Variable male potential rate of reproduction: high male mating capacity as an adaptation to parasite-induced excess of females?

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Numerous animals are known to harbour intracytoplasmic symbionts that gain transmission to a new host generation via female eggs and not male sperm. Bacteria of the genus *Wolbachia* are a typical example. They infect a large range of arthropod species and manipulate host reproduction in several ways. In terrestrial isopods (woodlice), *Wolbachia* are responsible for converting males into females (feminization (F)) in some species, or for infertility in certain host crosses in other species (cytoplasmic incompatibility (CI)). *Wolbachia* with the F phenotype impose a strong excess of females on their host populations, while *Wolbachia* expressing CI do not. Here, we test the possibility that male mating capacity (MC) is correlated with *Wolbachia*-induced phenotype. We show that males of isopod hosts harbouring F *Wolbachia* possess a strong MC (i.e. are able to mate with several females in a short time), while those of species harbouring CI *Wolbachia* possess a weaker MC. This pattern may be explained either by the selection of high MC following the increase in female-biased sex ratios, or because the F phenotype would lead to population extinction in species where MC is not sufficiently high. This last hypotheses is nevertheless more constrained by population structure.

Keywords: Oniscidea; male mating capacity; *Wolbachia*; feminization; cytoplasmic incompatibility

1. INTRODUCTION

Parasitism is one of the numerous factors explaining the variation in reproductive effort and reproductive success of living organisms (e.g. Polak & Starmer 1998; Altizer & Oberhauser 1999; Bollache *et al.* 2002), with different mechanisms and proximate or ultimate causes (Worden *et al.* 2000; Combes 2001; Hurd 2001). In some cases, parasites decrease the reproductive effort of their hosts as host resources are reallocated to parasites (Combes 2001). However, parasite-induced risk of mortality may also increase reproductive effort (e.g. Polak & Starmer 1998). Numerous animals harbour endoparasites or endosymbionts which gain transmission to a new host generation via female eggs. In this case, reproduction of the host is an intimate component of parasite fitness. Among these symbionts, the bacteria of the genus *Wolbachia* are a typical example. *Wolbachia* are intracytoplasmic inherited bacteria which infect a large number of arthropod and nematode species (Rousset *et al.* 1992; Werren *et al.* 1995; Bandi *et al.* 1998). These bacteria are able to alter the reproductive biology of their hosts in many ways, all increasing their spread within host populations (Werren & O'Neill 1997; Stouthamer *et al.* 1999; Dedeine *et al.* 2001). The impact of such 'reproductive parasites' on host reproductive effort or strategy has been investigated rarely (e.g. Wade & Chang 1995; Jiggins *et al.* 2000). A number of these parasites distort the sex ratio of their host, and

have, through the modification of this trait, the potential to induce evolutionary changes in host reproductive strategy.

In terrestrial isopods (woodlice), *Wolbachia* induce two different effects depending on the *Wolbachia* strain and the host species: feminization (F) and cytoplasmic incompatibility (CI). *Wolbachia* express an F phenotype in most woodlice species, inducing reversion of genotypic males into functional females (Martin *et al.* 1973; Juchault & Legrand 1979; Juchault *et al.* 1993; Bouchon *et al.* 1998; Rigaud *et al.* 1999a). Because of the maternal transmission of *Wolbachia*, infected mothers produce highly female-biased progenies, and *Wolbachia* are always associated with an overall female-biased sex ratio in their hosts (e.g. Juchault *et al.* 1993; Moreau & Rigaud 2000). However, two species are known to harbour a *Wolbachia* expressing CI (Legrand *et al.* 1978; Moret *et al.* 2001). In this case, the offspring of crosses between uninfected mothers and infected fathers die early in development, whereas all other crosses are fertile. Contrary to F *Wolbachia*, CI *Wolbachia* do not induce an excess of females (e.g. Moret *et al.* 2001), but spread in populations by reducing the reproductive success of uninfected females (reviewed in Hoffman & Turelli 1997). Although *Wolbachia* induce two phenotypes in terrestrial isopods, they are all phylogenetically closely related (Bouchon *et al.* 1998; Cordaux *et al.* 2001). *Wolbachia* inducing CI and those inducing F belong to a monophyletic group. Experimental cross-infections nevertheless revealed that the phenotype expressed by *Wolbachia* is due more to parasite characteristics than to host differences (e.g. Moret *et al.* 2001; Rigaud *et al.* 2001). In addition, the terrestrial isopods in

