Disrupting the timing of *Wolbachia*-induced male-killing

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Several lineages of maternally inherited symbionts have evolved the ability to kill infected females' sons, a phenomenon known as male-killing. Male-killing varies in its timing, from early (death during embryogenesis) to late (mortality of late larval instars). Following the observation that treatment of male-killer infected adult females *Hypolimnas bolina* with tetracycline, a bacteriostatic antibiotic, produces a delay in the timing of male death, we hypothesized that early male-killers possess the ability to kill males through bacterial activity outside of embryogenesis. We verified this hypothesis by showing that treatment of surviving larvae with the bacteriocidal antibiotic rifampicin rescues males. This discounted the hypothesis that delayed death occurred due to postponed effects of toxins produced at earlier stages, and thus supported the importance of bacterial activity in the larval phase in delayed male-killing. These results argue against the view that early male-killing is achieved by specifically targeting an early developmental process within the sex determination pathway.

Keywords: *Wolbachia*; male-killing; sexual parasitism; sex-ratio distorters; genetic conflicts; mechanism

1. INTRODUCTION

Several lineages of micro-organisms have evolved the capacity to kill infected females' sons, a phenomenon known as male-killing (Hurst *et al.* 2003). This is best interpreted as a consequence of the sex-specific selective pressures associated with maternal inheritance, making male hosts an evolutionary 'dead end'. Past work has characterized male-killing by the timing of male death (Hurst 1991). In 'early male-killing', male death occurs during the embryonic stages. These cases have to date been associated with bacterial infections. In 'late male-killing', male death occurs during late larval instars. These cases have been primarily associated with intracellular eukaryotic infections (microsporidia) and observed most commonly in mosquitoes. Recently, late male-killing has been observed in the tea tortrix moth, *Homona magnanima*, and the agent, although transferable through inoculation, is unlikely to be bacterial (the trait is not sensitive to antibiotics; Morimoto *et al.* 2001).

The timing of male-killing has been held to be adaptive (Hurst 1991). In the case of late male-killing, male death is associated with the rupture of larval cuticle and the release of infectious spores into the environment. The late timing is considered to allow time for the titre of infectious spores to build. In the case of early male-killing, there is little or no horizontal transmission, and the advantage of male-killing derives from various processes of fitness compensation (resource reallocation, inbreeding avoidance) where male-death can increase the fitness of infected females' daughters, allowing male-killers to spread in the population. The earlier the male death occurs, it is held, the higher the advantage that can accrue through fitness compensation.

What is unclear is the mechanism of male-killing. This process must clearly involve some interaction with the host sex determination either as a cue for toxin production by the bacterium or as a target of a constitutively produced toxin. To date, this question has only been studied in the model organism *Drosophila*, where male-killing by *Spiroplasma* bacteria were observed to require a functioning host system of dosage compensation (Veneti *et al.* 2005). The dosage compensation system is a downstream element of sexual differentiation, and was considered most likely to be a cue instigating male-killing rather than a target of the male-killer itself.

In this paper, we examine the process of male-killing in the butterfly, *Hypolimnas bolina*. In this species, *Wolbachia* bacteria (strain name: wBol1) cause the death of male hosts during embryogenesis (Dyson *et al.* 2002; Mitsuhashi *et al.* 2004; Charlat *et al.* 2005). We use antibiotic intervention to examine whether male-killing can be achieved through bacterial actions outside of embryogenesis. We first demonstrate that treatment of adult females with tetracycline (a bacteriostatic factor; Baron 1996) delays male-killing, while rifampicin, a more efficient (bacteriocidal) antibiotic rescues males completely. We further examine whether delayed male-killing represents a more protracted death associated with weakened bacterial activity in the embryo (as seen in some haplodiploids, where *Wolbachia* can cause mortality even in the F2; Vala *et al.* 2000), or is actively induced by live *Wolbachia* during the larval phase. We conclude that the killing mechanism is active, at least in part, during the larval phase, ruling out early stages of sex determination as the (sole) point of interaction between male-killer and host.

2. MATERIAL AND METHODS

(a) *Lines used, rearing procedures*
The experiments presented here are based on lines collected throughout 2005, on the island of Moorea in French Polynesia, where the experiments took place (at the Gump station, University of California Berkeley in French Polynesia). Some matrilines were maintained in the laboratory for several generations, with inbreeding depression avoided by mating females with unrelated males (from lab stock or from the wild) at every generation. Details of the lines used in each experiment are given in §3 (tables 1 and 2). Females were induced to oviposit and larvae reared to adult as described elsewhere (Charlat *et al.* 2005). Embryo death was scored 5–6 days following oviposition by counting all larvae and unhatched eggs. Sex ratio was estimated by sexing all adults at emergence based on wing colour patterns.

