THE ROYAL SOCIETY

doi 10.1098/rspb.2000.1166

Male-killing *Wolbachia* in a flour beetle

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The bacteria in the genus *Wolbachia* are cytoplasmically inherited symbionts of arthropods. Infection often causes profound changes in host reproduction, enhancing bacterial transmission and spread in a population. The reproductive alterations known to result from *Wolbachia* infection include cytoplasmic incompatibility (CI), parthenogenesis, feminization of genetic males, fecundity enhancement, male killing and, perhaps, lethality. Here, we report male killing in a third insect, the black flour beetle *Tribolium madens*, based on highly female-biased sex ratios of progeny from females infected with *Wolbachia*. The bias is cytoplasmic in nature as shown by repeated backcrossing of infected females with males of a naturally uninfected strain. Infection also lowers the egg hatch rates significantly to approximately half of those observed for uninfected females. Treatment of the host with antibiotics eliminated infection, reverted the sex ratio to unbiased levels and increased the percentage hatch. Typically *Wolbachia* infection is transmitted from mother to progeny, regardless of the sex of the progeny; however, infected *T. madens* males are never found. Virgin females are sterile, suggesting that the sex-ratio distortion in *T. madens* results from embryonic male killing rather than parthenogenesis. Based on DNA sequence data, the male-killing strain of *Wolbachia* in *T. madens* was indistinguishable from the CI-inducing *Wolbachia* in *Tribolium confusum*, a closely related beetle. Our findings suggest that host–symbiont interaction effects may play an important role in the induction of *Wolbachia* reproductive phenotypes.

Keywords: male killing; *Wolbachia*; *Tribolium madens*; endosymbiont; cytoplasmic incompatibility

1. INTRODUCTION

Intracellular rickettsial bacteria of the genus *Wolbachia* are found in numerous host species from a variety of insect orders, mites and isopod crustaceans (e.g. Stevens 1993; Johanowicz & Hoy 1996; Bouchon *et al*. 1998). The mosquito-borne nematode that causes dog heartworm harbours a close relative of *Wolbachia* (Sironi *et al*. 1995), and *Wolbachia* occurs in at least nine other nematodes (Bandi *et al*. 1998). The notion that these bacteria are widespread is supported by many surveys, e.g. *Wolbachia* has been found to infect over 16% of New World insect species (Werren *et al*. 1995*a*) and 50% of Indonesian ant species (Wenseleers *et al*. 1998). Because infection can have significant consequences on host fitness, it has been suggested that *Wolbachia* could be involved in speciation events (Coyne 1992; Hurst & Schilthuizen 1998) and have potential application in the control of arthropod pests (Sinkins *et al*. 1997).

Infection with *Wolbachia* is associated with the induction of five reproductive phenotypes in arthropods. Thelytokous parthenogenesis is the production of all-female progeny from unfertilized eggs. It occurs in several species of parasitic wasps and appears to be restricted to the chalcidoid and cynipoid Hymenoptera (Stouthamer *et al*. 1993; Stouthamer 1997). Feminizing *Wolbachia* turn genetic males into functional females and are known from terrestrial isopods (Bouchon *et al*. 1998). However, despite its potentially widespread distribution in diplodiploid insects, *Wolbachia* does not exhibit these two phenotypes. Cytoplasmic incompatibility (CI) is expressed as lower fitness or sterility of the cross when infected males mate with uninfected females or females carrying a different genetic strain of *Wolbachia*. Enhanced fecundity and possibly lethality have also been reported (Girin & Bouletreau 1995; Min & Benzer 1997).

An additional phenotype, male killing, has been reported in two species of insect (Hurst *et al*. 1999). Here we report that a third insect host, the black flour beetle *Tribolium madens*, exhibits *Wolbachia*-induced sex-ratio distortion caused by male killing. In a closely related species, the confused flour beetle *Tribolium confusum*, *Wolbachia* is known to induce CI (Wade & Stevens 1985). The phylogeny of male-killing *Wolbachia* in *T. madens* supports the suggestion that male-killing behaviour is easily evolved in symbionts of invertebrates.

