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Wolbachia infection suppresses both host defence and parasitoid counter-defence

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Endosymbiotic bacteria in the genus *Wolbachia* have been linked to several types of reproductive parasitism, which enhance their own transmission, while their direct effects on the host vary from beneficial to neutral or detrimental. Here, we report negative effects of infection on immunity-related traits of *Drosophila simulans* and the parasitoid wasp *Leptopilina heterotoma*. Infected *D. simulans* showed a reduced ability to encapsulate parasitoid eggs, compared to a tetracycline-treated, bacterium-free line. Challenging the two lines with a fungal pathogen, *Beauveria bassiana*, on the other hand, revealed no differences in survival. Moreover, elimination of *Wolbachia* was beneficial for the parasitoid wasp, as eggs laid by uninfected females suffered significantly lower encapsulation rates. We discuss possible origins of these fitness costs and their implications for infection dynamics and the interactions between host species.

Keywords: *Wolbachia*; *Drosophila simulans*; fitness costs; parasitoid; resistance; encapsulation

1. INTRODUCTION

Wolbachia is a maternally inherited, intracellular symbiotic bacterium, extremely widespread among arthropods (Werren *et al.* 1995*a*), and has been associated with various reproductive manipulations in the host that enhance its own transmission, such as parthenogenesis, male-killing, feminization of genetic males and cytoplasmic incompatibility (CI; Stouthamer *et al.* 1999). In hosts exhibiting CI (the most commonly observed effect of *Wolbachia*), crosses between infected males and uninfected females fail to produce viable offspring, which offers a considerable fitness advantage to infected females (Hoffmann & Turelli 1997). *Wolbachia* symbionts exhibit a great degree of evolutionary plasticity, inducing effects that range from beneficial to neutral and detrimental.

Despite the evidence for rare horizontal transmission of *Wolbachia* (O'Neill *et al.* 1992; Werren *et al.* 1995*b*; Huigens *et al.* 2004*a*), transmission is principally vertical (from mother to offspring). Strictly vertically transmitted obligate symbionts are generally expected to evolve towards benign associations, with neutral or beneficial effects on host fitness, as their survival and reproduction completely depends on those of the host (Ewald 1987; Lipsitch *et al.* 1995). However, in several cases costs of *Wolbachia* infection have been identified. *Wolbachia* can afford to have negative fitness effects on its host due to the advantage it gains from reproductive parasitism. *Wolbachia* can be maintained in host populations by increasing the proportion of females produced by infected hosts (parthenogenesis, feminization, male-killing) or by decreasing the fitness of uninfected ones (CI), rather than increasing the fitness of the host (mutualism; Werren & O'Neill 1997).

Fitness costs associated with the presence of the symbiont can, however, play an important role in determining infection dynamics. So far, such effects have been observed on parameters such as body size (Hoffmann & Turelli 1988), fecundity (Hoffmann & Turelli 1988; Hoffmann *et al.* 1990; Stouthamer & Luck 1993; Johanowicz & Hoy 1999; Fleury *et al.* 2000), survival (Fleury *et al.* 2000; Tagami *et al.* 2001; Fry *et al.* 2004), larval competitive ability (Huigens *et al.* 2004*b*), male fertility and sperm cyst production (Snook *et al.* 2000). We attempted to identify effects of the symbiont on the immune response of *Drosophila simulans* and the counter-defence of its parasitoid *Leptopilina heterotoma* (in both species *Wolbachia* causes CI (Binnington & Hoffmann 1989; Louis & Nigro 1989; Vavre *et al.* 1999)).

To determine whether *Wolbachia* affects the ability of its *D. simulans* host to mount an efficient immune response against the parasitoid we subjected *Wolbachia*-infected and uninfected larvae to parasitism by *L. heterotoma* and recorded the encapsulation rates achieved by the host. In this experiment, we used infected and uninfected *L. heterotoma* females, in order to additionally examine whether *Wolbachia* affects the ability of the parasitoid to evade the immune response of its host. Moreover, in a separate experiment, we attempted to identify effects of *Wolbachia* on another aspect of the immune system of the *D. simulans* host, by subjecting flies with or without the symbiont to infection by the entomopathogenic fungus *Beauveria bassiana* and comparing their survival. We also investigated the effects of *Wolbachia* on several fitness parameters of *D. simulans* (body size, development time, number of ovarioles and fecundity).

