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Wolbachia Density and Host Fitness Components in *Muscidifurax uniraptor* (Hymenoptera: Pteromalidae)

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Intracellular bacteria of the genus *Wolbachia* are found in a variety of arthropod hosts, where they cause various reproductive disorders. Attempts to study the fitness advantages and disadvantages of carrying these symbionts have yielded contradicting results. Using various doses of the antibiotic rifampicin, we were able to manipulate the density of *Wolbachia* in the uniparental parasitoid *Muscidifurax uniraptor* (Hymenoptera: Pteromalidae). The effect of different titers of the symbiont on the fecundity, reproductive rate, longevity, survival rate, and sex ratio of the host was measured. The data gathered show that following antibiotic treatments, the percentage of males rises at low doses of rifampicin and then drops again. The total sex ratio of offspring produced by treated mothers was positively correlated with the numbers of *Wolbachia* found in eggs laid by these females. No significant effects were detected with regard to the other studied fitness components. It is concluded that in *M. uniraptor, Wolbachia* are not posing any burden on the life history trait studied.

***Key Words: Muscidifurax uniraptor*; *Wolbachia pipientis*; endosymbionts; fitness; parthenogenesis; sex ratio; *Wolbachia* density.**

INTRODUCTION

Wolbachia are intracellular bacteria that are known to cause reproductive and sex ratio disorders in many insects and other arthropods (Werren, 1997, for review). The bacteria have been found in over 16% of the insects surveyed (Werren *et al.,* 1995) and are transovarially transmitted. Three major phenomena are known to be associated with the presence of *Wolbachia:*

(1) Cytoplasmic incompatibility (CI), which occurs between infected and uninfected strains. This phenomenon has been described in many insect species and several mites (O'Neill & Karr, 1990; Breeuwer and Werren, 1990);

(2) Feminization diversion of genetic males into phenotypic females, which has been described in several isopod species (Rigaud *et al.,* 1991); and

(3) Parthenogenetic production of female offspring without fertilization by males, which has been found only in species of parasitic Hymenoptera (Stouthamer, 1997, for review).

Different CI types caused by three *Wolbachia* variants have been described in wild populations of *Drosophila simulans:* wRi, wHa, and wNo (Clancy and Hoffmann, 1996, for review). A thorough study could detect no effect of the most common *Wolbachia* variant (wRi) on the number of progeny and developmental time of its *D. simulans* host (Hoffmann *et al.,* 1990). However, these authors did find a significant reduction of up to 18% in fecundity of infected lines when compared to uninfected ones in the laboratory (but no similar reduction in fitness was found in the field). Studying parthenogenesis-inducing *Wolbachia* (PI), Stouthamer and Luck (1993) also found an increase of fecundity in three *Trichogramma* species when the host was freed from its symbionts. In contrast, the Indo-pacific *Wolbachia* variants wHa and wNo were found to exert no deleterious physiological effects on the fitness of their *D. simulans* female hosts (Poinsot and Mercot, 1997). Moreover, three generations after tetracycline treatment, cured stocks had productivity significantly lower than that of the corresponding infected stock. These differences were apparently temporary, as they were no longer significant after 14 generations (Poinsot and Mercot, 1997). Reduction in fitness following the loss of *Wolbachia* was found in other cases of CI, such as *Nasonia vitripennis* (Stolk and Stouthamer, 1996) and *Tribolium confusum* (Wade and Chang, 1995), and in the PI-carrying parasitoid *Apoanagyrus diversicornis* (Pijls *et al.,* 1996).

Wolbachia infection was found to have no detectable effect on the fecundity of various *Drosophila* species (Giordano *et al.,* 1995; Bourtzis *et al.,* 1996). Measuring total progeny and longevity of *Muscidifurax uniraptor* and *M. raptor,* Stouthamer *et al.* (1994) also failed to

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detect a significant influence of an antibiotic treatment and concluded "the negative impact of the symbionts on this species is limited."

