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Effect of intracellular *Wolbachia* on interspecific crosses between *Drosophila melanogaster* and *Drosophila simulans*

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ABSTRACT. *Wolbachia* are bacteria that live inside the cells of a large number of invertebrate hosts and are transmitted from infected females to their offspring. Their presence is associated with cytoplasmic incompatibility in several species of *Drosophila*. Cytoplasmic incompatibility results when the sperm of infected males fertilize eggs of uninfected females, causing more or less intense embryonic mortality (unidirectional incompatibility). This phenomenon also appears in crosses between populations infected with different *Wolbachia* strains (bidirectional incompatibility). The influence of *Wolbachia* infection on host populations has attracted attention as a potentially rapid mechanism for development of reproductive isolation and subsequent speciation. We examined the influence of this bacterium on reproductive isolation in interspecific crosses between *Drosophila melanogaster* and *D. simulans*. We found that *Wolbachia* infection negatively affected these two species in homospecific crosses. However, in interspecific crosses, it only influenced sexual isolation, as infected females more frequently hybridized than females free of infection; postzygotic reduction of fitness (bidirectional cytoplasmic incompatibility) was not detected. This would be explained by the existence of several modes of rescue systems in these two species, reducing cytoplasmic incompatibility

between them. *Wolbachia* does not appear to cause reproductive isolation between these two species.

Key words: *Wolbachia*; Interspecific crosses; *Drosophila melanogaster*; *Drosophila simulans*

INTRODUCTION

Wolbachia pipientis are a group of maternally transmitted intracellular alpha-proteobacteria (Hoffmann and Turelli, 1988; Binnington and Hoffmann, 1989), that are frequently found in a wide range of arthropod and nematode species (Stouthamer et al., 1999; Dobson et al., 2002; McGraw and O'Neill, 2004).

Infection by this microorganism is associated with cytoplasmic incompatibility (CI), manifested as parthenogenesis, male feminization and embryonic lethality in crosses between infected males and uninfected females (unidirectional incompatibility), or between populations infected with different strains of *Wolbachia* (bidirectional incompatibility) (O'Neill and Karr, 1990; Werren and Jaenike, 1995; Moret et al., 2001; Stevens et al., 2001; Clark et al., 2003). Two *Wolbachia* strains are compatible with each other only if they harbor the same compatibility type.

The bacterium can be removed by physical treatment (high temperature) or chemical treatment (antibiotics such as tetracycline). When *Wolbachia* is removed from the populations, the negative effect of CI disappears, showing that infection is responsible for the negative effects in incompatible crosses (Koukou et al., 2006).

The molecular mechanisms of CI are unknown; *Wolbachia* modifies sperm development in infected males at the level of chromosomal condensation, pro-nucleus modifications and altered structures (Callaini et al., 1997; Presgraves, 2000; Tram et al., 2003; Riparbelli et al., 2007). However, if females are also infected with the same strain of *Wolbachia*, these modifications are restored, and the embryo develops normally (Starr and Cline, 2002; Veneti et al., 2003).

Several authors postulate the existence of two bacterial functions: *mod* (for modification), which acts on males, and *resc* (for rescue), which is expressed in the germinal and/or early embryos of infected females, neutralizing the sperm modifications (Presgraves, 2000; Charlat et al., 2001; Veneti et al., 2003). Thus, the compatibility types are defined by a given *mod resc* pair.

Other authors conjecture about the interactions between the host-symbiont genotypes, and the combination of environmental and physiological factors to determine the positive or negative effects of infection and to explain the phenotypic variability of CI from homospecific crosses (Reynolds et al., 2003; Dean, 2006; Dowling et al., 2007; Iturbe-Ormaetxe and O'Neill, 2007; Yamada et al., 2007; Xi et al., 2008).

Wolbachia manipulates host reproductive biology for its own benefit. Because the bacterium is transmitted by females only, infected cytoplasms are selected for, allowing the bacterium to spread through the population and then maintain itself. The fitness of uninfected females is lower than that of infected females due to the existence of CI, and the bacterium tends to spread rapidly through host populations (Turelli and Hoffmann, 1991).

