Wolbachia infection in the terrestrial isopod *Oniscus asellus*: sex ratio distortion and effect on fecundity

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Maternally inherited *Wolbachia* bacteria are widespread in arthropods where they are responsible for various reproductive alterations. In terrestrial isopods (woodlice), *Wolbachia* may induce feminization or cytoplasmic incompatibility (CI), but their effect remains unknown in most host species. To increase our understanding of host/symbiont interactions in terrestrial isopods, the effect of *Wolbachia* was investigated in the oniscidean *Oniscus asellus*, mainly to discriminate between feminization and CI. The *Wolbachia* infection was not linked with a CI phenomenon, but females infected with *Wolbachia* produced female-biased broods compared with uninfected females. The fecundity of infected females was slightly lower than that of uninfected, but the number of young at the adult stage was similar between the two female categories. The experimental transfer of the symbiont into uninfected strains showed that *Wolbachia* was responsible for the feminization of a number of genetic males. In female-biased broods, *Wolbachia* were vertically transmitted to around 88% of the offspring, but the transmission rate was lower in the few male-biased progenies. The feminizing activity of these symbionts was not systematic, as many phenotypic males were infected. These results contrasted with what is known in another woodlouse species, and indicated that feminization has evolved in different ways in terrestrial isopods.

Keywords: Crustacea, cytoplasmic bacteria, feminization, Oniscidea, sex determination.

Introduction

Because of anisogamy, vertically transmitted cytoplasmic genes (organelles, endosymbionts, etc.) tend to be inherited exclusively from the mothers and have zero fitness in males. A Fisherian sex ratio (stable 1:1 sex ratio) is therefore not in the evolutionary interest of cytoplasmic elements, because their spread can increase if they favour the female sex in their host (Cosmides & Tooby, 1981). Among other strategies (reviewed by Werren & O'Neill, 1997), inherited cytoplasmic elements might induce the production of female-biased sex ratios.

A variety of such cytoplasmic sex ratio distorters is known in animals (Hurst, 1993). These include some endosymbionts that produce feminization (Rigaud, 1997; Kageyama *et al.*, 1998). This phenomenon has been discovered in the terrestrial isopod (woodlouse) *Armadillidium vulgare*, a species where sex is normally determined by heterochromosomes (female heterogamety: females WZ and males ZZ). In this species, the

microorganism responsible for feminization is a bacterium of the genus Wolbachia (Martin et al., 1973; Rousset et al., 1992). All infected individuals develop a female phenotype whatever their sex chromosome composition, and transmit the symbiont to most of their offspring (around 90% on average), generating female-biased sex ratios. In the wild, all infected females found so far have been of a ZZ (male) chromosomal composition, and only the uninfected offspring develop a male phenotype. In such infected strains, the sex ratio therefore depends only on the bacterial transmission rate. To put it in another way, the cytoplasmic Wolbachia represent the only female determinant in infected strains, whereas the male determinants are the Z chromosomes and autosomal genes repressing the transmission of the symbiont (Rigaud & Juchault, 1992).

Wolbachia have recently been found in the majority of terrestrial isopods (Juchault *et al.*, 1994; Bouchon *et al.*, 1998), most of the time inducing feminization, with the exception of *Porcellio dilatatus*, where *Wolbachia*-induced cytoplasmic incompatibility (CI), a *Wolbachia* reproductive manipulation common in insects, is seen (Werren & O'Neill, 1997). However, the effects

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Wolbachia has on host reproduction remain unknown in most isopods. The knowledge of these effects is important in understanding the evolution of host/endosymbiont relationships: to determine if feminization evolved in the same way in all infected species of this group, or if CI is more widespread in isopods. In these respects, the *Wolbachia* infection in *Oniscus asellus* is of interest. In this species, *Wolbachia* are present in several populations, there is no obvious sex ratio distortion linked with the infection, and some males are infected (Bouchon *et al.*, 1998). The aim of the present study is to investigate the effects of *Wolbachia* on *O. asellus* reproduction, mainly to discriminate between the possibilities of feminization and CI.

Materials and methods

Study in a natural population

Animals were collected in spring 1995 in Quincay (Vienne, France). The 14 females found gravid were reared in the laboratory at 20°C, L:D 18:6, on moistened soil with dead leaves and carrots as food. Each female was allowed to produce successive broods without remating (sperm are stored) until the end of the reproductive season. Offspring were reared in wider boxes to avoid competition, and were sorted by sex after sexual maturity, usually 3 months after birth. Each mother was dissected and DNA was extracted from the ovaries and the nerve chord (Bouchon et al., 1998). The presence/absence of Wolbachia was tested by PCR amplification of the bacterial 16S ribosomal gene, using specific primers, according to Bouchon et al. (1998). When no amplification was obtained, the quality of DNA template was tested using mitochondrial primers (Bouchon et al., 1998).