All the experiments conducted on PI microorganisms so far correlate fitness traits with a dose of antibiotics that induces the production of males. However, it has been shown with CI-inducing symbionts that the influence of *Wolbachia* depends on its density within the host rather than being an all or none effect (Boyle *et al.*, 1993; Breeuwer and Werren, 1993; Clancy and Hoffmann, 1998).

Species of the genus *Muscidifurax* (Hymenoptera: Pteromalidae) are pupal parasitoids of synanthropic filth-breeding Diptera and are among the principal natural enemies of the housefly (*Musca domestica*) and the stable fly (*Stomoxys calcitrans*). Parasitoids in that genus are produced and sold by commercial insectaries for fly control on poultry and dairy farms (Rutz and Patterson, 1990). *Muscidifurax uniraptor* is a parthenogenetic species in which *Wolbachia* endosymbionts were found to be the cause of uniparental reproduction (Stouthamer *et al.*, 1993).

Usually, the effect of a symbiont on the fitness of its host is determined by comparing infected and uninfected hosts with identical genetic backgrounds. In species where uniparental reproduction is fixed in the population and uninfected lines can not be established, it is possible to measure the influence of *Wolbachia* on several life history traits of its host by comparing antibiotic-fed vs honey-fed females (Stouthamer, 1997). The present experiments address two major questions:

(1) What is the effect of various doses of the antibiotic rifampicin on *Wolbachia* density and consequently on the sex ratio produced?

(2) What is the effect of different *Wolbachia* densities on selected fitness components of the host?

MATERIAL AND METHODS

Strains

The uniparental species *M. uniraptor* was imported from J. H. Werren, Rochester, New York, in 1997. *Muscidifurax uniraptor* is known only from Puerto Rico (Kogan and Legner, 1970) and was originally collected in that island (Legner, 1988). The wasps were kept in the laboratory on *Musca domestica* pupae in a rearing chamber under standard conditions of 25 ± 2°C and 50% RH.

Antibiotic Treatments

A stock concentration of 100 mg rifampicin (Sigma) in 1 ml honey was prepared and diluted to 0.1, 1.0, and 10 mg/ml. To each dilution, a drop of 5% carmozine food color in 40% sucrose solution was added. Pure honey with food color was used as a control. *Muscidifurax uniraptor* females (0–24 h old) were starved overnight and then randomly assigned and allowed to feed on four antibiotic treatments and a control for 24 h as described by Zchori-Fein *et al.* (1995). In studies conducted on *Muscidifurax*, where the antibiotic treatments were used to cure hosts from *Wolbachia*, the treatment itself did not have a significant adverse effect on the wasps (Stouthamer *et al.*, 1994; Horjus and Stouthamer, 1995).

Egg Collection and Fixation

The effect of the antibiotic rifampicin on *Wolbachia* density was tested on eggs deposited 4 days after treatment. We choose to count *Wolbachia* density on the fourth day because, as sex ratio results indicate, the antibiotic effect is stable from Day 3 to Day 7 post-treatment. To maintain equal conditions, the wasps in all experiments were kept with 20 fresh hosts for the first 3 days prior to egg collection. To collect eggs in early developmental stages, four wasps, 4 days post-treatment, were placed in a 50-ml plastic vial covered with a foam plug. Housefly pupae were inserted in holes in the plug so that their tops were exposed and available for oviposition. The pupae were replaced every 2 h, and the parasitized hosts were kept at 4°C until egg collection. Under a microscope, using an entomological needle, the front of the pupa shell was cracked and removed. The eggs were carefully collected with a fine brush into a "basket" soaked in PBS 1X (50 mM Na_2HPO_4, 50 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, pH 7.8) that was set on ice in order to arrest egg development. The basket was made of the upper part of a 0.5-ml plastic tube that was sealed with a fine mesh. Dechorionation of eggs was performed by soaking the basket in 50% commercial bleach (3.7% activity) for 10 min, or until the eggs sank to the bottom, and then soaking for 5 min in 0.7% NaCl/0.5 Triton X-100 solution to remove bleach. The basket was then dropped into a scintillation vial filled with 3 ml heptane (Scharlau)/3ml methanol (Frutarom) and shaken to remove the egg content, and then the basket was taken out. After 10 min of fixation, all the dechorionated eggs sank to the methanol fraction. The removal of the top (heptane) fraction with all the chrionated eggs in the interface was followed by two methanol washes. The eggs were then transferred to a 1.5-ml tube with methanol and kept refrigerated until staining.