Several models and hypotheses regarding the dynamics of the infection in populations suggest that the expansion of *Wolbachia* can be due to a possible symbiotic parasite-host ef-

fect. However, there are few examples providing direct fitness benefits of the bacterium for its hosts (Vavre et al., 1999; Dedeine et al., 2001; de Crespigny et al., 2006; Weeks et al., 2007), and the majority of reports indicate neutral (Poinsot and Mercot, 1997; Harcombe and Hoffmann, 2004) or negative effects in several biological traits (Hoffmann et al., 1990; Clancy and Hoffmann, 1997; McGraw et al., 2002).

The influence of *Wolbachia* infection on host populations has attracted attention as a potentially rapid mechanism for the development of reproductive isolation and subsequent speciation (Breeuwer and Werren, 1990; Coyne, 1992). Consistent with this possibility, is that besides CI in incompatible crosses, bidirectional incompatibility has been found between geographical races of *Culex pipiens*, between different geographical populations of *D. simulans* (O'Neill and Karr, 1990) and between closely related species of *Nasonia* (Breeuwer and Werren, 1990). To determine the possible role of *Wolbachia* in facilitating speciation events, in relation to the development of reproductive isolation, incompatibility between closely related species must be determined (Werren and Jaenike, 1995).

Drosophila melanogaster and *D. simulans* are cosmopolitan sibling species, infected with different strains of *Wolbachia*, between which reproductive isolation exists, based on both the limitation of interspecific mating or sexual isolation (above all among *simulans* females with *melanogaster* males) and on the unfeasibility of hybrid descent or reproductive isolation (*melanogaster* females with *simulans* males produce only females, while reciprocal crosses produce basically males, and occasionally some females) and the sterility of all the progeny.

The aim of the present study was to determine the effects of *Wolbachia* infection on the reproductive isolation and biological efficacy of these two species of *Drosophila* in interspecific crosses. Homospecific crosses were performed as control, to compare the effect of the bacterium on the different crosses and species.

MATERIAL AND METHODS

The test was carried out in two experiments, one for homospecific and other for interspecific crosses. In both experiments, the same populations were used: a population of *D. simulans*, captured from Sanabria (Zamora, Spain) in 2003, infected with the *wRi* strain of *Wolbachia*, and a population of *D. melanogaster*, captured in Asturias (Spain) in 1999, infected with *wMel* strain.

The flies were reared on a standard medium made up of 100 g baker yeast, 100 g sugar, 12 g agar, 2 g salt, and 5 mL propionic acid per liter of water.

Wolbachia was removed using tetracycline. Flies of both species were reared on standard medium containing tetracycline at the concentrations of 0.25 g/L, 0.8 g/L, and 1 g/L in three consecutive generations. To avoid inbreeding, the flies came from the progeny of 20 bottles at least, with 30 pairs of parents each. The flies were renovated by random mass culture.

After antibiotic treatment, the flies were reared on standard medium without tetracycline for three generations before the start of the experiment to avoid the possible effect of the treatment on fitness. *D. melanogaster* and *D. simulans* infected and "cured" were supported in two culture chambers isolated at 21°C and with 12-h light:dark cycles.

To test the efficacy of the antibiotic in removing the infection, DNA from 50 flies (25 of each sex) was extracted individually and purified, as described by O'Neill et al. (1992).

The presence of *Wolbachia* was confirmed by polymerase chain reaction using the primers of the 16S rDNA partial sequence of the *Wolbachia* strain associated with *Drosophila* (Gomez-Valero et al., 2004), and was checked at the beginning and end of each experiment.

Changes in fitness induced by *Wolbachia* were estimated from intra- and interspecific crosses between males and females infected or cured in all possible combinations.

Homospecific crosses

A male and a female, both newly emerged (1-day-old virgins), were placed in a vial with food. Five days later, the flies were discarded, and the vials were examined to detect the presence of progeny. The number of pairs analyzed was 50 per combination and species.

The fitness elements estimated were:

- Fertility: Number of females leaving descent.
- Productivity: Number of live progeny per couple. We believe that this parameter reflects female fecundity and the egg-adult viability from the progeny.

The number of adults emerged was counted every day, until their total emergence (around a week).

Interspecific crosses

Crosses between *D. melanogaster* and *D. simulans* were performed by the no-choice method in both directions and in all the possible combinations of males and females, infected or cured.

Ten females of *D. melanogaster* or *D. simulans* were placed with 10 males from the other species, all recently emerged (1-day-old virgins). Five days later, the males were discarded and each female replaced in a new vial.