2. METHODS

We used the sex-ratio strain (SR) of *T. madens*, which is noted to have female-biased sex ratios. Two uninfected strains, TSR and NSR, were also used. TSR was cured of *Wolbachia* as a result of tetracycline treatment (0.15% by weight tetracycline added to flour medium) of a subpopulation of the SR strain. The NSR strain, which is naturally uninfected with *Wolbachia*, exhibits a sex ratio close to 1:1 (pupae sexed from stock population, 133 females:121 males) ($\chi^2 = 0.57$ and $p > 0.05$). Female-biased sex ratios had been observed in the stocks of the infected strain for several months prior to the beginning of the experiments (1834 females:62 males) ($\chi^2 = 1656.11$ and $p < 0.0001$). The *Wolbachia* infection status of the three beetle strains was confirmed by polymerase chain reaction (PCR) amplification of bacterial DNA using specific primers (O'Neill *et al*. 1992). Individual insects were dissected and their gonads removed and used to extract DNA for the PCR reactions with *Wolbachia*-specific primers in order to confirm their infection status (O'Neill *et al*. 1992; Fialho & Stevens 1997).

Specific experiments are described below and their statistical analyses are given in § 3. Prior to analysis, the data were tested for

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Received 30 November 1999 *Accepted* 11 January 2000

fit to the assumption of a normal distribution. Parametric or non-parametric tests were then used in the analysis as appropriate.

(a) *Sex ratio and percentage hatch experiment*

We assayed the sex ratio and percentage of eggs hatched for *ca.* 30 single-pair matings for each of seven different crosses (male × female: SR × SR, NSR × SR, TSR × SR, NSR × NSR, SR × NSR, TSR × TSR and SR × TSR). Virgin females were allowed to mate with single males and lay eggs in 1 dram vials containing 1 g flour medium (95% by weight very fine-sifted whole wheat flour plus 5% dried, powdered brewer's yeast) (Fialho & Stevens 1996) for two four-day periods. Eggs were collected at the end of each laying period, counted and transferred to empty vials. Hatched larvae from each mating were counted, transferred to vials with 8 g of medium and allowed to develop to pupae, which were sexed before eclosion. Data on the egg hatch rates and sex ratio were obtained for each single-pair mating. At the end of the experiment male and female parents were tested with the PCR using *Wolbachia*-specific primers in order to confirm their infection status. The SR strain demonstrated incomplete transmission and the few SR female parents that tested negative for *Wolbachia* were excluded from the analyses examining the effect of *Wolbachia* infection on the sex ratio and percentage hatch.

(b) *Backcross experiment*

In order to distinguish between nuclear and cytoplasmic effects, we backcrossed females of the SR strain with NSR males for seven generations and recorded the number of male and female pupae produced at each generation. After seven generations of backcrossing, the replacement of the maternal nuclear genome of the SR strain with that of the NSR strain is expected to be 99.2%. Each backcrossed line was established by allowing ten virgin SR females to mass mate with five to ten virgin NSR males in a vial with 8 g of standard medium for ten days. The adults were then removed. The progeny developed into pupae and were differentiated by sex. Subsequent generations were established with ten virgin females from the previous generation mated with five to ten virgin NSR males.

At generation 3, five new lines, one derived from each of the five original lines, were established in the same manner. At the fifth generation of inbreeding, two lines showed a decline in the sex ratio. We tested 12 females from each of these two lines and one of the highly female-biased lines for infection using the PCR.

(c) *DNA sequence analysis*

In order to study the phylogenetic relationship of the male-killing strain of *Wolbachia*, we sequenced a total of *ca.* 2500 nucleotides from four *Wolbachia* DNA regions. Bacteria were sequenced from at least two and as many as four individuals of *T. madens* as well as *T. confusum*, a closely related species. Both forward and reverse sequences were determined and compared. Sequence data from both strands were obtained for *ca.* 1000 bp of the *ftsZ* cell division gene (Werren *et al.* 1995*b*), almost 350 bp of the *wsp* gene (Zhou *et al.* 1998), *ca.* 850 bp of the *groE* intergenic region (Masui *et al.* 1997) and over 200 bp of an internal transcribed spacer (ITS) region flanked by the 23S and 5S rDNA genes (Fialho & Stevens 1997). DNA sequences were obtained by direct cycle sequencing of the PCR product (Fialho & Stevens 1997).

(d) *Infertility of virgin females and paternal genetic contribution*

Although *Wolbachia*-induced parthenogenesis has only been reported in haplodiploid insects (Stouthamer 1997), we empirically tested the possibility that it is the cause of the sex-ratio distortion in *T. madens*. Thirty virgin SR females were maintained in individual vials containing standard flour medium. After a period of six months the vials were checked for reproduction.

In addition, we tested for a paternal genetic contribution to the progeny of SR females by mating them with males of a closely related species (*Tribolium audax*). These two species hybridize and have a species-diagnostic difference in a ribosomal DNA ITS (ITS2) (Porter & Collins 1991), which yields products of different sizes for these two species.