Eggs Staining and Image Analysis

Eggs were rehydrated through the following methanol series: (a) 75% methanol/25% PBS 1X with 0.01% Triton X-100, (b) 50% methanol/50% PBS with 0.01% Triton X-100, (c) 25% methanol/75% PBS with 0.01% Triton X-100, (d) 100% PBS with 0.01% Triton X-100. For mounting, the eggs were soaked in 1 mg/ml DAPI (Sigma) for a few minutes and transferred onto a mi-

croscope slide to which a drop of 80% glycerol/TBST 1X was added. The slide was covered with a coverslip and sealed with clear nail polish.

At least 10 eggs were analyzed for each treatment using a Zeiss 410 LSM confocal microscope and an IPLab image analysis software (Vaytek). For each egg, 20 1-μm-thick sections of the posterior pole were taken and the number of pixels from an arbitrarily chosen 20 μm^2 was measured. Pixel counting was performed using a specific script that marks the clear stained regions and ignores the unspecified background. To estimate the amount of light emitted from one *Wolbachia*, 10 clear spots of stained bacteria were measured. The average number of pixels was found to be 12,442.7 ($\pm$3565.4). The number of pixels from each egg was divided by the pixels value of one *Wolbachia*, and an estimated average number of *Wolbachia* from 10 eggs was determined.

Fitness Determination

Following treatment with the various antibiotic concentrations, wasps were numbered and individually placed in 5-ml glass tubes containing 20 hosts each and stopped with a cotton plug. Each wasp was transferred daily to a tube with fresh hosts until she died. Upon emergence, the offspring (F1) of each mother were counted and sexed. For each treatment, the mothers' longevity and the number and sex ratio of offspring produced were calculated.

To determine female fecundity, at least 10 wasps from each treatment were placed individually in small glass tubes with about 20 hosts and allowed to lay eggs for 3 successive days. The hosts were replaced daily, and each day the pupae were cracked open and the number of laid eggs was determined. To determine survival from egg to adult the above experiment was repeated but instead of opening the hosts they were kept in the rearing room, offspring were counted upon emergence and survival rate to adulthood was calculated. This experiment was replicated three times.

Data Analysis

Analysis of variance (JMP, SAS Institute, 1996) and the Fisher protected least significant difference (LSD) tests (Steel and Torrie, 1980; JMP, SAS Institute, 1996) were used for analyzing treatment effects on wasp fitness. Error variances were homogenized by data transformation when ANOVA of the residuals indicated a significant F statistic (Levene test, Milliken and Johnson, 1984). Unless otherwise noted, tests of significance used a probability level of 0.05. Back-transformed means and 1 SE are presented.

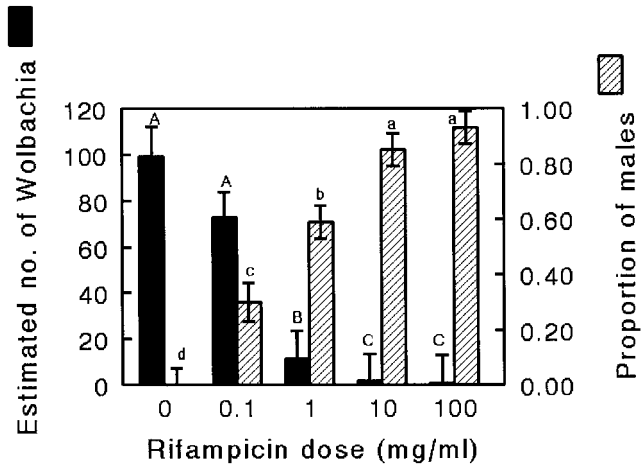

FIG. 1. Effect of antibiotic dose on the mean proportion of males ($\pm$SEM) produced by *M. uniraptor* females after antibiotic treatment and on the mean number ($\pm$SEM) of *Wolbachia* in 0- to 2-h-old eggs laid by *M. uniraptor* females 4 days post-treatment. Different letters indicate significant differences (protected LSD, $P < 0.05$); uppercase for the number of *Wolbachia* and lowercase for the proportion of males.

