Male-Killing Wolbachia in Drosophila: A Temperature-Sensitive Trait With a Threshold Bacterial Density

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ABSTRACT

Inherited microorganisms that disturb the reproduction of their host have been characterized from a number of host taxa. To understand the general principles underlying the genetic and mechanistic basis of interactions, study of different agents in model host species is required. To this end, the nature and genetics of the maternally inherited sex-ratio trait of Drosophila bifasciata were investigated. Successful curing of affected lines with antibiotics demonstrated this trait was associated with the presence of a bacterium, and molecular systematic analysis demonstrated an association between the presence of the trait and infection with an A group Wolbachia. The penetrance and heritability of the trait did not vary with maternal age. Exposure to elevated temperatures did reduce trait penetrance but did not affect heritability. Examination of the effect of temperature on bacterial density in eggs revealed a decrease in bacterial density following exposure of the parent to elevated temperature, consistent with the hypothesis that male killing in D. bifasciata requires a threshold density of Wolbachia within eggs. The male offspring produced following exposure to elevated temperatures were infected with Wolbachia on emergence as adults. Crossing studies demonstrated a weak cytoplasmic incompatibility phenotype exhibited by Wolbachia in these males. The results are discussed with respect to the incidence of male killing within the clad Wolbachia, the general nature of Wolbachia-host interactions, and the prospects for using this association to investigate the mechanism of male killing.

MANY species of invertebrates are host to inherited microorganisms. These genetic elements may be either beneficial (as is the case for Buchnera and its aphid host) or parasitic (such as the sex-ratio distorting microorganisms of many insects). There has been a growing appreciation that these “extra” genetic elements may be of great importance in host biology and evolution. Beneficial symbionts are important in the nutritional ecology of the hosts (DOUGLAS 1989). Parasitic ones may be important as agents driving evolutionary change (HURST et al. 1996), such as the evolution of the sex determination system of the host (RIGAUD and JUCHAULT 1993). Additionally, divergence of allopatric populations in nuclear genes that have coevolved with symbionts (EHMAN et al. 1990), or divergence of allopatric strains with respect to the strain of cytoplasmic incompatibility inducing Wolbachia (BREEWER and WERREN 1990), may be important in speciation.

There is a growing need to develop model systems in which to investigate the genetic and mechanistic bases of the interactions between inherited microorganisms and their hosts. Study of a variety of infections within model host species such as Drosophila is required to examine the generality or specificity of any observations. To date, two main interactions have been studied within this genus. First, there is the interaction between Drosophila and Wolbachia that causes cytoplasmic incompatibility (TURELLI and HOFFMAN 1991; BRESSAC and ROUSSET 1993; BRAIG et al. 1994; GIORDANO et al. 1995; BOURTZIS et al. 1996, 1998; CALLANI et al. 1996, 1997; DOBSON et al. 1999). Second, there is the association between members of the willistoni group and the male-killing bacterium Spiroplasma poulsonii (MALOGOLOWINKIN 1958, 1959; MALOGOLOWINKIN et al. 1959; POULSON and SAKAGUCHI 1961; SAKAGUCHI and POULSON 1961; OISHI 1971; HACKETT et al. 1986; EBERT 1991; WILLIAMSON et al. 1999). Records of other male-killing infections in Drosophila are known (MAGNI 1953; CAVALCANTI et al. 1957; POULSON 1966), but the agents remain uncharacterized and the systems unstudied.

We therefore examined the male-killing trait of Drosophila bifasciata. The male-killing trait born by 5–7% of female D. bifasciata was one of the first inherited infections to be observed (MAGNI 1953). It is characterized by the production of all, or nearly all, female broods associated with low egg hatch rates, with the condition being inherited by 99% of daughters. In the single host line investigated, IKEDA (1970) found a small effect of infection on lifetime reproductive success. However, the etiology of this trait is uncertain, as are nearly all aspects
of the interaction between the agent and the host. Microinjection experiments showed the trait could be transferred artificially between lines, confirming a microbial basis to the trait (Leventhal 1968). No organisms being readily visible within the hemolymph of sex-ratio matrilines, the trait was considered most likely to be caused by an inherited virus, although infection with intracellular bacteria could not be ruled out in this system (Leventhal 1968).