(b) *Antibiotic interventions*
Two antibiotics were used in this study: tetracycline and rifampicin. While both molecules are known to be active against intracellular bacteria, their mode of actions and efficiencies are distinct

Table 1. Embryo mortality and F1 adult sex ratio following various treatments on the adult mothers. (N_{line}, number of treated matrilines.)

treatment no.	infection	treatment on mother	larvae	unhatched eggs	adult females	adult males	N_{line}
1	*w*Bol1	none	804	582	130	0	7
2	*w*Bol1	tetracycline	558	42	61	8	6
3	*w*Bol1	rifampicin	294	15	44	48	2
4	uninfected	none	1027	54	74	66	8

Table 2. Embryo mortality and F1 adult sex ratio following presence or absence of rifampicin treatment on the larvae produced by tetracycline-treated adult mothers. (N_{line}, number of matrilines.)

treatment no.	infection	treatment on larvae	larvae	unhatched eggs	adult females	adult males	N_{line}
5	*w*Bol1	none	544	41	56	8	5
6	*w*Bol1	rifampicin	362	40	27	37	3
7	uninfected	none	206	13	17	22	3
8	uninfected	rifampicin	690	60	42	41	4

(Baron 1996). Tetracycline inhibits translation and has a bacteriostatic effect. In other words, it does not kill bacterial cells but prevents cell division. On the contrary, rifampicin, an inhibitor of transcription, is bacteriocidic resulting in the death of *Wolbachia* and hence far more effective elimination of the infection.

In the first experiment, we treated infected adult females with either tetracycline or rifampicin. Following a slightly modified version of an earlier protocol (Mitsuhashi *et al.* 2004), antibiotic treatment was delivered to adults by feeding them individually on 15% w/v sugar solution with 0.1% w/v antibiotics for a minimum of 4 days before oviposition, without alternative food source. When necessary, the proboscis was manually unfolded to ensure the uptake of a daily dose of antibiotics. Oviposition from these females was then induced as above and the timing and penetrance of male-killing were ascertained from embryo mortality and F1 adult sex ratio.

In the second experiment, we investigated the effect of adding a larval treatment with rifampicin to treatment of the maternal adult with tetracycline. Infected adult females were fed on tetracycline as before, and then a subset of clutches allowed to feed on rifampicin as larvae. To treat larvae, 5 ml of a 0.1% w/v antibiotic solution was sprayed on fresh leaves every time larvae were transferred to new boxes (i.e. every 2–4 days depending on the larval stage) with one box containing not more than 20 larvae. As a control against endogenous effects of rifampicin on the host, this experiment was repeated on uninfected females.

(c) *Infection status of females*
Prior to the experiments, the status of the lines used was determined by PCR assay following Charlat *et al.* (2005). In brief, DNA extracts were quality assessed using a general 'metazoan' PCR of the COI mitochondrial gene using the primer pair LCO/HCO (Folmer *et al.* 1994), and amplifiable template was then assayed for the presence of *w*Bol1 (the male-killing *Wolbachia*) using B-clade specific *wsp* primers (wsp81f and wsp522r; Zhou *et al.* 1998). The effect of tetracycline and rifampicin on infection presence was also ascertained on a subset of the experiment through PCR assay in the generations following treatment.

(d) *Statistical analysis*
Differences in embryo mortality or adult sex ratio between the various treatments were ascertained using Pearson's χ^2 test with Yates' continuity correction, computed in R (R Development Core Team 2005). Homogeneity among broods within a given treatment was ascertained before pooling the data.

3. RESULTS
Consistent with an earlier report (Mitsuhashi *et al.* 2004), tetracycline treatment on adult females did result in the production of some male progeny (table 1: sex ratio in treatment 1 versus 2, $\chi_1^2 = 12.8$, $p = 0.0003$), although at a much lower level than in the absence of

infection (table 1: sex ratio in treatment 2 versus 4, $\chi_1^2 = 24.0$, $p = 9.6 \times 10^{-7}$). Tetracycline increased survival of male progeny through embryogenesis (table 1, hatch rate in treatment 1 versus 2: $\chi_1^2 = 236.3$, $p < 2.2 \times 10^{-16}$) with a shift to a similar level to that seen in uninfected females' broods (table 1, hatch rate in treatment 2 versus 4, $\chi_1^2 = 2.5$, $p = 0.11$, n.s.). Thus, tetracycline treatment on adult females produced delayed male death in their progeny, rather than full male rescue; the infection was converted into an incompletely penetrant late male-killer.