RESULTS

An increase in antibiotic dose resulted in a significant reduction in estimated densities of *Wolbachia* in *M. uniraptor* eggs ($F_{4,47} = 31.28$, $P < 0.001$; Fig. 1). As expected, a significant positive relationship was detected between antibiotic dose and the proportion of male offspring produced by the treated females ($F_{4,46} = 35.87$, $P < 0.001$). Whereas only about 30% of the progeny of 0.1 mg/ml rifamipicin-fed females were males, 60 to 93% males were produced at higher doses (Fig. 1). The differences between all five treatments were highly significant except the one between 10 and 100 mg/ml.

The offspring sex ratio was strongly affected by the post-treatment time, yet it did so differently in the various treatments (i.e., significant treatment by oviposition day interactions, $F_{84,521} = 2.48$, $P < 0.001$). When mothers were treated with 0.1 mg/ml rifampicin, they produced about an equal number of male and female offspring for 7 days and only females developed thereafter (Fig. 2). In the 1.0 and 10 mg/ml treatments, females produced predominantly male offspring until the 8th and 17th days, respectively. The sex ratio was female-biased thereafter. In contrast, 100 mg/ml fed mothers produced almost only males from the 2nd to the 15th day and male progeny were dominant throughout the experiment. Interestingly, in all treatments, at least some females were produced on the first day of oviposition (Fig. 2).

Variations in *Wolbachia* density, induced by antibiotic treatment, did not have a significant effect on the fecundity or longevity of the females, the number of mature offspring produced per female per day, or their

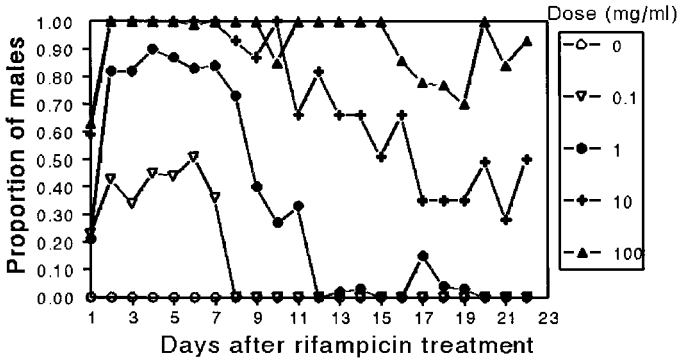

FIG. 2. Daily proportion of male progeny produced by rifampicin-treated *M. uniraptor* females following antibiotic treatment.

survival to adulthood ($F_{4,55} = 2.27$, $P > 0.07$; $F_{4,142} = 1.90$, $P > 0.11$; $F_{4,47} = 0.62$, $P > 0.65$; and $F_{4,47} = 1.80$, $P > 0.15$, respectively). Furthermore, the response of none of these fitness components showed a consistent, dose-dependent trend (Figs. 3a–3d).

DISCUSSION

So far, studies concerning the influence of *Wolbachia* on their hosts compared either bacterial density with CI levels (Boyle *et al.*, 1993; Breeuwer and Werren, 1993; Bourtzis *et al.*, 1996; Clancy and Hoffmann, 1996) or an antibiotic treatment with fitness measures (Stouthamer *et al.*, 1994; Poinsot and Mercot, 1997). Here, for the first time, the effect of symbiont density on both host fitness and sex ratio was studied.