Studies of male-killing infections since this time have typically revealed them to be curable with antibiotics (Hurst et al. 1997b). Molecular systematic analysis has implicated bacteria from four different divisions as the causal agents of male killing (Werren et al. 1986, 1994; Hurst et al. 1997a, 1999a,b; Williamson et al. 1999). This diversity casts doubt upon the original conjecture that the trait in D. bifasciata was likely to be viral.

In this study, we first examined the cause of male killing in D. bifasciata. We showed it is susceptible to antibiotics and is thus of bacterial and not viral origin. We subsequently showed that it is associated with the presence of Wolbachia. We determined the phylogenetic position of this strain within the genus Wolbachia to ascertain the nearest relatives of this male killing strain. We then investigated aspects of the interaction between the male-killing Wolbachia and its host. First, we examined the effect of age and environmental temperature on the condition. These variables are known to affect the penetrance of Wolbachia-induced cytoplasmic incompatibility (Hoffmann et al. 1986; Turelli and Hoffmann 1995), and temperature has been observed to affect sex ratio in previous D. bifasciata lines (Magni 1953). Second, we tested the hypothesis that male killing by Wolbachia is a threshold effect, requiring a critical bacterial density. Studies of cytoplasmic incompatibility-inducing Wolbachia have suggested that Wolbachia density may be associated with the strength of the phenotype produced (Boyle et al. 1993; Breeuwer and Werren 1993; Bressac and Roussel 1993; Solignac et al. 1994; Sinkins et al. 1995; Bourtzis et al. 1996; Perrot-Minnot and Werren 1999), and this hypothesis has also been raised (but not tested) for male killing in D. bifasciata (Magni 1957). We therefore used microscopy to ascertain whether exposure to high temperatures reduced the density of bacteria as well as induced the survival of male offspring. Last, we investigated whether male D. bifasciata that have inherited the bacterium but survive bear an infection capable of inducing the phenotype of cytoplasmic incompatibility when the fly host has developed to sexual maturity.

MATERIALS AND METHODS

Source of male-killing lines: Eighty wild female D. bifasciata were collected by Dr. Katsura Beppu from Shiga-Kogen, Nagano, Japan, in July 1998. These were brought into the laboratory, where they were maintained individually in vials at 18°C on a modified cornmeal-agar diet (70 g sucrose, 60 g maize meal, 60 g wheat bran, 15 g yeast extract, 8 g agar, 4 g nipapin in a total volume of 1 liter). These were checked for the presence of the male-killing trait through scoring of the sex ratio produced by each of these females. Of the 80 lines, 5 produced a strong female bias. Individual females from the sex-ratio lines were outcrossed to maintain the trait, and this has now been stably maintained through >10 generations in the laboratory, with only a low level of reversion to production of a normal sex ratio. In line with the observations of Magni, the female sex ratio was associated with high embryonic mortality (egg hatch rate = 39.9%, average from 54 crosses).

Cause of trait: We tested the hypothesis that the cause of this trait was bacterial through attempting to cure the trait by administration of antibiotics. To this end, individual female flies of similar age showing the sex-ratio trait were allowed to lay eggs on either normal medium or normal medium supplemented with antibiotic (0.025% w/v of either tetracycline or rifampin). The sex ratios produced by progeny emerging from the normal and antibiotic-supplemented media were then assessed. As a control against endogenous effects of antibiotics on the sex ratio, “normal” flies were also allowed to oviposit on standard and antibiotic-supplemented medium, and the sex ratios produced by the emerging females were monitored.

We tested for the presence of Wolbachia in our samples using a PCR-based assay. Tests were conducted for the presence of Wolbachia 16S rDNA. In brief, genomic DNA was purified via phenol-chloroform extraction of proteinase K-digested individual fly abdomen macerates from each of the five male-killing lines, from two lines cured through administration of antibiotics, from four individuals from a line that had lost the sex-ratio trait through inefficient transmission, and from five uninfected lines. This DNA was then used as template in a PCR reaction using the primer pairs 16SAf/16SBf and 16SAr/16SBr of Werren et al. (1995) that amplify a 259-bp portion of the 16S rDNA of A and B group Wolbachia, respectively. Products were run out on a 1% agarose gel.

To test for the presence of other bacteria in the infected lines, the full 16S rDNA of two specimens was amplified using the primers that amplify 16S rDNA from a broad spectrum of eubacteria (Weisburg et al. 1991). The products from these two specimens were electrophoresed and purified using the QIAGEN (Chatsworth, CA) gel-purification kit and subsequently cloned into pGEM T-vector. Escherichia coli DH5a was then transformed with the resulting plasmids, the E. coli being grown for <1 hr before plating to prevent duplication. White colonies were then picked and replated on new LB-carbenicillin plates to remove colonies from the uninfected PCR product and untransformed plasmid contaminating the original plate surface. These were checked for Wolbachia 16S rDNA inserts via PCR using primers 16SAf and 16SAr that specifically amplify the 16S rDNA of A group Wolbachia (see above).