The elements of fitness estimated were: frequency of hybridization (number of females engendering progeny) and productivity, number of live progeny per couple, following the same protocol for homospecific crosses. The total number of pairs analyzed was about 250, in a two-block design.

RESULTS

Homospecific crosses

Drosophila melanogaster

- Fertility: The percentage of females producing progeny was higher than 90% in all crosses, with no significant differences being detected among these ($\chi^2 = 4.05$, d.f. = 3; P = 0.2), according to a test of variance for homogeneity of binomial distribution (Snedecor and Cochran, 1967).
- Productivity: The average numbers of descendants per couple, the standard error and the number of mothers (in parentheses) are presented in Table 1. The effect of the infection on both sexes was estimated by two-way ANOVA and the F values appear in Table 2.

Table 1. Number of progeny per female in homo- and heterospecific crosses.

Cross (female x male)	C x C	C x I	I x C	I x I
M x M	34.53 ± 1.38 (46)	27.93 ± 1.41 (45)	60.43 ± 3.51 (47)	75.50 ± 6.96 (46)
S x S	53.20 ± 3.76 (46)	19.53 ± 3.52 (45)	111.03 ± 4.14 (47)	86.03 ± 4.47 (48)
M x S	17.32 ± 0.99 (77)	21.97 ± 0.71 (114)	22.27 ± 0.83 (166)	29.45 ± 1.04 (149)
S x M	47.40 ± 5.30 (22)	37.27 ± 3.17 (28)	50.20 ± 5.17 (43)	41.80 ± 4.97 (50)

M = *Drosophila melanogaster*; S = *D. simulans*; C = cured; I = infected. Values are reported as means ± standard error. Number of females is shown in parentheses.

Table 2. F values of two-way ANOVA of homo- and heterospecific crosses.

Source of variation	Cross (female x male)					
	Productivity				% Hybridization	
	M x M	S x S	M x S	S x M	M x S	S x M
Females	83.49^{a}	171.02^{a}	49.13^{a}	0.52^{d}	67.03^{a}	21.41^{a}
Males	1.11^{d}	23.49^{a}	44.04^{a}	3.34^{d}	0.05^{d}	5.01^{c}
Interaction	7.76^{b}	2.02^{d}	4.81^{c}	0.03^{d}	0.05^{d}	0.65^{d}
Degrees of freedom	(1,180)	(1,182)	(1,502)	(1,139)	(1,4)	(1,4)

M = *Drosophila melanogaster*; S = *D. simulans*. a < 0.001; b < 0.01; c < 0.05; d > 0.05.

Differences between females depending on the infection were detected, indicating that cured females had reduced productivity compared with infected females, and there was an interaction between sexes, which may be due to the lower value observed from the incompatible cross (females cured with males infected). No differences were detected between males, indicating that there was no effect of the antibiotic in this sex.

Drosophila simulans

- Fertility: The percentage of females that produced progeny was higher than 92% in all cases, with no significant differences being detected among these ($\chi^2 = 4.25$, d.f. = 3; P= 0.25).
- Productivity: Table 1 shows the mean values of progeny per female, the standard error and the number of mothers (in parentheses). Two-way ANOVA (Table 2) detected differences between females and males depending on infection, but no interaction. The lower value corresponds to incompatible crosses (females cured and males infected). In general, infected females have greater productivity than those free of infection, above all in crosses between females infected and males "cured" suggesting no effect of tetracycline in this sex.

Interspecific crosses

D. melanogaster females x *D. simulans* males

- Frequency of hybridization: Table 3 shows the percentages of females producing hybrid descent, as well as the number of mothers in parentheses. Each percentage corresponds to the mean of the two experimental blocks. No differences between the two blocks were detected.

Table 3. Percentages of females producing hybrid progeny.

Cross (female x male)	C x C	C x I	I x C	I x I
M x S	32.22 (239)	43.18 (264)	62.17 (267)	61.11 (244)
S x M	9.17 (240)	11.07 (253)	15.09 (285)	18.18 (275)

M = *Drosophila melanogaster*; S = *D. simulans*; C = cured; I = infected. Number of females analyzed in heterospecific crosses is shown in parentheses.