Table 1. *Mean sex ratios and mean percentages of eggs hatched for the various crosses*

male parent	female parent	number of crosses	sex ratio	total hatch (%)	hatch in trial 1 (%)	hatch in trial 2 (%)
SR	SR	24	0.85	0.46	0.44	0.50
TSR	SR	21	0.95	0.42	0.45	0.42
NSR	SR	21	0.93	0.40	0.45	0.36
SR	TSR	23	0.52	0.68	0.74	0.65
TSR	TSR	28	0.50	0.69	0.74	0.67
SR	NSR	30	0.54	0.69	0.72	0.62
NSR	NSR	30	0.60	0.59	0.66	0.49
all infected		66	0.91	0.43	0.44	0.43
all uninfected		111	0.54	0.66	0.71	0.61

3. RESULTS

(a) *Sex ratio and percentage hatch experiment*

The mean sex ratio (proportion of females) and mean percentage of eggs hatched for the between-strain crosses are shown in table 1. The sex ratio produced by infected SR females (SR × SR, TSR × SR and NSR × SR) was significantly more female biased than that of both TSR and NSR females mated with SR, TSR or NSR males (Wilcoxon two-sample test, $z = 9.97$ and $p < 0.0001$). The sex ratio of TSR females (SR × TSR and TSR × TSR crosses) was not significantly different from 0.5 (estimate 0.51) (*t*-test, $t = 0.41$ and $p = 0.69$).

The sex ratio of the NSR females was slightly female biased (estimate = 0.57) (*t*-test, $t = 3.55$ and $p < 0.05$) and the crosses with infected females (SR × SR, NSR × SR and TSR × SR) produced a mean sex ratio that was significantly greater than 0.5 (estimate = 0.91) (Wilcoxon signed-ranks test = 1103.5 and $p < 0.0001$). Note that only the crosses with highly biased sex ratios (mean sex ratio > 0.85) and all-female progeny were those in which the female parent belonged to the infected strain (SR × SR, NSR × SR and TSR × SR). In these crosses, 50, 67 and 81% of matings produced all-female progeny, respectively. The sex ratios of the crosses involving SR females are shown in figure 1.

The egg hatch rates of the SR females with all-daughter progenies (mated to single SR, NSR and TSR males) were compared with those of the TSR females (mated to single SR and TSR males). If *Wolbachia* is killing male embryos, the hatch rates from the SR females should be approximately half that of infected females. The egg hatch rates of the SR females with all-female progeny were slightly higher than half those

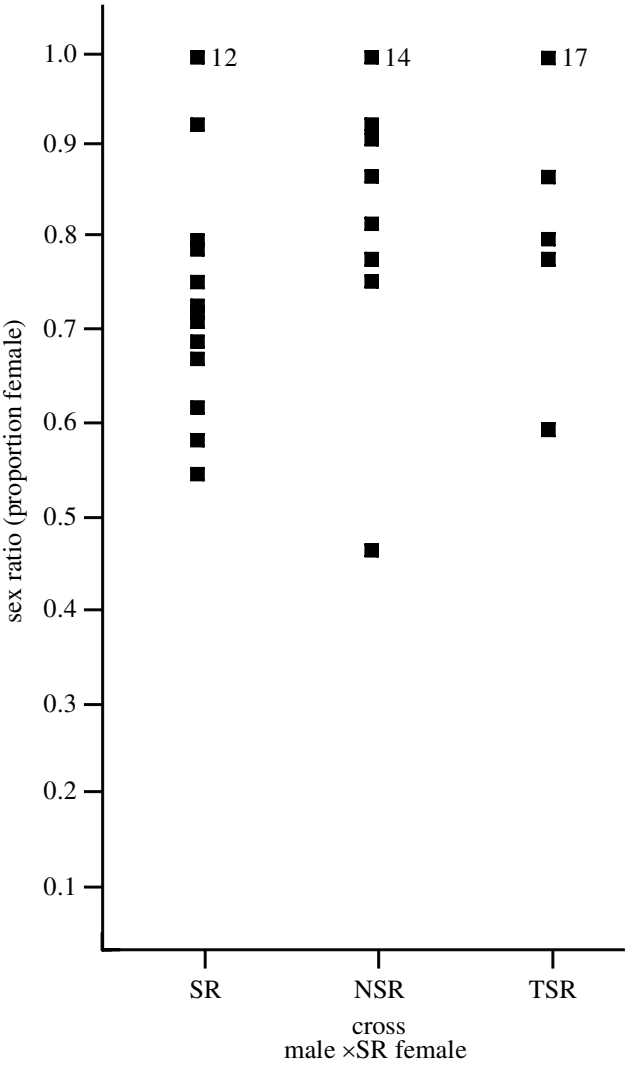

Figure 1. Sex ratio of the progeny of SR females singly mated to each of three types of males. The number of crosses with a sex ratio of unity is indicated next to a sex ratio of unity for each cross.