That antibiotic treatment had a negative effect on wasp fitness is unlikely because antibiotic doses of 10 and 100 mg/ml (an order of magnitude difference) did not differ in their effect on the wasps. Other studies similarly found no negative effect of antibiotic treatment (Stouthamer *et al.*, 1994; Horjus and Stouthamer, 1995).

The most striking effect of *Wolbachia* densities is on the sex ratio produced by treated mothers. As *Wolbachia* densities are reduced by antibiotic treatments, the offspring's sex ratio shifts to become more male biased. These results clearly demonstrate that, like in CI bacteria, the influence of the PI symbiont is not an "all or none" effect, but rather is a matter of titer. A correlation between *Wolbachia* density and cytoplasmic incompatibility levels has been demonstrated in several studies (Breeuwer and Werren, 1990; Boyle *et al.*, 1993; O'Neill and Karr, 1990; Bourtzis *et al.*, 1996). Our results show that *Wolbachia* density in the host varies with rifampicin dose. This correlation allows the use of antibiotic treatments to investigate the effect of *Wolbachia* density on host fitness.

We found no negative effect of *Wolbachia* density on longevity, fecundity, survival, or reproductive rate of its *M. uniraptor* host. This result is in agreement with a mathematical model that predicts that: "the closer the parasite is to a vertical transmission, as in *Wolbachia*, the more likely it is to evolve less harm to its host" (Prout, 1994). Our results also agree with other studies where hosts were treated with antibiotics and various fitness traits, such as longevity, fecundity, and developmental time, were compared between *Wolbachia*-

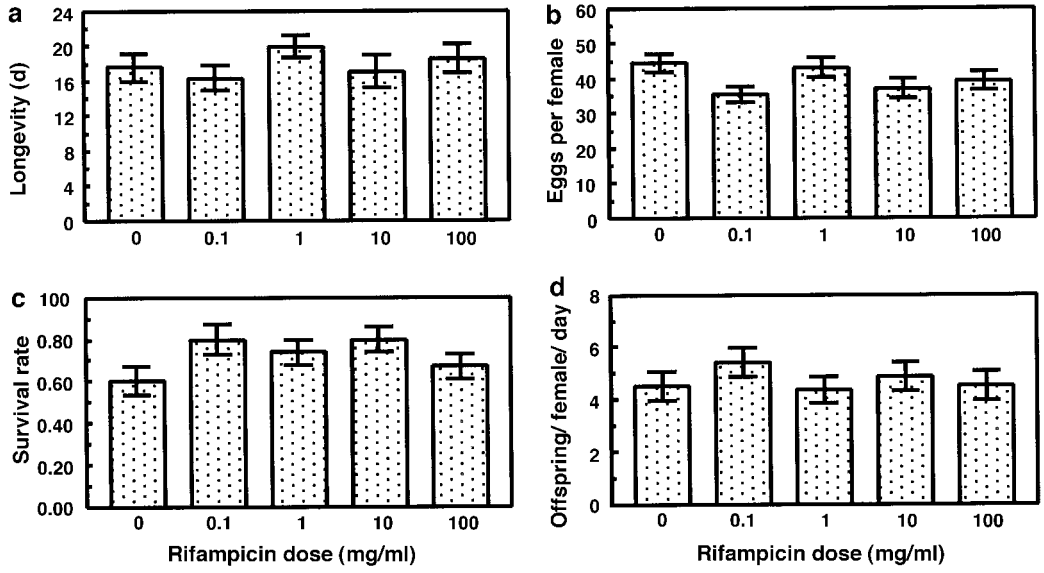

FIG. 3. Effect of antibiotic treatment on fitness components in *M. uniraptor*. (a) Mean longevity (±SEM) of females following antibiotic treatments. (b) Mean number of eggs (±SEM) laid by females during 3 days following antibiotic treatments. (c) Mean survival rate (±SEM) from egg to adults of offspring during 3 days following antibiotic treatments of parents. (d) Mean number of adults (±SEM) produced by females following antibiotic treatments.