To assess the phylogenetic position of the Wolbachia, the sequence of the wsp gene was obtained. In brief, a portion of the wsp gene was amplified from two specimens using primer pair 81F/691R (Zhou et al. 1998). This was gel purified and then directly sequenced using the original primer pair. The sequence obtained was aligned to almost all available and unique wsp sequences from A group Wolbachia and two outgroup taxa. The alignment was produced taking into account the coding structure of the genes. Two regions could not be aligned with absolute certainty and were thus excluded from further analysis (positions 217–255 and 525–585); the alignment, including information on sequence accession numbers, is available from the EMBL alignment database under accession no. D841548. Phylogenetic analysis was then performed on the aligned sequences with the program PAUP*, written
by D. L. Swofford, using the likelihood criterion. For initial tree estimation, we employed different substitution models such as modifications of the Hasegawa-Kishino-Yano (Hasegawa et al. 1985) or the general time-reversible substitution model (Lanave et al. 1984). These modifications consisted of the consideration of gamma-distributed rate heterogeneity across sites and, in addition, a proportion of invariant positions. All models yielded identical tree topologies. Their likelihood scores could thus be compared using likelihood-ratio statistics (Yang et al. 1995). On the basis of this approach, the general time-reversible model with rate heterogeneity was identified to provide the best description of the data (detailed results not shown). This model was subsequently used for nonparametric bootstrapping (Felsenstein 1985) to assess the robustness of the inferred topologies. For further details on the methods used, see Schulenburg et al. (2000).

Effect of age and temperature on the male-killing phenotype: Age effects and temperature effects on the male-killing phenotype were examined. For age effects, female flies from male-killing lines were kept virgin for 10 days, then placed with males and allowed to oviposit on standard cornmeal agar at 18° in groups of 10, with vials being changed every 2 days over a period of 40 days. Two different male-killer lines were investigated, and three replicates performed per line.

To investigate the effect of maternal age on the rate of inheritance of the trait by F1 females, and the penetrance in these females, 17 daughters conceived 9–12 days post-first maternal mating, 17 conceived 21–24 days post-first mating, and 16 conceived 29–36 days postmating were crossed 10 days following emergence as adult, and the sex ratios produced were monitored over 16 days.

To examine temperature effects, a second trial was simultaneously set up as for the age experiment (two different male-killer lines, three replicates per line), but the flies were transferred to a 26° incubator for oviposition 8 days subsequent to the start of the experiment and then allowed to oviposit as before for a further 28 days. After this time, they were returned to 18° and monitored as before. All progeny were reared at 18°.

To investigate the effect of progressive maternal exposure to elevated temperature on the sex ratio produced by F1 female progeny, individual F1 female flies produced at different maternal ages, following different levels of maternal exposure to elevated temperature, were crossed as in the age experiment, and the sex ratio produced was monitored. The flies produced 9–12 days post-first mating represent flies whose parents experienced 0–4 days of exposure to elevated temperature, those oviposited 21–24 days post-first mating represent flies whose parents experienced 12–16 days of elevated temperatures, and those oviposited 29–36 days post-mating represent flies whose parents experienced 20–28 days of elevated temperature.

Association of elevated temperature with bacterial density in eggs: To investigate whether the effect of temperature was associated with reduced bacterial load of eggs, adult female D. bifasciata bearing the male-killing trait were obtained from a single vial and allowed to age for 10 days at 18° before being mated. Five females were then either placed at 26° for 7–10 days or at 18° for a similar period (no exposure to elevated temperatures).

