to opposite sides of the dish and a male was then placed between them. Behavioural sequences were recorded using a programmable WorkAbout recorder (Psion, UK). Behaviour was classified as three types: (i) non-interactive activity: locomotion, immobility; (ii) non-sexual interactions: brief (<1 s) reciprocal antennae contact or antennae contact on the body; and (iii) sexual interactions: long

male exploration of female body with antennae (assessment); male mounting on female's dorsal surface (first step of the mating sequence); female's response to mounting; copulation sequence *per se* (see Mead, 1973). The criteria analysed for each item were (1) individual exhibiting the item; (2) the number of occurrences and (3) the duration (NB: some non-sexual interactions were too brief to be measured). Replicates where no sexual interaction was observed were removed from the analysis. At the end of each replicate, females that received at least one complete mating sequence were dissected to check whether they had been inseminated or not (see above).

For experiment 2, the same procedure was repeated to compare behaviours of experimentally transinfected WZ females and uninfected ones (see above).

All statistical tests were performed using the JMP software (Version 3.2.2, SAS Institute Inc.).

Results

Mating success of uninfected vs. infected females

A significant difference in insemination rates of different female types, expressed either as the number of mated females or the number of inseminated genital apertures (one given female can be inseminated in only one genital aperture), was observed only in the 'choice 1' experiment, involving infected neo-females (Table 1). In this last series, we found three replicates where no female were inseminated, one replicate where only the infected female was inseminated, eight replicates where only the uninfected female was inseminated, and 12 replicates where both females were inseminated (among which seven replicates where males fully inseminated both females, i.e. in the two genital apertures).

In the 'choice 2' experiment, there was no difference between sperm provided by males to experimentally infected or uninfected females (Table 1). In the two experiments involving naturally infected neo-females, males inseminated much more sperm within uninfected females (Table 1). In the 'choice 1' experiment, this difference was always found, even when males fully inseminated the two female types (Table 1). To strengthen this observation, a new experiment was made ('serial' experiment), where infected neo-females were mated before uninfected females. Infected neo-females were placed for 12 h with a male, as described in the 'no choice' experiment, females were dissected, and sperm counted when insemination occurred in the two genital apertures (11 cases out of 34 replicates). In this last case, an uninfected female was placed immediately with the same male, in the same conditions. When fully inseminated (nine cases out of 11 replicates), the sperm of this last female was counted. Even in these conditions, males provided less sperm to infected females (700.4 ± 61.2 sperm per 60 μL per genital

Table 1 Mating parameters in females according to their *Wolbachia* infection status and the type of experiment ('No Choice' = one male: one uninfected female, or one male: one naturally infected neo-female; 'Choice 1' = one male: one uninfected female: one naturally infected neo-female; 'Choice 2' = one male: one uninfected female: one experimentally infected female).

		Female type		
Experiment	Measures	Uninfected	Infected	*P*
'No choice'	% females inseminated (*n*)	81.0% (21)	66.7% (27)	0.27[a]
	% G.A. inseminated (*n*)	76.2% (42)	66.7% (54)	0.17[a]
	Nb sperm/60 μL/G.A.	786.7 ± 50.8	396.6 ± 26.4	<0.0001[b]
'Choice 1'	% females inseminated (*n*)	83.3% (24)	54.2% (24)	0.04[c]
	% G.A. inseminated (*n*)	79.1% (48)	47.9% (48)	0.02[c]
	Nb sperm/60 μL/G.A.*	948.9 ± 71.1	676.9 ± 43.1	0.0006[d]
'Choice 2'	% females inseminated (*n*)	66.7% (24)	70.8% (24)	0.57[c]
	% G.A. inseminated (*n*)	64.6% (48)	70.8% (48)	0.77[c]
	Nb sperm/60 μL/G.A.*	893.9 ± 70.0	848.2 ± 42.2	0.54[d]

[a]Pearson χ^2; [b]Wilcoxon rank-scores test; [c]Wilcoxon signed-rank test; [d]Paired *t*-test; *Measures made in replicates where all genital apertures of the two female types were inseminated (*n* = 7 replicates, 14 G.A. in each female type). G.A. Genital Aperture.

aperture) than to uninfected ones (1412.0 ± 151.7) (Wilcoxon signed-rank test; $P = 0.004$). The sperm number was different between the three experiments ('no choice', 'choice 1' and 'serial'), but always higher in uninfected females (two-way ANOVA: effect of the experiment: $F_{2,126} = 25.14$; $P < 0.0001$; effect of the female infection status: $F_{1,126} = 58.81$; $P < 0.0001$; effect of their interaction: $F_{2,126} = 4.38$; $P = 0.01$). A difference in the male weights cannot explain this variation (ANOVA: $F_{2,46} = 0.64$; $P = 0.53$), but these experiments were not made at the same time ('no choice' in March, 'choice 1' in April, and 'serial' in May). Although the three types of experiments were too different to allow a correct analysis, we could suggest an effect of the date, which could reflect a rhythm in male mating capacity.