infected and cured insects (e.g., Hoffmann *et al.*, 1990; Stolk and Stouthamer, 1996; Bourtzis *et al.*, 1996; Poinsot and Mercot, 1997). The majority of studies showed that the symbiont may have a minor negative or positive effect on its carrier, and in most cases, these differences are not large enough to pose strong selective pressure. However, the influence of the symbiont on other fitness components, such as resistance to parasitoids and tolerance to extreme temperatures, can not be ruled out. One such case is the *Wolbachia*-infected western alfalfa weevil, which is susceptible to the parasitoid *Microctonus aethiopoides*. In infected adult weevils, the parasitoid produces small and thin cocoons, pupal mortality rate is about 90%, and produced adults are nonreproductive. When reared on antibiotic-treated hosts, the parasitoid resumed normal development (Hsiao, 1996). The author concludes that ". . . the *Wolbachia* somehow alters the physiological conditions of the weevil to make it unsuitable for the development of the parasite, thereby contributing to the weevil's defense against an insect parasitoid."

Now that the volume of studies addressing the influence of *Wolbachia* on its host's fitness is growing, a pattern is starting to surface. It is hypothesized that *Wolbachia* is undergoing a shift in its effect on fitness components as a function of infection time: a relatively recent infection may result in a negative effect (i.e., 10–20% reduction in fecundity (Hoffmann *et al.*, 1990)), earlier infections will result in no effect as demonstrated in this study. Finally, *Wolbachia* may become a beneficial symbiont, as it appears to in the weevil system (Hsiao, 1996). This scenario may indicate an evolutionary transition toward mutualism, as it was the case in other endosymbionts (i.e., mitochondria). This hypothesis will be testable when the time of infection can be determined in a larger number of host-*Wolbachia* systems.

Results from the present study show that some female offspring were produced on the first day after treatment, regardless of the antibiotic dose their mothers received. A more prominent delay in the influence of antibiotic was found in the uniparental species *Aphytis lingnanensis* (Hymenoptera: Aphelinidae) (Zchori-Fein *et al.*, 1994, 1995) and *Apoanagyrus diversicornis* (Hymenoptera: Encyrtidae) (Pijls *et al.*, 1996), where the F1 offspring of antibiotic-treated mothers were mainly bacteria-free females, which, in turn, produced all-male broods. Similarly, Karr *et al.* (1998) found that repeated copulation by *Wolbachia*-infected male *D. simulans* significantly diminishes CI. These authors suggest that repeated copulation may reduce the time during spermatogenesis when *Wolbachia* can express CI. These data raise the possibility that *Wolbachia* produce a factor that travels through the cytoplasm and induces changes in the chromosomes regardless of the actual presence of the symbiont.

As discussed above, different antibiotic treatments yield differences in *Wolbachia* densities, and thelytoky is partially restored 16 days post-treatment even at the highest antibiotic dose, where *Wolbachia* were reduced to undetected levels. This partial elimination of the bacteria, combined with the possibility that *Wolbachia* may vary in their susceptibility to antibiotics in different organisms and under different environmental conditions, indicates that care must be taken when comparing fitness data between studies. Furthermore, studies that used different doses of antibiotic could not be compared unless the absence of the bacteria was demonstrated.

Another difficulty raised by the apparent buildup of *Wolbachia* density following treatments with low doses of rifampicin is that of results interpretation. For instance, we collected data on the developmental time of offspring produced by treated mothers. However, our results suggest that eggs laid on consecutive days carry a different load of symbionts (as shown by the fact that offspring sex ratio changed with time and was negatively correlated with *Wolbachia* density). To overcome this problem one should use higher antibiotic doses and verify the absence of *Wolbachia* by means of PCR or staining. Alternatively, *Wolbachia* levels should be determined for each individual and used as a covariant when fitness parameters are compared.

There is a growing amount of evidence indicating a minor, if any, influence of *Wolbachia* infection on host fitness. Yet, results of our study show that the effect of *Wolbachia* on offspring sex ratio in the parasitoid *M. uniraptor* is dose dependent and infection level is dynamic because of a rapid buildup after curing treatments.

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