Following this treatment, these female flies were allowed to oviposit, and the eggs were collected within 3 hr of oviposition. These eggs were subsequently manually dechorionated, then fixed and devitellinized through shaking in a 1:1 mix of methanol and heptane. Eggs that had sunk into the methanol phase (those successfully devitellinized) were serially rehydrated through 70% methanol in phosphate-buffered saline (PBS), 50% methanol/PBS, 30% methanol/PBS, and finally two rinses in 100% PBS. These eggs were subsequently placed on a slide, stained with 0.5% 4′,6-diamidino-2-phenylindole in Vectashield (Vector Laboratories, Burlingame, CA), covered, and squashed. They were then examined under ultraviolet light at X1000 magnification by a viewer blind to knowledge of the treatment of the parental flies. The density of extranuclear (bacterial) staining in the quarter of the embryo at the pole end (the area of densest infection; Hadfield and Axton 1999) was assessed. Assessment was qualitative only (i.e., relative to other embryos contemporaneously prepared and viewed), such that the viewer blind ranked slides as to density by reference between slides. The identity of the slides was then revealed, and the hypothesis that density differed between heat-exposed and nonheat-exposed individuals was tested nonparametrically.

 Compatibility of infected males with females: Females from male-killing lines were crossed to males from a wild-type (uninfected) population and maintained at 26° as above. Vials were changed daily, and progeny were reared at 18° on either normal medium or medium bearing antibiotics. A subset of adult male and female progeny reared through normal medium were then tested for the presence of Wolbachia using the assay based on 16S rDNA above. Remaining males (antibiotic treated and those reared through normal medium) were removed, allowed to mature for 7 days, and the compatibility of these males was tested by crossing to either uninfected or infected virgin females. To this end, eggs were collected from the above crosses, counted onto an agar plate, and left to hatch over 48 hr, and the hatching success of the eggs was recorded. In each case, compatibility was tested in at least 30 independent crosses, with an aim of scoring the hatchability of 100 eggs per cross. Additionally, to check against effects of antibiotics on egg hatch rate, the compatibility of males from uninfected lines with infected and uninfected females was tested.

RESULTS

Cause of trait: Addition of rifampin to the larval medium resulted in a change to the sex ratio produced by flies from sex-ratio lines, with males surviving to eclosion in normal numbers. However, addition of rifampin to the larval medium of normal flies produced no change to the sex ratio they produced (Table 1). The sex ratio

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<tr>
<td>The sex ratio (proportion male) produced by D. bifasciata of different maternal infection status, following rearing through either normal medium or antibiotic (rifampin/tetracycline)-supplemented medium</td>
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<tr>
<td>Larval medium</td>
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<td>Rifampin-Infected Control</td>
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produced following administration of rifampin to females from sex-ratio lines was homogeneous ($\chi^2 = 5.9, 9$ d.f., not significant (NS)), and significantly differed from that produced by flies from sex-ratio lines reared through standard medium ($\chi^2 = 374; 1$ d.f.; $P < 0.0001$). No effect of rifampin upon the sex ratio produced by normal flies could be detected. In a total of 11 crosses involving normal females reared through antibiotic, the sex ratios produced were homogeneous ($\chi^2 = 14.5, 10$ d.f., NS) and did not differ from the control ($\chi^2 = 0.047, 1$ d.f., NS). We thus conclude that the reversion effect is due to the presence of the rifampin and is particular to sex-ratio lines. Following treatment with rifampin, flies from sex-ratio lines produced sex ratios indistinguishable from those produced from normal lines ($\chi^2 = 0.23, 1$ d.f., NS).

Tetracycline produced a similar cure. The sex ratio produced following administration of tetracycline to females from sex-ratio lines was homogeneous ($\chi^2 = 8.4, 8$ d.f., NS) and significantly differed from that produced by flies from sex-ratio lines reared through standard medium ($\chi^2 = 208; 1$ d.f.; $P < 0.0001$). Further, no effect of tetracycline was observed on the sex ratios produced by normal flies (pooled data $\chi^2 = 0.46, 1$ d.f., NS). We again therefore conclude that the effect is due to the presence of the antibiotics and is particular to sex-ratio lines.

PCR assay for A group Wolbachia presence showed a perfect association between presence of Wolbachia and the male-killing infection of D. bifasciata. While template derived from flies from each of the five male-killing lines produced amplification product, no product was observed when template was derived from flies from naturally uninfected flies, flies from revertant lines, nor flies from antibiotic-cured lines.