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2. MATERIAL AND METHODS

(a) *Insect cultures*

The *D. simulans* Riverside cultures that were used in this study originated from a highly inbred population (sibling mating for more than 20 generations), infected with *Wolbachia* (W+). The original *L. heterotoma* population had also resulted from continuous inbreeding of W+ individuals (collected in Silwood Park, UK) for more than 20 generations (reared on *Drosophila subobscura*). *Wolbachia*-free (W−) fly and wasp lines were obtained by eliminating the bacterium with antibiotic treatment (O'Neill & Karr 1990) several generations before the start of the experiment. For *D. simulans*, 5 μg ml^{-1} tetracycline were added in the feeding medium of larvae and adults; for *L. heterotoma*, 5 μg ml^{-1} tetracycline were similarly added in the food of the *D. subobscura* hosts and also in the honey used for feeding the adults. Insect cultures were kept at 25 °C and 16L : 8D photoperiod.

(b) *Fungus culture*

Cultures of *B. bassiana* were generated by plating 5 μl of a stock solution (107 spores ml^{-1} in 25% glycerol, stored at −80 °C) onto a Petri dish with Sabouraud dextrose agar. The fungus was allowed to grow for three to four weeks at 29 °C. Then it was subcultured again, grown under the same conditions, and the resulting cultures were used in the experiment.

(c) *Molecular analysis for verification of* Wolbachia *infection status*

D. simulans and *L. heterotoma* cultures were repeatedly sampled and their infection status was verified by polymerase chain reaction (PCR). DNA was extracted from the insects using a DNAce Spin Tissue Kit (Bioline). *Wolbachia* was detected in *Drosophila* and *L. heterotoma* by PCR, using a set of specific primers, Wsp81 (forward) and Wsp691 (reverse), amplifying the *Wolbachia* surface protein gene *wsp* (Zhou *et al.* 1998). Also, a set of primers, specific to *Drosophila*, amplifying the subunit II of the cytochrome oxidase gene and another set, specific to *Leptopilina*, amplifying an internal transcribed spacer (ITS) region, were added as controls to the respective PCR reactions (primer sequences: Wsp81(forward): 5′-TGGTCCAATAAGTGATGAAGAAC-3′, Wsp69 (reverse): 5′-AAAAATTAAACGCTACTCCA-3′; COII (forward): 5′-CAAYTWATTGAAATAATTTGAAC-3′, COII(reverse): 5′-CCACAWATTTCTGAACATTG-3′; LhITS(forward): 5′-GTTGGCATATTGATAATCATC GG-3′, LhITS (reverse): 5′-TACAATTCCCGGACC AGCC-3′). PCR was performed using puReTaqTM Ready-To-GoTM PCR beads (Amersham Biosciences). All PCR tests confirmed the infection status of each insect line.

(d) *Measurement of fitness parameters*

The relative fitness of the W+ and W− *D. simulans* lines was assessed by comparing them in the following characteristics: development time (from hatching to eclosion), body size, early fecundity and ovariole number. Freshly laid eggs were collected from W+ and W− *D. simulans*, were individually placed in vials (2.5×7.5 cm) with feeding medium and the time of hatching, pupation and eclosion were recorded. When the adults eclosed, all females were mated to males of the same infection status and the number of eggs they laid between 4 and 6 days of age (48 h), as well as the number of ovarioles in each ovary were recorded. Ovariole number has been strongly associated with fecundity in *Drosophila* (Boulétreau-Merlé *et al.* 1982). The thorax length of all males and females was also measured. Thorax length was used as an index of body size but has also been directly associated with other fitness characteristics, such as ovariole number and fecundity (Santos *et al.* 1992). All procedures were carried out at 25 °C and 16L : 8D photoperiod. The results were analysed using generalized linear models.