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which CI and F are observed are also closely related (Michel-Salzat & Bouchon 2000). No congruence between phylogenetic position of either *Wolbachia* or the hosts was found to explain the distribution of *Wolbachia* manipulation types. However, F probably appeared secondarily compared with CI (Rousset *et al.* 1992). Different selective pressures have led *Wolbachia* expressing F or CI phenotypes to be selected in different isopod species, but the identification of these pressures remains unsolved.

Hypotheses explaining the maintenance of cytoplasmic sex ratio distorters (SRD), such as F *Wolbachia*, in host populations have been reviewed by Hatcher (2000). The maintenance and prevalence of these SRD could be explained by the product of different selection pressures, imposed by distinct host ecology or population genetics. In particular, in the context of SRD, a lack of males could induce a constraint on the SRD, in case of limited male mating capacity (MC) (Hatcher *et al.* 1999). High male MC could be important for the maintenance of F *Wolbachia* in populations, and a link should be found between the level of male MC and the phenotype induced by the parasites. We therefore tested this hypothesis, using experiments designed to evaluate male MC in different woodlice species, according to the variety of *Wolbachia* (F-inducing versus CI-inducing) they harbour.

2. MATERIAL AND METHODS

(a) *Strain maintenance and general methods*

Woodlice used for this study belong to seven different species of Oniscidea (Crustacea: Isopoda; Vandel 1962), chosen across the phylogenetic tree. These species are known to harbour *Wolbachia*: four species harbour *Wolbachia* with an F phenotype (Bouchon *et al.* 1998): *Armadillidium vulgare* (Latreille 1804), *A. nasatum* (Budde-Lund 1879), *Oniscus asellus* (Linné 1758) and *Philoscia muscorum* (Scopoli), whereas two species harbour *Wolbachia* with a CI phenotype (Legrand *et al.* 1978; Moret *et al.* 2001): *Porcellio dilatatus* (Brandt 1833) and *Cylisticus convexus* (De Geer 1878). In the last species, *Po. scaber* (Latreille 1804), *Wolbachia* is thought to induce F (SRD has been observed to be associated with the symbionts; J. Moreau, personal observation), but this has yet to be confirmed.

Animals came from strains uninfected by *Wolbachia*, initially caught in the wild, but were born in the laboratory during the spring prior to the experiments. They were reared 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 carrot provided *ad libitum*. Three months after their birth, the individuals can be sexed, and males and females were reared separately in boxes (26 cm × 13 cm × 8 cm) until sexual maturity to avoid sib-mating. All animals were 1 year old at the time of the experiments.

In most isopods species, mating receptivity of females depends on the reproductive cycle stage. For all species studied, except *Po. dilatatus*, mating occurs before the parturial moult when oocyte maturation is almost complete (Mead 1976; Lefebvre & Caubet 1999; Moreau & Rigaud 2000). This stage can be assessed by the appearance of white plates of calcium carbonate of a particular shape on the ventral surface, a few days before moulting (Moreau & Rigaud 2002). To obtain virgin females receptive to mating, females of each species were reared under simulating a photoperiod of 18 L : 6 D at 20 °C (Mocquard *et al.* 1989). Females of *Po. dilatatus* can mate at any time during

the intermoult. However, in order to avoid a bias in the experiments, females of this species were reared under the same conditions.

(b) *Evaluation of male MC*

The following experiments took place in a cylindrical box (diameter of 8 cm) with moistened soil and a piece of dead leaf and a slice of fresh carrot, at 20 °C over a 12 h period (time sufficient for mating (see Mead 1976)). To determine the MC of males, five receptive females were presented to a single male. This was repeated over 4 days, the five females being changed every day. Each male had therefore 20 females available for mating in 4 days. The mass (in mg ± 1 mg) was recorded for each individual by using a Sartorius precision balance and males were placed with females of approximately equal size (asymmetry random) to avoid any possibility of physical incompatibility in reproduction.