In woodlice, fecundity is positively correlated with female size (Sutton *et al.*, 1984). To test the effect of infection on fecundity and fertility, 50 gravid females (carrying eggs in their marsupium) were collected in May 1998 at the same site. Their size (body length without antennae) was measured using a binocular microscope with a graduated ocular. Fecundity was examined in 25 females by counting the number of freshly laid eggs. Fertility was measured in the remaining 25 females by counting the number of young reaching the sexual differentiation (around 2 months after their birth). The sex ratio was also assessed in the broods of these last 25 females. A cubic function of the size was used in the analysis, in order to obtain a linear relationship between size and fecundity. The infection by *Wolbachia* was checked for each female by PCR, after DNA extraction from one ovary and the nerve chord.

Injection experiments

Ovaries from 10 infected *O. asellus* females from Quinçay were dissected and homogenized in 100 μ L of Ringer solution. The suspension was filtered through 1.2 μ m pores. Using a Hamilton syringe with a fine glass needle, 1 μ L of the suspension was injected into the body cavity of recipient (uninfected) individuals. The recipients were females and males collected at Sepvret (Deux-Sèvres, France). These inoculated individuals were designated as the I animals. The remaining individuals were kept as control (U animals). The absence of *Wolbachia* has previously been investigated in this strain (negative PCR assays, Bouchon *et al.* [1998]) and was verified regularly during the experiments by checking U males and U females sampled randomly in the crosses.

Five months after injection, crosses were made to investigate the sex ratio distortion and the compatibility of crosses between infected and uninfected individuals. Pair crosses (male × female) of each of the four crossing types were made: $U \times U$, $U \times I$, $I \times U$, $I \times I$. The broods were analysed as described above. To obtain the second generation (G2) after injection, several sisters from three broods of the first generation were crossed with uninfected males. To obtain the third generation (G3), four sisters from 12 broods of the G2 were crossed with uninfected males.

The rate of vertical transmission of *Wolbachia* was investigated by extracting DNA of male and female offspring from broods with different sex ratios, and testing the presence/absence of *Wolbachia* by PCR. All strongly male-biased broods (>80% males) were tested, whereas the test was carried out only in eight G3 progenies sampled randomly from broods with femalebiased or balanced sex ratios.

All the females tested by PCR were previously implanted with an androgenic gland removed from uninfected males. This gland is responsible for the synthesis of the androgenic hormone (AH). When implanted in a female, the gland continues to produce AH and is able to masculinize females (Legrand, 1958). In *A. vulgare*, females infected with *Wolbachia* are insensitive to this masculinization, an effect interpreted as a competition on the target tissues between a bacterial product and the AH (Juchault & Legrand, 1985). The implant technique was similar to that described for *Wolbachia* injection, but the *Wolbachia* suspension was replaced by an androgenic gland previously dissected from a male.

Results

Wolbachia infection in the wild

Around half of the females collected in 1995 and in 1998 were infected by *Wolbachia* (χ^2 of difference from 1:1 ratio = 0.13; 1 d.f.; P > 0.05) (Table 1). The infected females produced broods with higher proportions of females than uninfected ones (two-way ANOVA after arcsin transformation of the data: $F_{1,35} = 12.56$; P < 0.001), whereas the sex ratio was neither significantly affected by the year of the sampling ($F_{1,35} = 3.73$; P = 0.062), nor by the interaction between the sample year and the infection status ($F_{1,35} = 3.42$; P = 0.073). When size was taken as a covariable, the uninfected females produced slightly more eggs than infected ones (Fig. 1a; ANCOVA with the number of eggs as dependent variable: effect of size: $F_{1,22} = 146.80$, P < 0.001; effect of infection: $F_{1,22} = 5.39$, $P^2 < 0.05$). However, there was no such difference in the numbers of young reaching the adult stage (Fig. 1b; ANCOVA with the number of young as dependent variable: effect of size: $F_{1,22} = 19.65$, P < 0.001; effect of infection: $F_{1,22} = 0.003$, P = 0.96).

Effect of Wolbachia injection on the sex ratio

The nine females and seven males tested in the I strains were found to be positive for the *Wolbachia* infection by PCR, whereas the 13 U females tested were negative.