Testing of cloned 16S rDNA obtained from a PCR that amplified this region from bacteria across the spectrum of the eubacteria confirmed the association between presence of Wolbachia and the trait. Fifty clones were obtained from two infected lines, and 45 of these were positive for the presence of Wolbachia. This strongly suggests that Wolbachia is uniquely associated with the male-killing trait. The wsp sequence obtained for this trait was 563 bp in length (EMBL accession no. AJ271121). Maximum-likelihood tree estimation using wsp sequence data clearly confirmed the presence of this strain in the A clade (Figure 1). The phylogenetic relationships among the major A group lineages and also between some of the closely related taxa are indicated by bootstrap analysis, based on the maximum-likelihood criterion, to be ambiguous. This is most likely due to similar factors, such as lack of sufficient phylogenetic information or the presence of high levels of homoplasy, which have been previously identified to be associated with uncertainties in the inferred wsp gene tree of B group Wolbachia (cf. Schulenburg et al. 2000). Such ambiguities also refer to the inferred phylogenetic position of the male-killing strain from D. bifasciata, which is here shown to form a monophyletic group with the symbions from two hymenopterans, Nasonia vitripennis and Coccidoxenoides peregrinus, the tsetse flies.
Glossina morsitans morsitans and G. morsitans centralis, and D. auvaria (Figure 1). Nevertheless, its relationship with these and also other Wolbachia strains requires further investigation via an analysis of additional gene regions. The Wolbachia in N. vitripennis is known to cause cytoplasmic incompatibility (Breeuwer et al. 1992), while that of C. peregrinus induces parthenogenesis in its host. The D. bifasciata Wolbachia is the only one within the clade to exhibit male killing.

**Effect of age and temperature on the male-killing phenotype:** At 18°C, no effect of maternal age on the penetrance of the male-killing trait was observed (Table 2). Males were not produced at any point in time in any of the six replicates. Further, there was no obvious effect of maternal age at the point of egg production on the inheritance and expression of the trait by F1 progeny (Table 3).

A significant progressive effect of elevated temperature (26°C) on the penetrance of the sex trait was observed. When male-killer-infected flies were placed at 26°C, no adult males were produced in the initial 4-day period. Subsequent to this, there was generally an increase in the survival of males over the next 8 days, and male survival continued until the reproducing females were returned to 18°C, after which male survival was again rare (Table 4).

Despite the magnitude of the decrease in the penetrance of the trait following exposure of females to elevated temperature, the effect of protracted exposure of parents to elevated temperature on the sex ratio produced by F1 flies was very small (Table 5). All the F1 flies clearly manifested the male-killing trait irrespective of parental age, and any effect of exposure of parents to elevated temperatures on the sex ratio produced by progeny is weak.

Considering the amalgamated data from progeny produced 21–24 and 31–38 days post-first maternal mating, there was no evidence for a difference in the probability of producing an all-female brood between F1 females whose parents had been exposed to elevated temperatures and those maintained at 18°C (Fisher’s exact test: 0.1 < P < 0.2, NS).

**Association of elevated temperature with bacterial load of eggs:** Bacteria could clearly be seen in all the eggs laid by male-killer-infected individuals, irrespective of exposure of the female parents to elevated temperature. When the eggs from different females were examined and ranked as to bacterial density, blind with respect to the time of parental exposure to high temperatures, a clear effect of maternal exposure to elevated temperatures on bacterial density within her eggs could be discerned. All five slides bearing eggs from females maintained at 18°C were ranked as bearing a higher density of bacteria than the five slides bearing eggs from females maintained at 26°C (Mann-Whitney test: W = 15, N1 = N2 = 5; P < 0.025).

**Compatibility of infected males with females:** Five
male and four female adult progeny derived from an infected line following exposure of the mother to elevated temperatures were tested for Wolbachia presence in PCR assay, and all nine individuals were found to be infected. Evidence of incompatibility between infected males and uninfected females was found, the data suggesting an ~25% decline in the viability of eggs from uninfected females when mated to infected males (Table 6).

The data being proportionate and not unimodal, nonparametric analyses of differences between medians were performed, which showed a marginally significant difference between the fertility of eggs sired from infected, antibiotic-cured, and naturally uninfected males when mated to uninfected females (Kruskal-Wallis test: $H = 6.72; 2$ d.f.; $P = 0.035$, with heterogeneity deriving from infected male crosses, $z = 2.58$). This heterogeneity is not present when these males are mated to infected females (Kruskal-Wallis test: $H = 1.65; 2$ d.f.; $P > 0.40$, NS).