After 12 h, females were dissected to control their mating status (see Moreau & Rigaud (2000) for the routine procedure). Insemination is internal and, as isopod females possess two genital apertures each independently linked to one ovary, a male must perform two successive inseminations to inseminate a female completely. Mated females are characterized by the presence of large white balls of sperm in their two genital ducts, while oviducts of unmated females are thin and transparent. The results were expressed as the number of oviducts rather than females inseminated, because a few females were found half inseminated.

At the end of the experiment, males were dissected to control for the presence of macro-parasites. Males with parasites (e.g. nematodes) were excluded from the analysis. Because of female availability in our laboratory rearing and the discarded males, the number of replicates varied from 4 to 16 males tested according to the species (table 2).

(c) *Sex ratio in the field as a function of* Wolbachia *prevalence*

To illustrate the relationship between F *Wolbachia* prevalence and the intensity of sex ratio bias experienced by the host species, samples of adults *P. muscorum* were taken at 11 locations around Poitiers (France) in April 2001. On each site, five squares of 1 m² were sampled randomly within a 10 m² area, and all animals present at the ground surface of these squares, or under the leaf-litter and dead wood, were collected with entomologist forceps. Immediately after collection, all animals were sexed, their moulting status (moulting versus non-moulting individuals) and the reproductive status of females were noted (i.e. gravid; receptive to mating; not receptive to mating). These parameters allowed us to calculate the proportion of individuals in reproductive 'time-in' condition (for males: non-moulting individuals; for females: non-moulting and non-gravid individuals receptive to mating; see Moreau & Rigaud 2000) and, therefore, to estimate the operational sex ratio (OSR): the ratio of males versus females actually able to reproduce (Kvarnemo & Ahnesjö 1996; Moreau & Rigaud 2000). The females were then dissected and DNA extracted from their gonads, to enable detection of *Wolbachia* parasites by PCR amplification of a part of the bacterial 16S rDNA (see Bouchon *et al.* (1998) for methods and controls avoiding false negative results).

(d) *Statistical analysis*

Comparison between species cannot be considered as statistically independent because similarities can result from either a

Table 1. Matrix of interspecific differences used for the Mantel tests.
(Below diagonal: genetic distances (as estimated by the number of nucleotide substitutions between sequences of mitochondrial LSU rRNA gene, Michel-Salzat & Bouchon (2000); D. Bouchon, personal communication). Above diagonal: differences in the average male MC (number of inseminated oviducts).)

	O. asellus	P. muscorum	A. nasatum	A. vulgare	C. convexus	Po. scaber	Po. dilatatus
O. asellus	—	2.6	4.3	6.8	1.8	5.3	3.3
P. muscorum	0.423	—	1.7	4.1	4.4	0.7	5.9
A. nasatum	0.358	0.341	—	2.5	6.1	0.9	7.6
A. vulgare	0.366	0.350	0.132	—	8.4	3.4	10.1
C. convexus	0.421	0.404	0.309	0.318	—	5.2	1.5
Po. scaber	0.378	0.360	0.266	0.275	0.301	—	6.7
Po. dilatatus	0.372	0.354	0.258	0.267	0.294	0.140	—

common ancestry or from convergent evolution (Pagel & Harvey 1988; Harvey & Pagel 1991). Methods exist to analyse such data (Harvey & Pagel 1991); however, they are only appropriate for large datasets, such as those found in comparative analyses from bibliographic data (e.g. Cézilly & Nager 1995). Indeed, the attempt we made with CAIC (Purvis & Rambaut 1995), using the method of independent contrast, revealed that our set of data was too small to be analysed in such a way (J. P. Randerson, personal communication). In addition, because of sample availability, it was impossible to perform this experiment with additional species. However, our data are relatively robust because they were obtained using the same protocol and, therefore, strictly describe the same parameter (which is not always the case in comparative analyses from bibliographic data). We found a compromise between correct comparative methods and 'classical' statistical methods by analysing our dataset according to Moore & Gotelli (1990). A Mantel test was used to test the relationship between differences in male MCs and species phylogenetic distances. The Mantel statistic was calculated using the software BIOM-PC 3.0, according to the method described in Sokal & Rohlf (1995). Two matrices were constructed: (i) a matrix of differences in MCs, by calculating differences between average MCs between each pair of species; and (ii) a matrix of genetic distances between each pair of species, calculated from the terrestrial isopod phylogenetic tree based on ribosomal gene sequences (Michel-Salzat & Bouchon 2000; D. Bouchon, personal communication). The number of available species being low, the significance of the Mantel test was given after a Monte-Carlo simulation (10 000 permutations).