Mating behaviour analysis

In experiment 1, where males had choice between one uninfected female and one naturally infected neo-female, *Wolbachia* infection status did not influence which female was first chosen for mounting (Table 2). Nevertheless, before the first mount, males had more interactions (nonsexual and assessments) with uninfected females than infected neo-females, although the time allocated to assessment was the same for both female types (Table 2). During the whole of the experiment (1 h), more uninfected females were mounted than infected ones (Table 2). More mountings attempts were also provided to uninfected females (borderline significance) (Table 2). Finally, the number of successful attempts (mounting followed by mating) was higher in unin-

Table 2 Items recorded in 21 replicates of the choice experiments (one male: one infected female: one uninfected female), in experiment 1 (infected females were naturally infected neo-females) and in experiment 2 (infected females were experimentally infected genetic females).

	Experiment 1			Experiment 2		
	Female type			Female type		
Items	WZ, Uninfected	ZZ, Infected	*P*	WZ, Uninfected	WZ, Infected	*P*
Number of experiments where the female was mounted first	13	8	0.12[a]	10	11	0.76[a]
% activity before the first mount*	0.62 ± 0.06	0.64 ± 0.04	0.83[b]	0.27 ± 0.04	0.38 ± 0.06	0.07[b]
Number of interaction given by the male before the first mount*	7.67 ± 1.39	5.81 ± 1.26	0.04[b]	14.71 ± 3.13	14.95 ± 3.07	0.66[b]
Duration (seconds) of male assessments before the first mount*	54.59 ± 25.50	24.81 ± 8.92	0.31[b]	17.73 ± 5.66	30.04 ± 12.90	0.93[b]
Number of females mounted at least once during 1 h	18	10	0.009[a]	12	14	0.53[a]
Total number of mounts observed during 1 h	38	23	0.05[c]	27	34	0.37[c]
Total number of mating observed during 1 h	20	6	0.04[a]	13	11	0.21[a]

[a]Pearson χ^2 test; [b]Wilcoxon signed-rank test; [c]observed vs. balanced expected frequencies χ^2 test; *Mean ± SEM.

Table 3 Behavioural items recorded after each mounting in 21 replicates of the choice experiment (one male: one infected female: one uninfected female), in experiment 1 (infected females were naturally infected neo-females) and in experiment 2 (infected females were experimentally infected genetic females).

Female behavioural response to mounting	Male behaviour after the female response	Occurrences of the behavioural sequence according to the female type			
		Experiment 1		Experiment 2	
		Uninfected	Infected	Uninfected	Infected
Rolling – Opening	Mating	20	6	13	11
Rolling – Opening	No mating	3	2	2	1
Other*	Mating	0	0	0	0
Other*	No mating	15	15	12	22

*Rolling without opening, immobilization, escape.

fected females (Table 2). We observed no female–female aggression nor interference before or during courtship (results not shown), and therefore the uninfected female advantage was not due to some direct form of female competition. In experiment 2, where males had choice between one uninfected female and one experimentally infected female, no significant differences were found between female types for all analysed items (Table 2).

The analysis of behaviours following the mounting revealed two categories of female response: a rolling immediately followed by a slight opening, which corresponded to the female's acceptance for mating (Mead, 1973), and three other behaviour types (Table 3). Indeed, these other behaviours systematically induced male retreat whatever the female type, while rolling-opening was often followed by copulation. These two categories of behaviours (rolling-opening vs. others) therefore led to very different mating success, within both infected females and uninfected females, and whatever the experiment (Pearson $\chi^2_1 = 11.22$; $P < 0.0001$ and $\chi^2_1 = 27.54$; $P < 0.0001$ for infected neo-females and uninfected females in experiment 1, and $\chi^2_1 = 29.81$; $P < 0.0001$ and $\chi^2_1 = 20.06$; $P < 0.0001$ for infected and uninfected females in experiment 2). In experiment 1, uninfected females were more likely to show a rolling-opening response compared to neo-females, but the test showed a borderline significance (Pearson $\chi^2_1 = 3.80$; $P = 0.05$). Such a difference was not found in experiment 2, involving experimentally infected females (Pearson $\chi^2_1 = 2.50$; $P = 0.11$). Interestingly, the infected female that did respond by rolling-opening had a similar mating success compared with that of uninfected females (Pearson $\chi^2_1 = 0.63$; $P = 0.43$ in experiment 1, and $\chi^2_1 = 0.17$; $P = 0.68$ in experiment 2).

Discussion

This study provides evidence for a lower mating success of *A. vulgare* neo-females infected by *Wolbachia* endosymbionts, relative to uninfected females. This difference was only significant when the two types of females were in competition for mating. It was probably not due to strain characteristics, since our line maintenance procedure should have minimized the genetic differences between the two strains.