Dynamics of the trait: Past work on the dynamics of obligately vertically transmitted male-killing bacteria models prevalence ($p$) in terms of three main parameters:

i. The efficiency with which the bacterium is transmitted from mother to daughter ($a$, where $a = 1$ for perfect transmission).

ii. The direct physiological impact of the bacterium on female host performance, $c$ (where $c = 0$ for no effect, $c > 0$ for a deleterious effect, and $c < 0$ for a beneficial effect). This effect is a result of either disruption or augmentation of host physiological efficiency.

iii. The indirect effect of the bacterium on female host performance, resulting from the death of their sibling males, $b$ ($b = 1$ for no indirect effect of male killing; $b > 1$ where male death enhances sibling female survival). Indirect effects on survival arise from cannibalism by these females upon their dead brothers, reduced competition for resources suffered by females because of the death of their brothers, or from reduced rates of inbreeding suffered by females as a result of death of brothers.

If these three parameters are the only factors in the dynamics of the bacterium, then

$$p' = \frac{p.a.b.(1 - c)}{[1 - p + p.b.(1 - a + a.(1 - c))]}.$$ 

And equilibrium prevalence is

$$p^* = \frac{a.b.(1 - c) - 1}{b.(1 - a + a.(1 - c) - 1)}.$$ (1)

Using this equation, we can examine the relationship between $b$ and $a$ for given male-killer equilibrium prevalence with given direct effects of the bacterium on infection. Following this study, and Ikeda (1970), equilibrium prevalence is in the region of 6%. Following Ikeda (1970), there is probably a direct cost to infection, with infected females in the laboratory on average producing between 90 and 100% of the progeny of uninfecteds (this assumes the infection in Ikeda’s study is the same as that in ours). For three different levels of cost, we plot the benefit of male killing required to maintain Wolbachia of different transmission efficiency at current levels in the field (Figure 2).

This model assumes no reduced fitness of uninfected females due to incompatibility with infected males in the population. We can augment this model with incompatibility between infected males and uninfected females. Two extra parameters are introduced:

i. The penetrance of incompatibility, $k$ ($k = 1$ if all progeny deriving from an infected male $\times$ uninfected female die; $k < 1$ if only a proportion do).

ii. The rate at which infected males die from male killing, $d$ ($d = 1$ if all males die; $d < 1$ if some males survive).

Prevalence of Wolbachia in the female and male populations is approximated by $p_*$ and $p_0$:

$$p_*' = \frac{(1 - c).p.a.b}{p.h((1 - c).a + (1 - a)(1 - k.p_h)) + (1 - p)(1 - k.p_h)}$$

$$p_0' = \frac{(1 - c).p.a.b.1(1 - d)}{p.h((1 - c).a.(1 - d) + (1 - a)(1 - k.p_h)) + (1 - p)(1 - k.p_h)}.$$
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The penetrance of male killing was analyzed across different values of the transmission efficiency for bacteria with different transmission efficiencies. In each case, the equilibrium prevalence in the absence of incompatibility (i.e., $d = 1$, fully penetrant male killing) is 6%, and this value is obtained for differing levels of transmission efficiency by manipulating the level of indirect benefit to male killing, $b$, using Equation 1 above for different values of $a$.

The effect of incompatibility on equilibrium prevalence is greatest where transmission efficiency is high and the penetrance of male killing is low (Figure 3). That is to say, the effect of incompatibility is greatest when sons from infected females are generated by failure of an infection to kill their male host, rather than the bacterium failing to transmit to male progeny. This makes intuitive sense; the proportion of males in the population that are infected (i.e., those that can generate incompatibility) is greatest when male-killing efficiency is low but transmission efficiency high, and it is only then that prevalence is significantly affected.

Reconciling the predictions of the simulation (male production must be mainly associated with inefficient transmission rather than incomplete penetrance) with the field situation in $D. bifasciata$ will represent a test of our understanding of the system. The observation of low equilibrium prevalence in natural populations leads to the prediction that infected males will be found at only a low rate in field collections.

DISCUSSION

The finding of a male-killing Wolbachia within another host insect species, and the observation that this strain derives from the A clade of Wolbachia, adds weight to the opinion that male-killing Wolbachia are common in insects. In the first place, the observation that these infections exist undetected at low prevalence suggests they will be found commonly in the future. In the second place, the observation that the male killer in $D. bifasciata$ is in the A group of Wolbachia, rather than the B group as is the case for the $A. bipunctata$ and $A. encedon$ male killers (Hurst et al. 1999a), suggests there are no phylogenetic bars to the incidence of the male killing phenotype within the genus Wolbachia. This combined with the host range of male killers so far observed suggests that infections bearing a "cost-free" Wolbachia with high transmission efficiency are likely to have high reproductive success 2% higher than that of uninfected females. If the Wolbachia has a direct cost, as suggested by Ikeda (1970), then the benefit deriving from male killing increases to 10–15%. These "benefits" must derive from the effect of death of male hosts on the survival of the host insect species, and so the effect of male killing increases to 10–15%. These "benefits" must derive from the effect of death of male hosts on the survival of the host insect species.