(e) *Parasitism of* D. simulans *by* L. heterotoma

In total 6400 *D. simulans* larvae (48 h old, of uniform size) were collected for parasitism by *L. heterotoma*, half from W+ and half from W− flies. The larvae were placed in plastic bottles (6×3 cm) with feeding medium, in groups of 40. Four W+ or W− *L. heterotoma* mated females (3 days old) were introduced in each bottle and were allowed to parasitize the larvae for 4 h. In total 160 bottles were set up (40 with W+/W+, 40 with W+/W−, 40 with W−/W+ and 40 with W−/W− flies/wasps). When the pupae had formed (4–5 days after parasitism) they were dissected and assigned to one of the following categories: (a) no parasitism (developing fly/no capsule), (b) successful parasitism (developing wasp) and (c) successful encapsulation of parasitoid egg (developing fly/capsule present), and the parasitism and encapsulation rate for each bottle were calculated (parasitism rate: $(b+c)/(a+b+c)$, encapsulation rate: $c/(b+c)$). All procedures were carried out at 25 °C and 16L : 8D. The data obtained were analysed using a generalized linear model with binomial errors and including host and parasitoid infection status as two-level factors.

(f) *Infection of* D. simulans *with* B. bassiana

Only males were used, as preliminary experiments had suggested that male and female *D. melanogaster* may respond differently to infection by *B. bassiana*. In total, 32 agar-lined pots (4.5×10 cm) were set up, 16 with W+ and 16 with W− flies (50 flies per pot). The flies were subject to *B. bassiana* infection 2–3 days after eclosion. Eight pots of each infection status were treated with the fungus and the remaining were left untreated and used as controls. The flies were anaesthetized with CO_2 and placed in a Petri dish containing a sporulating *B. bassiana* culture, which was then shaken for 10 to 15 s to ensure that all the flies had picked up fungal spores. The control flies were shaken in a clean Petri dish (lined with filter paper), to ensure that they received the same stress. Pots were renewed every 2 days and they were checked daily to record the number of dead individuals. The experiment was stopped when mortality levels in all pots had reached at least 75% (after about 60 days). All procedures were carried out at 25 °C and 16L : 8D photoperiod. Flies that were alive at the end of the experiment were treated as censored data. The mean time to death (T_m) for each pot was calculated from the parameters of the fitted Weibull distribution $[T_m = \alpha\Gamma(1/\gamma+1)]$ and these values were analysed with a two-way ANOVA using *Wolbachia* infection status and fungus infection status as two-level factors.

3. RESULTS

(a) *Effects of* Wolbachia *on* D. simulans *fitness parameters*

From the 94 eggs collected, emerged 23 and 20 W− males and females and 22 and 19 W+ males and females, respectively. The values of fitness parameters measured on

Table 1. Effects of *Wolbachia* infection on fitness characteristics of *D. simulans*.

	Wolbachia+			*Wolbachia*−			
	mean	s.e.	*n*	mean	s.e.	*n*	significance
females							
development time (h)	240.1	1.910	20	240.4	2.034	19	NS
thorax length (mm)	0.97	0.040	20	1.13	0.047	19	$p<0.001$
ovarioles (per ovary)	17.3	0.426	20	18.3	0.471	19	NS
fecundity (in 48 h)	46.9	2.602	20	60.2	1.957	19	$p<0.001$
males							
development time (h)	248.0	1.033	23	249.7	0.926	22	NS
thorax length (mm)	0.76	0.031	23	0.94	0.031	22	$p<0.001$

these individuals are summarized in table 1. Development time was longer for males than females ($p<0.001$), as is normally the case in *Drosophila*, but no effect of infection status was found ($p=0.40$). Also, larger flies took significantly longer to eclose ($p<0.001$). As expected, males were smaller than females ($p<0.001$), but it was also the presence of *Wolbachia* that strongly affected adult body size, with infected males and females having significantly shorter thoraces, by 19 and 14%, respectively, than uninfected ones ($p<0.001$).

Ovariole number was positively correlated with body size ($p=0.043$) but was not affected by *Wolbachia* infection status ($p=0.88$). However, fecundity of W− flies was higher by 28% with the main effect of infection status shown as highly significant ($p<0.001$). Fecundity was also positively correlated with ovariole number ($p=0.016$) and body size ($p=0.018$).

(b) *Effects of* Wolbachia *on* D. simulans *encapsulation rate*

The results of the parasitism experiment are presented in figure 1. *Wolbachia* infection status of both *D. simulans* and *L. heterotoma* had an effect on the encapsulation rates achieved by the fly larvae ($p<0.001$ for both factors). W− larvae achieved a 19.9% higher encapsulation rate than infected larvae (figure 1). From the wasp's point of view, eggs laid by W+ females suffered on average 22.6% more encapsulation of their eggs than those laid by their cured counterparts (figure 1). Parasitism rates were in all cases around 70% and were not affected by either the host's or the parasitoid's infection status ($p=0.52$ and $p=0.085$, respectively).