There was no significant effect of the cross type either on the average number of young produced per brood, or on the number of broods produced per female (Table 2). The size of the females was not measured during these experiments, so we can not estimate any effect of *Wolbachia* on female fertility. However, there was no strong decrease in fertility, typical of CI, in the cross type male I \times female U. The sex ratio in the broods of I females was significantly female-biased (Table 2). There was no significant effect of male infection on sex ratio.

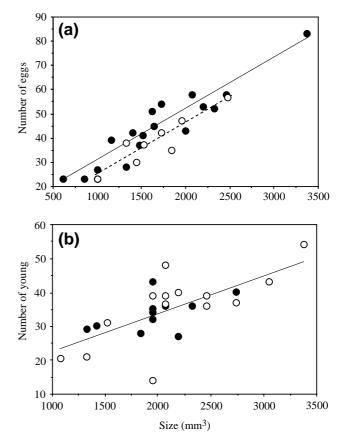


Fig. 1 Fecundity and fertility of *Oniscus asellus* females according to their size and their *Wolbachia* infection status. (a) Fecundity. Black circles and solid line: uninfected females $(y=0.021x + 8.49; r^2=0.877)$; open circles and dashed line: infected females $(y=0.021x + 3.147; r^2=0.833)$. (b) Fertility. Black circles: uninfected females; open circles: infected females. The relationship between size and fertility was $y=0.011x + 11.25; r^2=0.479$.

Table 1 Sex ratio and number of young in the broods of Oniscus asellus females
captured gravid in the wild, according to their Wolbachia infection status

	1995 sample		1998 sample	
	Wo+	Wo-	Wo+	Wo-
Number of females producing offspring	7	7	14	11
Total offspring	326	661	499	370
Male offspring	121	335	80	187
Number of brood per female (mean)	2.6	3.7	1	1
% males in the broods (mean)	37.1	50.7	16.0	50.5
(SE)	(5.2)	(2.7)	(3.9)	(2.3)
Number of young/brood (mean)	16.5	25.9	35.6	33.6
(SE)	(3.0)	(1.9)	(2.9)	1.5

	Cross type (male × female)				
	$I \times I$	$\mathbf{U} \times \mathbf{I}$	$I \times U$	$U \times U$	ANOVA
Number of crosses	12	11	12	10	
Total offspring	754	728	576	448	
Male offspring	167	176	267	230	
Brood per female (mean)	2.6	2.3	2.0	1.9	$F_{3,41} = 1.99; P = 0.13$
(SE)	(0.2)	(0.3)	(0.2)	(0.2)	-,
% males in the broods (mean)	22.1 ^a	24.2^{a}	46.3 ^b	(0.2) 51.3 ^b	$F_{3,41} = 15.46; P < 0.001;$
(SE)	(4.9)	(3.7)	(1.8)	(2.9)	-,
Young per brood (mean)	23.1	26.3	18.1	22.7	$F_{3,41} = 1.36; P = 0.270$
(SE)	(3.2)	(3.4)	(1.9)	(3.3)	- /

Table 2 The effect of Wolbachia injection on the sex ratio and the number of young per brood in an uninfected strain of Oniscus asellus

I: animals injected with Wolbachia; U: control uninfected animals.

†Test performed after arcsine transformation of the data.

^{a,b}Values followed by the same letter were not significantly different after PLSD posthoc test.

An anova revealed that the proportion of males significantly increased in the generations following injection in the I strains $(F_{2,86} = 6.85; P < 0.002)$ (Fig. 2). A Fisher PLSD test showed that the proportions of males in generations 2 and 3 did not significantly differ (P = 0.67), whereas the proportion of males was significantly lower in the first generation than in the second (P=0.002) or in the third (P=0.002). The increase in the male proportion in the second and third generations was mainly caused by the appearance of females producing male-biased broods and all-male broods (Fig. 2), a phenomenon not found in the first generation. The proportion of balanced sex ratio broods was also high in the third generation. There was no correlation between the sex ratios in G2 and G3 (Fig. 2): females issued from female-biased broods were able to produce female-biased progenies, as well as male-biased progenies, whereas some females from male-biased broods produced female-biased progenies.