To test the hypothesis that delayed male-killing was associated with knock down of the bacterium by a bacteriostatic intervention, rather than knock out, we compared the results of tetracycline treatment with treatment with rifampicin, a bacteriocidal antibiotic. As found for tetracycline, rifampicin increased survival of male progeny through embryogenesis (table 1, hatch rate in treatment 1 versus 3: $\chi_1^2 = 151.1$, $p < 2.2 \times 10^{-16}$) with a shift to a similar level to that seen in uninfected females' broods (table 1, hatch rate in treatment 3 versus 4: $\chi_1^2 = 0.002$, $p = 0.96$, n.s.). However, in contrast to the delayed male death phenotype associated with tetracycline treatment, rifampicin resulted in the full restoration of F1 male survival until the adult stage (table 1, sex ratio in treatment 1 versus 3: $\chi_1^2 = 83.5$, $p < 2.2 \times 10^{-16}$; $\chi_1^2 = 0.4$, $p = 0.54$, n.s.). Thus, we conclude that it is an incomplete knock down of *Wolbachia* by tetracycline that induces the delayed male-killer phenotype we see. Consistent with this interpretation, we observed that tetracycline does not cure the line of male-killing: infection in F1 females following treatment is detected both by PCR and breeding assays ($n = 6$ F1 females). In contrast, rifampicin produced a cure of the infection in F1 ($n = 2$ females).

There are two hypotheses that could account for the delayed male-killing phenotype. First, it may be that the activity of the male-killer is confined to embryos (e.g. some early aspect of sex determination), but the damage that occurs during embryogenesis is less substantial and this leads to a more protracted death.

Second, it may be that larval/pupal death is associated with the recovery of *Wolbachia* during these late developmental stages and activity of the male-killer in male larvae. We distinguished between these by employing a second bacteriocidal intervention during the larval phase. In brief, adult females were treated with tetracycline and their offspring were then treated with rifampicin as larvae. If late male-killing is a case of protracted male death due to reduced (but non-zero) *Wolbachia* virulence during embryogenesis alone, then rifampicin treatment of later developmental phases (larvae) should not alter the phenotype—it should remain as late male death. On the contrary, if the damage is caused by live and active *Wolbachia* during late developmental stages (larvae), then the bacteriocidal activity of rifampicin in the larval phase should result in full rescue of male larvae. We observed the latter (table 2). The sex ratio produced at the adult stage following rifampicin treatment of surviving larvae was not different from that observed in the absence of infection (table 2, sex ratio in treatment 6 versus 8: $\chi_1^2 = 0.72$, $p = 0.4$, n.s.), and deviated from the tetracycline only treatment (table 2, sex ratio in treatment 5 versus 6: $\chi_1^2 = 26.9$, $p = 2.2 \times 10^{-7}$). Control crosses on uninfected individuals demonstrated rifampicin had no sex-specific mortality effects in itself, with control and rifampicin treated broods from uninfected parents showing similar sex ratio (table 2, sex-ratio in treatment 7 versus 8: $\chi_1^2 = 0.2794$, $p = 0.5971$).

4. DISCUSSION

The main results of the present study can be summarized as follows. (i) tetracycline treatment of adult *H. bolina* females results in survival of male embryos through embryonic development, but death of males at later larval stages. This contrasts with rifampicin treatment of adults, which produces full curing of male-killing, (ii) following tetracycline treatment of adults, rifampicin treatment of larvae results in a removal of the delayed male death. These results demonstrate that (i) inhibiting bacteria can produce a delay in the timing of male death to the larval and/or pupal phases, (ii) male-killing action is not dependent upon activity in the embryo alone. Rather, *Wolbachia* actions in the larvae can also contribute to male death. Thus, male-killing activity is not confined to strict developmental stage.

Our results indicate that male-killing *Wolbachia* in *Hypolimnas* do not kill through a mechanism that functions solely in embryos. Application of a bacteriocidal antibiotic to larvae retrieved full male survival, and thus components of male-killing can be active in larvae. This rules out upstream elements of sex determination as the (sole) target of male-killing, as these are activated only early in embryogenesis. However, upstream elements of sex determination could still be involved in the activation of a toxin that can act later in development. Answers to the question of mechanism clearly await greater understanding of both bacteria and host genetics.

These results also suggest a new interpretation of late male-killing. Previously, late male-killing has been seen solely in terms of micro-organism interest. Under this view, late death is the optimal time for the release of propagules. While undoubtedly this works well for the microsporidial–mosquito system, our work suggests a second route to late male-killing that could apply for systems which do not have propagule survival in the wild. In this scenario, late male-killing is associated with a poorly functioning early male-killer (the analogue of our tetracycline-treated bacterium). Poor function could derive either from maladaptation on the part of the male-killer associated with a recent horizontal transfer, or modifiers in the host that have been selected to rescue males, but do not do so with complete penetrance. The latter would present an evolutionary transition from early to late male-killing.

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