All other statistical tests were performed using the JMP software, v. 3.2.2 (SAS Institute 1997). The sizes of males and females were entered in the models. Previous stepwise analyses (backward procedure) allowed simplification of the models by removing these sizes and their interaction with the factors under analysis ($p > 0.05$). Only the resulting models are presented here.

3. RESULTS

(a) *Male MC*

The Mantel statistics revealed no significant relationship between differences in the average MCs and phylogenetic distances ($r = -0.197$, $p > 0.20$; table 1). Therefore, species phylogenetically closely related did not share similar MCs, suggesting that this life-history trait is not constrained by phylogeny. Males of each species were able to inseminate several oviducts throughout trials (table 2). However, the average number of genital apertures insemi-

nated strongly varied among species (ANOVA $F_{6,62} = 8.23$, $p < 0.0001$). A Tukey–Kramer test revealed two groups: one consisted of three species harbouring F *Wolbachia* and *Po. scaber*, in which males had a high MC, and a second group consisted of the two species harbouring CI *Wolbachia*, in which males had a lower MC. One species (*O. asellus*) had an intermediate MC and did not differ significantly from both groups. An ANOVA with the 'species' factor nested within the 'phenotype' factor produced essentially the same results: the difference between phenotypes was significant ($p < 0.05$), even if there were still significant differences between species within each phenotype ($p < 0.01$; analysis not shown).

The dynamics of MC as a function of time varied between species: males of some species showed a severe decline in their MC after their first mating (e.g. *P. muscorum*, *A. nasatum*), while in some other species, the decline was less severe (*A. vulgare*, *O. asellus*) or no decline was observed (*Po. scaber*, *Po. dilatatus*) (figure 1; ANOVA for repeated measures: between group effect (species): $F_{6,62} = 8.36$; $p < 0.0001$; within group: effect of day: $F_{3,186} = 24.70$; $p < 0.0001$; interaction species × day: $F_{18,186} = 2.55$; $p < 0.001$).

(b) *Sex ratios in the field populations of* **Philoscia muscorum**

Global population sex ratio varied between sample sites, from 7.5% males to a balanced sex ratio (table 3; figure 2). *Wolbachia* prevalence was negatively and significantly correlated with both the sample sex ratio (figure 2; Spearman $r_s = -0.73$; $p = 0.01$) and our estimate of OSR (table 3; figure 2, $r_s = -0.67$; $p = 0.02$). This shows that high infection rate by F *Wolbachia* induces severe bias in the OSR (until there are 15% males for a prevalence of *ca.* 80% *Wolbachia* in females). These links between *Wolbachia* prevalence and sex ratio biases are in agreement with the few experimental data available for this species that suggest a strong penetrance of the F phenotype (infected females produced 100% daughters (Bouchon *et al.* 1998)).

4. DISCUSSION

Our results indicate that, in terrestrial isopods, male MC is quite high, but varies according to species. Our experiments may overestimate the real MC because the design favours encounters between males and females. It is probable that the real MC is lower in the field because, for example, males may incur costs in searching for mates

Table 2. Male MC according to species of oniscid and the manipulative phenotype of *Wolbachia*.

host species	Wo[a]	n[b]	MC[c]	T–K test[d]
A. vulgare	F	16	17.19 ± 1.22	A
A. nasatum	F	10	14.70 ± 1.74	A
P. muscorum	F	4	13.00 ± 1.29	A
O. asellus	F	10	10.40 ± 0.99	A,B
Po. scaber	F?	4	13.75 ± 3.75	A
Po. dilatatus	CI	13	7.08 ± 0.92	B
C. convexus	CI	12	8.58 ± 1.29	B