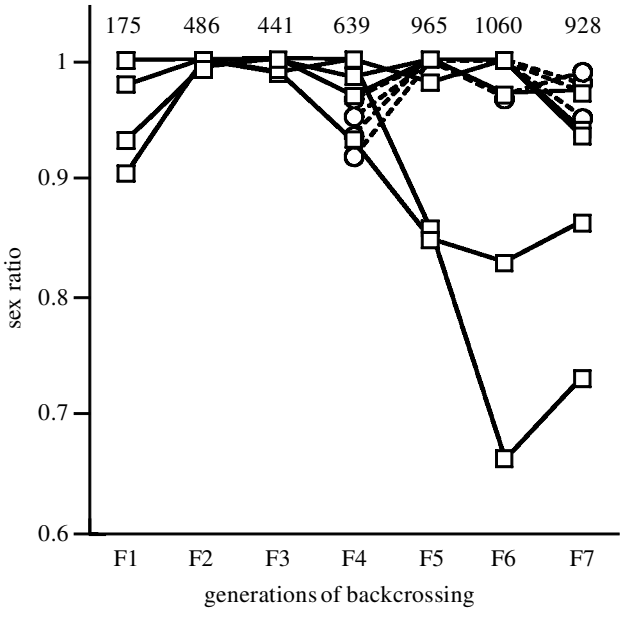

Figure 2. Sex ratio of the progeny of SR females over seven generations of repeated backcrossing to NSR males for five independent (squares) and five derived (circles; dashed lines) lines. The sample sizes are given as the total number of pupae sexed in all lines per generation.

observed for TSR females mated to SR males (*t*-test = 2.15, d.f. = 92 and $p = 0.04$). When considering both infected and uninfected females, the egg hatch rate was significantly higher in the first trial than in the second trial (mean difference = -0.07, $t = 0.71$, d.f. = 170 and $p = 0.0003$).

Further support for male killing comes from an examination of incomplete transmission. None of the few SR males carried *Wolbachia*, i.e. all were PCR negative. In addition, 11 out of the 77 SR female parents tested negative for *Wolbachia*. Most of these females produced unbiased sex ratios (average female bias in PCR-negative females = 0.65 and range = 0.39–1.0).

(b) *Backcross experiment*

Most of the backcrossed lines displayed extremely female-biased sex ratios (> 90% female) (figure 2) and maintained the bias until the end of the experiment (expected 99.2% NSR nuclear genes). However, two backcrossed lines showed a decline in the proportion of females from generation 4. This was probably the result of stochastic loss of infection in some of the parental females used in these lines. A diagnostic PCR indicated that the prevalence of *Wolbachia* was 75% (nine of the 12 females tested were infected) in both of the anomalous lines and 100% (12 of the 12 females tested were infected) in the highly female-biased control line. Further evidence that a change in the sex ratio is associated with loss of infection in these lines rather than a nuclear genetic effect comes from the maintenance of highly female-biased sex ratios in the two derived counterpart lines (figure 2).

(c) *DNA sequence analysis*

We did not find a single difference between the four sequenced genes obtained from the *T. madens* and *T. confusum Wolbachia*. Both the male-killing *Wolbachia* of *T. madens* and of the butterfly *Acraea encedon* belong to the same subgroup (Hurst *et al*. 1999). Male-killing *Wolbachia* are also found in the ladybird beetle *Adalia bipunctata* (Hurst *et al*. 1999). Phylogenetic analyses have been unable to distinguish whether the bacteria are monophyletic or whether male killing has repeatedly evolved (Hurst *et al*. 1999). DNA sequences for *wsp*, *ftsZ* and ITS are available in GenBank under accession numbers AF275546–AF275548 I was unable to find the *groE* intergenic sequences in the notes of the deceased first author.

(d) *Infertility of virgin females and paternal genetic contribution*

The possibility of parthenogenetic reproduction was ruled out because the 30 virgin SR females maintained in individual vials did not produce any progeny over a period of six months. The vials were checked periodically for the presence of progeny; however, we did not sift the vials to check for eggs. Unmated females of *T. confusum* are known to lay a few eggs that never hatch. Finally,

using the species-diagnostic difference in ITS2, the progeny resulting from crosses between *T. madens* females and *T. audax* males were found to have both paternal and maternal genes.

4. DISCUSSION

The reversion of the sex ratio to 1:1 levels following antibiotic treatment suggests *Wolbachia* as the causative agent of the highly female-biased sex ratio. Several lines of evidence implicate male killing as the mechanism of sex-ratio distortion.