Two behavioural components are involved in this relative mating deficiency. First, males interacted more with uninfected females. This active discrimination could be due to differences in pheromones, since preliminary gas chromatography results suggest differences in cuticular components between uninfected females and infected neo-females (F. Lefebvre, A. Bertin & Y. Caubet, unpublished data). This discrimination by males may explain why males made more mating attempts with uninfected females. The second behavioural component involved is the female's response to male mating attempts. Infected neo-females often responded inappropriately to allow the continuation of the mating sequence.

The absence of differences between uninfected and experimentally infected WZ females showed that infection alone cannot explain the differences between infected neo-females and uninfected females. We suggest that these differences were more due to the peculiar genotype of infected neo-females (they are in fact genetic males feminized by *Wolbachia*, lacking the W chromosome), even if an interaction between the two phenomena cannot be dismissed. The absence of the W chromosome in the infected neo-females is therefore likely to influence a female's mating behaviour and hence her attractiveness. The behavioural difference between infected neo-females and uninfected females was nevertheless not qualitative: the same behaviours were observed, but in different proportions. We could hypothesize that gene(s) necessary for mating behaviour regulation could be located on the W chromosome, but behaviour is also influenced by autosomes, as is the case for other reproductive phenomena (Souty-Grosset *et al.*, 1994).

Males also provided less sperm to infected neo-females. The reason for this remains to be explained, but this observation may strengthen the fact that infected females

were less attractive to males than their uninfected counterparts. Alternatively, since females are probably not totally passive during sperm transfer, slight behavioural differences between WZ females and feminized males during the mating sequence, not detected in the present study, could explain this male 'deficiency'. Sperm limitation of infected females could have consequences for their fertility, but this remains to be shown since our estimation of sperm number suggests that average ejaculate size greatly exceeds the number of eggs that a female can lay.

Lower mating rate of *Wolbachia*-infected females has already been demonstrated in natural populations of the butterfly *Acraea encedon*, in which the symbiont induces male-killing (Jiggins *et al.*, 2000). However, this was only observed when *Wolbachia* reach high prevalence in populations, and therefore when the sex ratio is extremely female-biased (around 97% females). In *A. vulgare*, differences in mating rate were significant only when males had a choice of partner, but the sex ratio used in our experiments was not as female-biased as those observed in *Acraea*.

Under panmictic conditions, females infected with *Wolbachia* deterministically spread because on average they produce more daughters than uninfected females (Taylor, 1990). The choice-dependent characteristics of the decreased mating rate in infected females might affect *Wolbachia* population dynamics (Randerson *et al.*, 2000). If male mate choice is frequency independent, the decreased mating rate of neo-females could affect the initial conditions of the invasive process and, in an extreme outcome, prevent the infection from becoming established. However, if male mate choice is frequency-dependent, the choice would not affect the initial invasion conditions, but rather the infection frequency in the population (Randerson *et al.*, 2000). We believe that the reproductive traits of terrestrial isopods are likely to result in frequency-dependent processes for the following reasons. The appearance of the infection at low prevalence in an uninfected population would not affect the overall 1:1 sex ratio because infected females are rare. Under such a sex ratio, strong male–male competition is suspected because of the high potential reproductive rate of males relative to females (Kvarnemo & Ahnesjo, 1996; Moreau & Rigaud, 2000). We therefore expect that all females can be mated at this point, including the rare infected ones, because many mating attempts are possible with many different males. Therefore, the mating disadvantage of infected females should not be consequential when *Wolbachia* are rare: the proportion of infected females should increase and the sex ratio would tend to evolve toward a female-bias (Taylor, 1990). As infected female proportion increases, males become rarer, and mating attempts per female would decrease. Our results suggest that reduced mating success is not high enough to reverse the relative proportions of daughters produced

by the two female types, since the mating differential is around 30%. However, this difference could be higher in natural conditions because the small mating area used in this study tends to maximize the encounters between individuals, therefore optimizing mating probabilities. In the wild, the probability of encountering a male is probably lower and the multiplicity of mating attempts is probably reduced. Also, the possibility exists for selection to favour males that choose to mate with uninfected females (see Moreau & Rigaud, 2000, for data supporting this in woodlice, and Jiggins *et al.*, 2000, for data in butterflies), because many attempts on infected females to obtain only a few successes could be costly for males, and because of the greater number of sons (i.e. the rarer sex) they produce (Pomiankowski & Hurst, 1999). At this point, low mating frequency of infected females could be a barrier to *Wolbachia* spreading and fixation in the population. This type of frequency-dependent dynamics could therefore maintain a polymorphism in *Wolbachia* infection, by a process similar to the one modelled by Randerson *et al.* (2000).

Acknowledgments

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