These results suggest that the presence of the symbiont in *D. simulans* significantly reduces the efficiency of the host's cellular immune response against the eggs deposited by *L. heterotoma*. In addition, it appears that the presence of *Wolbachia* in *L. heterotoma* causes a decrease in the survival probability of its offspring in the host, as a result of increased encapsulation.

(c) *Effects of* Wolbachia *on survival of* D. simulans *infected by* B. bassiana

As expected, fungal treatment significantly reduced survival of *D. simulans* ($p<0.001$), but neither the main effect of *Wolbachia* infection nor the interaction between the two treatments was significant ($p=0.59$ and $p=0.39$, respectively; figure 2). Therefore, there is no indication that the presence of *Wolbachia* has an effect on the immune response of *D. simulans* to fungal infection.

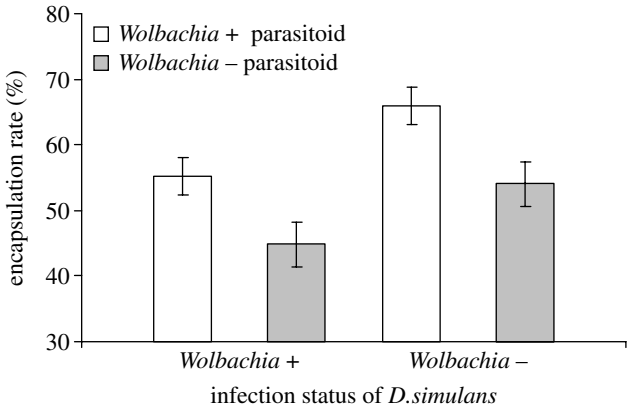

Figure 1. Mean proportion of eggs laid by *Wolbachia*-infected and uninfected *L. heterotoma* females that were encapsulated by *Wolbachia*-uninfected and infected *D. simulans* hosts. Error bars represent 1 s.e.

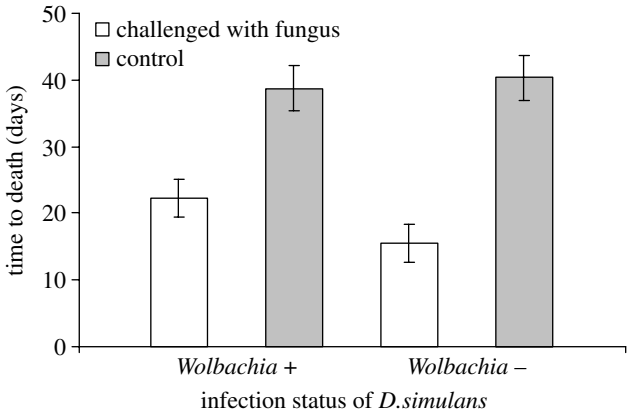

Figure 2. Survival of *Wolbachia*-infected and uninfected *D. simulans* males that had or had not been treated with the fungus *B. bassiana*. Error bars represent 1 s.e.

4. DISCUSSION

Wolbachia infection has adverse effects, including effects on immunity-related traits, on two interacting host species, *D. simulans* and *L. heterotoma*. In the former, the presence of the symbiont is associated with smaller body size, reduced fecundity and a decline in the encapsulation response of the larvae against deposited parasitoid eggs. Similar fitness costs of *Wolbachia* infection on body size and fecundity have already been identified in *D. simulans* (Hoffmann & Turelli 1988; Hoffmann *et al.* 1990). However, a reduced efficiency of the cellular immune response of W+ flies against parasitoid eggs is reported

here for the first time. In other organisms carrying vertically transmitted symbionts the opposite pattern has been observed, e.g. the presence of maternally inherited secondary symbionts in the pea aphid increases the host's resistance to parasitoid wasps (Oliver *et al.* 2003; Ferrari *et al.* 2004). This discrepancy can be seen as a result of the different strategies adopted by symbionts in order to invade host populations. While symbionts such as those in the pea aphid may spread by increasing their host's fitness, *Wolbachia* relies on manipulating host reproduction. The decrease in parasitoid resistance conferred by *Wolbachia* reduces the fitness of both the host and the symbiont, although for *Wolbachia* this cost is potentially offset by the effects of reproductive parasitism. Studies on *Drosophila* have shown that parasitoids (mostly *Leptopilina* and *Asobara* spp.) are a major source of mortality in natural populations (Fleury *et al.* 2004). Given the high prevalence of parasitoids in nature and the strong selective pressures, therefore, imposed on their hosts, this suggests that *Wolbachia* may substantially affect the population dynamics of its hosts by influencing such interspecific interactions.