Wolbachia infection is transmitted from mother to progeny, regardless of sex. The sex-biased pattern of inheritance we observed is consistent with male lethality. The few males produced by infected SR females were most likely those that escaped lethality due to a lack of *Wolbachia*. Incomplete transmission has been reported for *Wolbachia* (Hoffman *et al*. 1990) as well as other male-killing infections (Ebbert 1993). Male-lethal infections involving death during embryogenesis are often associated with egg hatch rates that are approximately half or lower than normal (Hurst *et al*. 1992, 1996; Ebbert 1993). The reversion to a sex ratio of unity and increased egg hatch rates following antibiotic treatment not only provide strong support for male killing but also argue against meiotic drive as an alternative explanation for the sex-ratio distortion. The persistence of female bias after multiple generations of repeated backcrossing further indicates that a cytoplasmically inherited micro-organism is involved in the sex-ratio distortion observed in *T. madens*.

The data reported here suggest that the CI-inducing and male-killing *Wolbachia* strains are very closely related despite being associated with different phenotypes in these host species. *Wolbachia* causes complete CI in the flour beetle *T. confusum* (Wade & Stevens 1985). Crosses between infected males and uninfected females are sterile. Host–symbiont phylogenetic concordance does not necessarily imply transmission by descent. *T. madens* and *T. confusum* belong to two distinct species groups that diverged in the Upper Cretaceous period *ca*. 60 million years (Myr) before present (Hinton 1948). The estimated silent substitution rate for bacterial protein-coding regions is 0.7–0.8% Myr^{-1} (Ochman & Wilson 1987). Considering their identical sequences, it is unlikely that the *Wolbachia* strains represent infection by a common ancestor. The identical sequence across several genes indicates that horizontal transmission rather than common infection from the time of co-speciation is likely. Such horizontal (interspecies) transmission of *Wolbachia*, which has been used to explain the incongruencies in *Wolbachia*–arthropod phylogenies (O'Neill *et al*. 1992), is an alternative explanation. *T. confusum* and *T. madens* are nearly cosmopolitan today and can coexist in stored grain piles. No infected *T. madens* males have ever been found. Thus, it was not possible to determine whether the male-killing strain of *Wolbachia* is also capable of inducing CI in this species. Transfer of infection by microinjection may help determine whether these different reproductive phenotypes are related to the genetics of these bacterial strains or the 'host environment' in which they occur, or are the result of an interaction between host and symbiont effects.

The results presented here indicate that *Wolbachia* infection in *T. madens* is associated with embryonic male killing, a phenomenon that is known to occur in only two other insect hosts (Hurst *et al*. 1999). Unlike CI, which has been associated exclusively with *Wolbachia* infections, male killing is caused by micro-organisms in three distinct and diverse bacterial taxa (Hurst 1991; Ebbert 1993; Werren *et al*. 1994; Hurst *et al*. 1997). In fact, it has been postulated that there are no phylogenetic constraints to the evolution of this trait (Hurst *et al*. 1997); however, several factors are likely to affect its evolution, including the host population structure, transmission efficiency and local competition. Apart from the estimates of transmission efficiency (85%) in this study, relevant information is not available for *T. madens*.

The sequence data indicate that the male-killing *Wolbachia* of *T. madens* belong to the same subgroup as the male-killing *Wolbachia* of the butterfly *Acraea encedon* (Hurst *et al*. 1999). However, at present, phylogenetic analysis is unable to resolve whether these bacteria are monophyletic with male-killing *Wolbachia* in the ladybird beetle *Adalia bipunctata* or if male-killing has repeatedly evolved.

Although other rickettsial bacteria have been found to kill males (Werren *et al*. 1994; Roberts *et al*. 1997), the discovery of male-killing *Wolbachia* provides additional support for the hypothesis that male killing should evolve easily and expands the repertoire of reproductive alterations caused by these bacteria. Our finding suggests that host–symbiont interaction effects may play an important role in the induction of *Wolbachia* reproductive phenotypes.

This paper is dedicated to Rob's parents Luzia and Juarez Fialho, his sister Monica and M. Upton. Sincere thanks go out to Rob's many friends and colleagues who helped with this project, including C. W. Kilpatrick, M. Ebbert, R. Giordano, J. Schall and S. Perkins. Special thanks are also given to M. Upton. Insect stocks were kindly provided by N. White, A. Sokoloff, D. Ugarkovic and E. Petitpierre. An anonymous reviewer made thoughtful comments on the manuscript. This research was supported by US National Science Foundation grants IBN-9724037 and DEB-9407217 to L.S.

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