Three replicates were performed for each strain. Sex ratio is given as proportion male, with sample size in parentheses. —, no data.

### TABLE 4

The sex ratio produced by adults of two lines of $D. bifasciata$ infected with male-killing Wolbachia over time since start of reproduction, with adults being placed at 26°C from day 9 through day 37.

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<tbody>
<tr>
<td>37-1</td>
<td>0 (8)</td>
<td>0 (194)</td>
<td>0 (42)</td>
<td>0.06 (69)</td>
<td>0.09 (23)</td>
<td>0 (2)</td>
<td>—</td>
<td>—</td>
<td>0 (1)</td>
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<td>—</td>
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<tr>
<td>37-2</td>
<td>0.03 (40)</td>
<td>0 (96)</td>
<td>0 (48)</td>
<td>0.07 (44)</td>
<td>0.24 (38)</td>
<td>0.33 (15)</td>
<td>—</td>
<td>—</td>
<td>—</td>
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<tr>
<td>37-3</td>
<td>—</td>
<td>0 (17)</td>
<td>0 (10)</td>
<td>0.42 (33)</td>
<td>0.45 (29)</td>
<td>0.35 (17)</td>
<td>0.64 (22)</td>
<td>0.34 (38)</td>
<td>0.58 (24)</td>
<td>0 (35)</td>
<td>0.17 (125)</td>
</tr>
<tr>
<td>58-2</td>
<td>—</td>
<td>—</td>
<td>0 (59)</td>
<td>0.15 (153)</td>
<td>0.26 (159)</td>
<td>0.25 (32)</td>
<td>0.38 (13)</td>
<td>0.42 (48)</td>
<td>0.50 (2)</td>
<td>0 (4)</td>
<td>0 (19)</td>
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<tr>
<td>58-3</td>
<td>0 (5)</td>
<td>0 (90)</td>
<td>0 (95)</td>
<td>0.10 (62)</td>
<td>0.11 (54)</td>
<td>0.39 (31)</td>
<td>0.18 (17)</td>
<td>0.09 (22)</td>
<td>0.12 (17)</td>
<td>0 (33)</td>
<td>0 (89)</td>
</tr>
</tbody>
</table>

The sex ratio produced by adults of two lines of $D. bifasciata$ infected with male-killing Wolbachia over time since start of reproduction, with adults being placed at 26°C from day 9 through day 37.
of their female siblings. In Drosophila, there are two potential ways in which this may occur. First, females may suffer from mating with sibling males in the wild, and male killing may prevent this from occurring. Second, siblings may compete for food, and male death may lessen the intensity of this competition. It is not possible at present to tell the relative importance of these factors. With respect to sibling competition, it is notable that D. bifasciata lays its eggs in sap fluxes, an environment that may be limiting in terms of resources (Kimura et al. 1977).

Our study found no effect of age on the penetrance of the trait, but a strong effect of temperature on this trait. This differs subtly from Wolbachia-caused cytoplasmic incompatibility, where both age and temperature effects are observed. The greater sensitivity of cytoplasmic incompatibility (CI) to host age may derive from the fact that CI is an effect that derives from the effect of Wolbachia on males, and more particularly is associated with a modification of sperm produced by Wolbachia, rather than the presence of Wolbachia itself. In male killing, the bacterium remains alive within the zygote and, it is therefore presumed, has a greater time span in which to effect its phenotype.

Our observation of an association between decreased bacterial density and decreased penetrance of the trait following exposure to elevated temperature is in accord with observations made for cytoplasmic incompatibility. While we cannot rule out the hypothesis that the association is noncausal (temperature could affect Wolbachia in a generally deleterious manner that feeds back onto both bacterial density and the ability of the bacterium to kill males), the data are clearly consistent with the requirement of a threshold density of Wolbachia within embryos to affect the phenotype. The requirement for a threshold density may reflect either a requirement for a given titer of a chemical produced by Wolbachia to effect embryonic death or sufficient bacterial numbers to produce pathology. Detailed analysis, however, awaits formal test of the hypothesis that male death requires high bacterial density. This will require examination of the effect of artificial selection on bacterial density upon the sex ratio produced by infected flies.