Wolbachia transmission rates after transinfection

The infection rate in female offspring was high (91.1% of females tested harboured *Wolbachia*) and homogeneous between broods whatever their sex ratio ($\chi^2_{10} = 15.46$; P > 0.05) (Table 3). In male-biased progenies, the rare females all harboured *Wolbachia*. Such a complete infection in females was also found in half of the remaining progenies. Numerous males were infected, but the infection rate differed among broods ($\chi^2_{12} = 55.68$; P < 0.01): the lower the *Wolbachia* transmission rate in males, the lower the proportion of males in the broods (y = -0.88x + 100.7; $r^2 = 0.74$; $F_{1,12} = 31.68$; P < 0.001). Moreover, we found no infection in males from the all-male progenies (Table 4). In the

progenies without male-biased sex ratios, the *Wolbachia* transmission rates were similar between male and female offspring ($\chi_1^2 = 0.84$; P > 0.05). Here, 87.7% offspring inherited *Wolbachia*.

Before the PCR test, the females were implanted with an androgenic gland. One or two moults after the implantation, all these females developed male secondary sexual characters (male differentiation of the second pleopod pair, bristles on pereiopods, etc.), regardless of their infection status. This masculinization indicated that *Wolbachia* were therefore unable to overcome the androgenic hormone in *O. asellus*, unlike the situation observed in *A. vulgare*.

Discussion

Results reported in this paper show that Wolbachia endosymbionts induce feminization, but not CI, in Oniscus asellus. A sex ratio distortion was found associated with the Wolbachia infection in the wild, and symbiont injection in uninfected strains induced female-biased sex ratios. The sex ratio varied strongly in the generations following the experimental transfer, and became less female-biased. This was caused by both imperfect feminization and loss of transmission. Paradoxically, this incomplete feminization gives the best argument for the occurrence of feminization. It has previously been showed that O. asellus is a species with female heterogamety (Juchault & Rigaud, 1995). The females producing only sons are therefore genetic males (i.e. ZZ) feminized by Wolbachia, that failed to transmit symbionts to the following generation. In the absence of the feminizing activity of the cytoplasmic element, the offspring develop a phenotype consistent with their male (ZZ) genotype. Therefore, the increase of male proportion



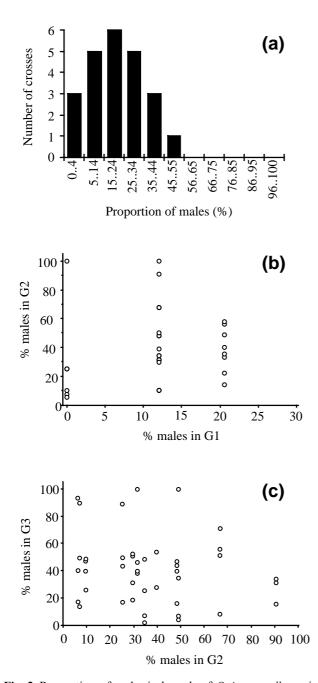


Fig. 2 Proportion of males in broods of *Oniscus asellus* uninfected females injected with *Wolbachia*, in the three generations following injection. (a) First generation (G1) after injection. Mean proportion of males \pm SE = 23.1% \pm 3.0. (b) Proportion of males in G2 plotted against proportion of males in G1. Several sisters of three G1 broods were crossed to obtain G2. Mean proportion of males \pm SE = 44.0% \pm 5.3 in G2. (c) Proportion of males in G3 plotted against proportion of males in G2. From two to four sisters from several G2 broods were crossed to obtain G3. $r^2 = 0.03$; y = -0.17x + 47.52 ($F_{1,41} = 1.10$; P = 0.30). Mean proportion of males \pm SE = 42.5% \pm 3.8 in G3.

	Males	tested	Females tested		
Brood no.	Wo-	Wo+	Wo-	Wo+	Total offspring (males; females)
1	_		3	6	2; 28
2	1	2	3	12	4; 50
3	2	7	3	19	12; 105
4			0	8	4; 29
5	0	2	0	11	4; 14
6	1	11	0	20	15; 45
7	2	6	0	9	18; 19
8			2	8	54; 56
9	6	4	0	3	32; 4
10	4	6			25; 3
11	5	0	0	4	79; 7
12	7	3	0	3	38; 3
13	5	0			10; 0
14	10	0			88; 0
15	10	0			59; 0
16	10	0	—		36; 0

-, test not done; Wo-, individual uninfected by *Wolbachia* (negative PCR test); Wo+, individual infected by *Wolbachia* (positive PCR test).