The origin of these fitness effects may be a physiological cost of bacterial burden that leads to a reduced availability of resources for growth, egg production and immune functions. Another possible interpretation of the impairment of cellular immunity is that *Wolbachia* is interfering directly with elements of the host immune system. Such an effect could be the result of an attempt on the part of the symbiont to actively suppress host immunity in order to protect itself. However, there is evidence against the existence of such mechanisms. It has been found, for example, that *Wolbachia* does not affect the expression levels of genes encoding antimicrobial peptides in the hosts *D. simulans* and *Aedes albopictus* (Bourtzis *et al.* 2000), which suggests that the bacterium survives in the host by evading rather than actively suppressing host immunity. On the other hand, studies on the evolution of the *wsp* gene in *Wolbachia* have revealed that the outer membrane protein of the bacterium is under strong positive selection, especially concerning the regions of the protein that come into contact with the host (Jiggins *et al.* 2002), indicating important symbiont–host interactions rather than mere evasion.

Whatever the origin of this effect it does not appear to be reflected on the anti-fungal response of *D. simulans*. One explanation for this discrepancy could lie in the relative costs of the activation of the anti-parasitoid and anti-fungal responses. It is possible that the resource requirements for mounting a response against fungal pathogens are lower than those of anti-parasitoid cellular immunity, which has already been shown to be a costly system (Kraaijeveld *et al.* 2002). On the other hand, the uniformly low survival of *Wolbachia*-infected and uninfected flies after fungal treatment may imply that the spore dose applied was too high for any subtle effects of the symbiont to be observed.

Wolbachia infection also reduces the ability of the parasitoid to protect its eggs against the host's cellular immune response. Successful evasion of host immunity is invariably necessary for the survival of the parasitoid, unless the host is completely incapable of mounting an immune response. In *Leptopilina*, this involves the injection of virus-like particles (VLPs) in the host upon oviposition, which render host lamellocytes unable to encapsulate the deposited egg (Labrosse *et al.* 2003). The presence of *Wolbachia* in the parasitoid may have an effect on the production of VLPs, with detrimental effects on offspring survival. Other important physiological costs of *Wolbachia* infection in *L. heterotoma* have been identified, namely reduced fecundity, adult survival and locomotor activity, indicating that *Wolbachia* infection in this species is among the most harmful studied so far (Fleury *et al.* 2000).

Wolbachia is harmful to both *D. simulans* and *L. heterotoma* and its effects may have important implications for infection dynamics. Reproductive parasitism greatly enhances *Wolbachia* transmission, and infection is therefore predicted to spread rapidly. Although in many *Wolbachia*-carrying populations infection indeed appears to be at or near fixation, in others it occurs at various frequencies (Hoffmann *et al.* 1994; Breeuwer & Jacobs 1996). Fitness costs of infection suffered by the host are believed to be among the factors responsible for such variable infection levels (Hoffmann *et al.* 1990; Nigro 1991); others include imperfect transmission of the symbiont to the offspring (Turelli & Hoffmann 1995), low levels of expression of reproductive effects (Mercot *et al.* 1995; Bourtzis *et al.* 1996), non-random mating (Vala *et al.* 2004) and environmental curing (Stevens & Wicklow 1992). Fitness costs will tend to keep the prevalence of infection at lower levels than those predicted by CI effects alone and may reduce the rate at which the symbiont invades populations. It has been shown that when the CI-inducing symbiont has negative fitness effects on the host, an unstable equilibrium frequency of infection exists, above which the infection is driven to fixation, and below which it is lost (Caspari & Watson 1959; Hoffmann *et al.* 1990). On the other hand, fitness costs are not expected to influence the ultimate infection frequency (stable equilibrium), which is rather more likely to be determined by maternal transmission efficiency. Fitness costs may play a more important role in haplodiploid (e.g. *Leptopilina*) than diploid (e.g. *Drosophila*) systems, as the unstable equilibrium is predicted to be higher in the former (Vavre *et al.* 2000).

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