To estimate the effect of the infection in both sexes, the percentages of hybridization of each block were angular transformed and analyzed by two-way ANOVA (Table 2). Only differences between females were detected. *Wolbachia* increased the frequency of hybridization of females regardless of male infection.

- Productivity: All progeny were females. Table 1 shows the mean values of descendants per female from the two blocks, the standard error and the number of mothers.

The result of two-way ANOVA (Table 2) indicates differences between females and males and slight interaction between species. Infection increased productivity in both sexes. However, the differences between the mean values are not very substantial. CI was not found in any of the crosses.

D. simulans females x *D. melanogaster* males

- Frequency of hybridization: The mean percentages of the two blocks of females producing hybrid progeny are presented in Table 3, along with the number of females on which each value is based (in parentheses). No differences between the two blocks were detected.

ANOVA of angular transformed data detected differences between females, indicating that infected females hybridized more than cured females. The males showed similar values regardless of the presence of *Wolbachia*. No interaction between species was detected either.

- Productivity: The progeny was composed of males, with the occasional appearance of some females. Table 1 shows the mean values of descendants per female, the standard error and the number of females producing hybrid progeny from the two blocks. The analysis of the data did not detect differences between females and males or interaction between them. CI was not found in any of the crosses.

DISCUSSION

It is perfectly well documented that *Wolbachia* uses the host to achieve its expansion, using diverse evolutionary strategies. The results of this study indicate that the different effects of *Wolbachia* infection depend on species, sex and cross.

In homospecific crosses, the parasite does not affect male or female fertility; however, in general, the productivity of infected females is greater than in non-infected females. This phenomenon can be considered as a form of mutualism for the two organisms in order to increase their fitness (Weeks et al., 2007). Cytoplasmic incompatibility is always detected in incompatible crosses in the two species, especially in *D. simulans*.

Surprisingly, infected *D. simulans* females demonstrated the highest productivity when mated with uninfected males, showing a negative effect of the parasite in males. Similar results were obtained by Fry et al. (2004), suggesting the involvement of different degrees of fitness in the two sexes in the evolution of both organisms.

The different effects of infection in homospecific crosses in both species may be due to the interaction between the genomes of the parasite and the host (Reynolds et al., 2003; Dean, 2006; Dowling et al., 2007; Iturbe-Ormaetxe and O'Neill, 2007; Yamada et al., 2007; Xi et al., 2008). The genome of *Drosophila* plays an important role in the response to the effect of the bacterium on changes in biological efficacy, as has been shown by several authors (Fry and Rand, 2002; Charlat et al., 2003; McGraw and O'Neill, 2004). These interactions may explain why there are polymorphic populations in both events: presence of *Wolbachia* and sensitivity to their effects. However, the polymorphism also may be due to reduction of fitness of infected individuals, natural curing (Stevens and Wicklow, 1992) and stochastic loss of bacteria within infected individuals (Werren and Jaenike, 1995).

In interspecific crosses, infected females show a greater frequency of hybridization than uninfected females, regardless of the presence of the bacterium in the males. This fact could be due to *Wolbachia* increasing female receptivity, because the speed in mating has been associated with the frequency of hybridization between this two species (Carracedo et al., 1987).

With regard to productivity, the effect of infection may be considered non-significant, due to the similar values from the different crosses (Table 1). Therefore, the most important result is that no cytoplasmic incompatibility was detected in interspecific crosses. This result is surprising because both species are infected with different strains of *Wolbachia* (Reynolds and Hoffmann, 2002; Charlat et al., 2003), and therefore, the crosses of non-infected males (C x C and I x C) should display more productivity, because they are compatible.

These results may be explained by either of two hypotheses proposed by Charlat et al. (2001) to understand the existence of different compatibility types: 1) CI might have emerged many times independently, giving rise to different CI systems *mod-resc* pairs, and the some rescue system of females could inhibit the modification of several systems of males, and 2) alternatively, the different CI systems existing today may derive from one or a few ancestral ones. In this case, bidirectional incompatible strains must have evolved from compatible predecessors, making some crosses between related and isolated species compatible. In this sense, some *Wolbachia* strains that are unable to induce CI in incompatible crosses, can rescue it (Bourtzis et al., 1998; Merçot and Poinsot, 1998).

Our results do not support the notion that *Wolbachia* plays any role (at least at the postzygotic level) in the reproductive isolation between these two species.

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