observed in generations following the transfer is not caused by the decrease in bacterial feminizing activity. The occurrence of ZZ females coupled with a constant incomplete feminization could be responsible for this. In other words, when feminization is incomplete in the broods of a ZZ neo-female, the entire brood can be male, whereas this is impossible in the first generation after inoculation, because recipient females are all WZ and can produce only a maximum of 50% males. No male-biased broods were observed in the wild. Our study was unable to discriminate between whether females naturally infected by Wolbachia are WZ infected with weak feminizers, or ZZ with strong feminizers. So we can not estimate if the Z chromosome reached fixation in lineages infected by Wolbachia, as is the case in A. vulgare (Rigaud et al., 1991). The inefficiency of feminization is probably not caused by symbiont strains differing in their feminizing ability, because there was no correlation in the male proportions between two generations after transinfection. Genetic control by the host cannot be dismissed, but, again, the lack of a correlation in sex ratios between these two generations indicates that there is no simple genetic mechanism involved. Furthermore, the recipient strains came from an uninfected population, where genes resisting feminization should not be selected.

The type of feminization is different from that found in Armadillidium vulgare. The main difference is that males can be infected in O. asellus. There was therefore no direct relationship between the Wolbachia transmission rate and the sex ratio, and the feminizing capacity of the symbiont and its transmission rate probably evolved separately (Hatcher & Dunn, 1995). Such a situation has already been found in the amphipod Gammarus duebeni infected with a microsporidian feminizing parasite: not all embryos inheriting the parasite were feminized (Terry et al., 1998). The cause of this inefficiency is unknown. In insects, variation in Wolbachia dosage induces variation in cytoplasmic incompatibility expression: the more numerous are Wolbachia in their male hosts, the more intense is the incompatibility in crosses (e.g. Breeuwer & Werren, 1993; Bressac & Rousset, 1993). Differences in Wolbachia-induced feminization may simply result from differences in bacterial dosage, the embryos harbouring a low dose of symbionts developing a phenotype consistent with their nuclear genotype, and those harbouring a high dose developing a female phenotype. However, staining methods generally used to detect Wolbachia (DAPI, Lacmoid) are inadequate in isopods (T. Rigaud, unpubl. obs.), and a reliable quantification method to test this hypothesis is still lacking. In addition, another phenomenon might explain the differences in feminization. This study showed that O. asellus females were sensitive to the masculinizing effect of the androgenic hormone, even when they possessed Wolbachia. The Wolbachia have therefore no effect on the hormone once the hormone is produced. The situation is different in A. vulgare where Wolbachia override the effect of the hormone. This effect can be seen as a backup system for the symbiont: if Wolbachia do not completely suppress differentiation of the androgenic gland, this second effect suppresses the effect of the hormone, and a male phenotype cannot appear. In O. asellus, there is no such 'second effect': once the gland develops, the development of a male phenotype is irreversible. We suggest that Wolbachia in A. vulgare evolved this 'second effect' to increase its feminizing power, an evolutionary state not reached in O. asellus.

Differences in host-symbiont relationships are probably responsible for the differences in feminization type between *A. vulgare* and *O. asellus*. Because both *Wolbachia* and hosts are genetically different in the two systems (Bouchon *et al.*, 1998), it is hard to distinguish which genome (host or symbiont) of the association has the main role in controlling these effects. However, nuclear resistance alleles are known in *A. vulgare*. These alleles either overcome the symbiotic suppression of the androgenic gland differentiation (but in turn the *Wolbachia* is able to surpass this effect by suppressing the effect of the hormone; see Rigaud & Juchault, 1993), or restrain the transmission of *Wolba-chia* (Rigaud & Juchault, 1992). So, perhaps the *Wolbachia* of *O. asellus* did not evolve a total efficiency of action/transmission because of the lack of host resistance. The coevolutionary scenario would therefore be of a 'Red Queen' type, the efficiency of feminization increasing only after the resistance of the host. Such a hypothetical scenario must be confirmed. Trans-specific infections could help to test this hypothesis, by following the progress of the infection after inoculation of different *Wolbachia* types in different female hosts.

Finally, our results suggest that *Wolbachia* induces a slight negative effect on host fecundity in O. asellus, an effect already found in Drosophila simulans (Hoffmann et al., 1990). But a negative effect on host fitness is not a general effect of Wolbachia, because positive effects on fecundity have also been reported (Stolk & Stouthamer, 1995; Poinsot & Merçot, 1997; Vavre et al., 1999). Furthermore, this negative effect was not found when the adult offspring of infected vs. uninfected O. asellus females were compared. This could suggest that a better survival of infected offspring could counterbalance the negative effect of the bacteria on fecundity. However, the difference in fecundity found in this study was weak, and we may conclude that Wolbachia has no obvious effect on the host other than sex ratio distortion.

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