Beyond this, the data on temperature and bacterial density also show that the heritability of the temperature effect is low. Daughters that were produced following exposure to elevated temperatures themselves laid near

### TABLE 5

<table>
<thead>
<tr>
<th>Maternal age at laying of egg (days after first reproduction)</th>
<th>Period of exposure of mother to elevated temperature (days)</th>
<th>Females producing all female broods</th>
<th>Females producing males (sex ratio produced)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9–13</td>
<td>0–4</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>22–25</td>
<td>12–26</td>
<td>15</td>
<td>1 (2 males/129 progeny)</td>
</tr>
<tr>
<td>30–37</td>
<td>20–28</td>
<td>10</td>
<td>2 (1 male/95 progeny; 2 males/147 progeny)</td>
</tr>
</tbody>
</table>

The sex ratio produced by F1 females from sex-ratio lines following exposure of the maternal female to elevated temperatures for differing periods of time.

### TABLE 6

<table>
<thead>
<tr>
<th>Male</th>
<th>Female</th>
<th>Infected</th>
<th>Antibiotic cured</th>
<th>Uninfected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infected</td>
<td>0.346 (44)</td>
<td>0.395 (23)</td>
<td>0.399 (34)</td>
<td></td>
</tr>
<tr>
<td>Uninfected</td>
<td>0.492 (45)</td>
<td>0.663 (27)</td>
<td>0.666 (30)</td>
<td></td>
</tr>
</tbody>
</table>

The mean egg hatch rate produced by females of different infection status mated to infected males produced by maternal exposure to elevated temperatures, antibiotic-cured sibs of these males, and uninfected males.

Hatch rate given as proportion hatched; number of crosses examined in parentheses.

**Figure 2**—The indirect benefit of male killing (b) required to maintain a male-killing bacterium at 6% equilibrium presence across a range of transmission efficiencies for three different levels of direct (physiological) cost to infection. +, c = 0.1; ○, c = 0.05; □, c = 0. The model assumes no incompatibility.
Wolbachia on emergence as adults. This result is compatible with the inheritance studies showing that adult female sibs of these males exhibit the male-killing trait. These males exhibited some cytoplasmic incompatibility in crosses with uninfected females (~25% drop in egg hatch rate). Analysis of the dynamics of strains causing joint male killing and CI showed that incompatibility at this level had only a minor effect on equilibrium prevalence if the penetrance of the male-killing phenotype was high. This is because the majority of males in the population then derive from either uninfected females or from infected females via inefficient transmission. However, when the penetrance of male killing is low compared to bacterial transmission efficiency, appreciable numbers of infected males arise, and significant effects on prevalence would be observed.

We can imagine several possible explanations for the lack of a strong CI phenotype. One possibility is that both host and Wolbachia strains are competent to produce CI, but the effect of heat treatment used to generate the males is such as to reduce the ability of the Wolbachia to induce CI. Effects of temperature on penetrance of CI are well known. Alternatively, the data may reflect the true level of CI in the interaction. In this case, there are two possibilities. First, \emph{D. bifasciata} may be a host in which CI by Wolbachia is generally weak. This would be comparable to the interaction between \emph{D. melanogaster} and Wolbachia, where strains naturally present in \emph{D. melanogaster} do not produce CI in this species, but will in \emph{D. simulans} (Boyle et al. 1993). Second, the Wolbachia present in \emph{D. bifasciata} may not be competent to produce CI, although \emph{D. bifasciata} itself is a competent host. Wolbachia of weak or no effect in competent hosts are known from \emph{D. simulans} (Gior-dano et al. 1995; Hoffmann et al. 1996; Bourtzis et al. 1998; Mercot and Poinso). 

In addition to the above, the finding of a male-killing Wolbachia in Drosophila will open up research into the mechanism of male killing by Wolbachia. Using the Drosophila model system, it should be possible to examine the cues affecting male killing. It will also allow comparative studies into the mechanism of male killing by different bacteria. Beyond this, comparison of the mechanism of Wolbachia male killing to that of cytoplasmic incompatibility and parthenogenesis induction can be undertaken. Cytoplasmic incompatibility and parthenogenesis induction both involve manipulation of host chromosomes (Breeuwer and Werren 1990; Callaini et al. 1994; Stouthamer and Kazmer 1994). Is the death of male embryos brought about by Wolbachia similarly derived?

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