[a] *Wolbachia*-induced phenotype (F?, feminization hypothesized).
[b] Number of males tested.
[c] Number of genital apertures inseminated by a male (mean ± s.e.m.).
[d] Tukey–Kramer *post hoc* test. Each letter denotes groups where the values were not significantly different ($p < 0.05$).
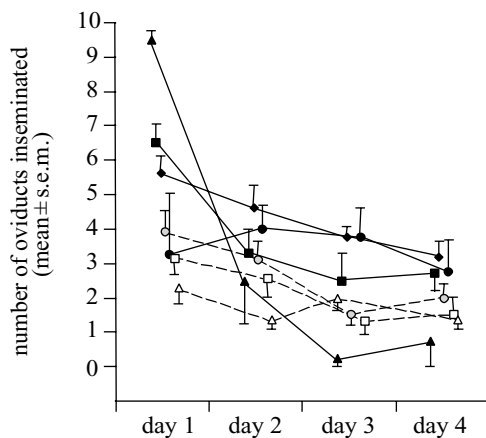

Figure 1. Male MC (number of female oviducts a male has inseminated) as a function of time for seven species of terrestrial isopods. Filled triangles, *Philoscia muscorum*; filled squares, *Armadillidium nasatum*; filled diamonds, *A. vulgare*; filled circles, *Porcellio scaber*; shaded circles, *Oniscus asellus*; open squares, *Cylisticus convexus*; open triangles, *Po. dilatatus*. Each day, five females (therefore 10 potential inseminations) were provided to each male (see text). The patterns of lines and symbols are arranged according to the groups determined in table 2.

(Gibson & Langen 1996). However, this is true for all species tested here, because they were tested under the same conditions.

The classification of species according to male MC fits better with the type of *Wolbachia* they harbour than with their phylogenetic relationships. Males of species infected by F *Wolbachia* have a stronger MC (double in most cases) than males of species infected by *Wolbachia* inducing CI. The present study also shows that F *Wolbachia* may induce severe bias in the OSR when its prevalence is high (see also Juchault *et al.* (1993) and Moreau & Rigaud (2000) for examples of population sex ratio distortion in other species). In cases of low prevalence, the overall sex ratio is balanced and the OSR is slightly male-biased. This is also probably the case in species without F *Wolbachia*, such as *C. convexus* and *Po. dilatatus*, because sex ratios are not female-biased in the field (Vandel 1962). *Oniscus asellus* was found to have an intermediate male MC. This species is known to harbour F *Wolbachia*, but both the vertical transmission rate of the parasite and the penetrance of the F were weaker than those of *A. vulgare* and

other species, such as *P. muscorum* (see Rigaud *et al.* (1999*a*) for a more complete description, and Bouchon *et al.* (1998) and Juchault *et al.* (1993) for comparisons).

This study therefore emphasizes the relationship between the *Wolbachia* phenotype and male MC in the host species. Because our experiment was designed to reveal correlations, the two traits might not be causally linked together, but might be linked to a third one, such as an ecological constraint. However, population dynamics, densities and/or habitat are very variable between woodlice species (Vandel 1962), and we found no sound ecological links within the groups revealed by our ANOVA analysis. The relationship between high MC and parasite-induced phenotype more probably means that strong male MC has either been selected as a result of the appearance of F *Wolbachia* in the population, or was an intrinsic characteristic of the species that has allowed the selection of an F phenotype in *Wolbachia*. Our results can assess that variation in male MC is not a proximal response to the infection or an excess of females, as our experiments were all conducted with individuals from uninfected strains and because we artificially imposed an excess of females on all species. Therefore, it cannot be directly compared to the increase in fertility observed in males of *Tribolium* beetles infected with CI *Wolbachia* (Wade & Chang 1995). High male MC in woodlice is therefore either the result of selection after a long coevolutionary history between host and symbionts, or a characteristic that preceded the appearance of *Wolbachia* (i.e. constraint by phylogeny). The poor relationship between woodlice phylogeny and male MC suggests that the first hypothesis is more convincing.

Two hypotheses can be proposed to explain how selection of high male MC has followed the appearance of F *Wolbachia* in woodlice. First, a low male MC can be seen as a factor limiting the selection of an F phenotype in the symbionts: a theoretical analysis indicates that parasitic sex ratio distortion can drive the extinction of a population if male MC is limited (Hatcher *et al.* 1999). Considering that the CI phenotype is ancestral to *Wolbachia* (*Wolbachia* involved in CI are found in each of three main lineages identified by rDNA sequencing (see Rousset *et al.* 1992)), we can reasonably assume that the original infection in terrestrial isopods was by a *Wolbachia* inducing CI. Randerson *et al.* (2001) have shown theoretically that a variant causing F is often favoured if it appears in populations infected by CI *Wolbachia*. The results obtained in the

Table 3. Distribution of *Philoscia muscorum* individuals, according to sex, reproductive status (time-in (TI) versus time-out (TO); see text) and *Wolbachia* prevalence in the different sample sites.

(*n*, number of females tested by PCR for the presence of *Wolbachia*.)

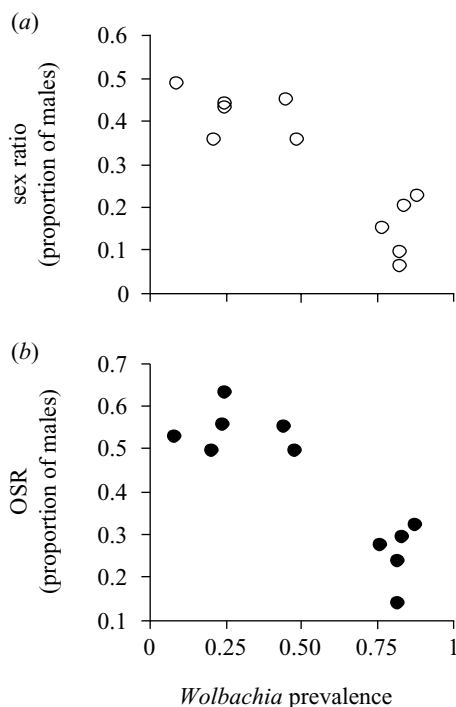| location	males TI	males TO	females TI	females TO	*Wo* prevalence	*n*
A	21	1	48	31	0.82	76
B	42	0	36	6	0.07	40
C	9	0	50	23	0.81	69
D	40	2	31	18	0.43	49
E	16	0	40	41	0.75	79
F	68	2	66	55	0.19	121
G	32	0	31	23	0.47	54
H	37	0	28	16	0.23	44
I	18	0	10	12	0.23	22
J	3	0	9	28	0.81	37
K	11	0	22	13	0.86	35

Figure 2. (*a*) Sex ratio and (*b*) OSR variations as a function of *Wolbachia* prevalence (proportion of infected females) in *Philoscia muscorum*.

present study nevertheless suggest that the only populations harbouring F symbionts that can be maintained are those where the selection of high male MC is possible. This hypothesis is constrained by the population structure: if too low, a continuous supply of migrants may counterbalance male rareness, especially if the selection of F *Wolbachia* is not too strong. Populations of woodlice are often substantially structured (e.g. Gentile & Sbordoni 1998; Bilton *et al.* 1999; Rigaud *et al.* 1999b), making this hypothesis possible, at least in species where the F phenotype of *Wolbachia* is very strong. The second hypothesis is that selection for high male MC is increased in populations with female-biased sex ratios. In this case, the more the sex ratio is female-biased, the higher the male MC should be, which is consistent with our interspecific comparison (see results with *O. asellus*). This hypothesis is much less constrained by the pattern of population struc-

ture, making it more likely. These two hypotheses are nevertheless non-exclusive.

We therefore propose that the variation in phenotypes of *Wolbachia* endosymbionts infecting terrestrial isopods may explain a part of the variation in male MC of their hosts. This illustrates how reproductive parasites, such as sex ratio distorting *Wolbachia*, may have an impact on the evolution of their host, outside their effect on sex determination.

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