Evolution, 59(4), 2005, pp. 838–848

EXPRESSION AND MODULATION OF EMBRYONIC MALE-KILLING IN *DROSOPHILA INNUBILA*: OPPORTUNITIES FOR MULTILEVEL SELECTION

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Abstract.—Organisms and the symbionts they harbor may experience opposing forces of selection. In particular, the contrasting inheritance patterns of maternally transmitted symbionts and their host's nuclear genes can engender conflict among organizational levels over the optimal host offspring sex ratio. This study uses a male-killing *Wolbachia* endosymbiont and its host *Drosophila innubila* to experimentally address the potential for multilevel selection in a host-symbiont system. We show that bacterial density can vary among infected females, and that females with a higher density have a more female-biased offspring sex ratio. Furthermore, bacterial density is an epigenetic and heritable trait: females with a low bacterial load have daughters with a lower-than-average bacterial density, whose offspring then experience less severe male-killing. For infected sons, the probability of embryonic mortality increases with the bacterial density in their mothers. The frequency distribution of *Wolbachia* density among individual *D. innubila* females, and therefore the dynamics of infection within populations of these flies, results both from processes affecting the growth and regulation of bacterial populations within cytoplasmic lineages and from selection among cytoplasmic lineages that vary in bacterial density. Estimates of effective population size of *Wolbachia* within cytoplasmic lineages and of *D. innubila* at the host population level suggest that selection among cytoplasmic lineages is likely to overwhelm the results of selection within lineages.

Key words.—Antibiotic curing, bacterial density, quantitative polymerase chain reaction, sex-ratio distortion, *Wolbachia*.

Received August 26, 2004. Accepted January 11, 2005.

While selection on most traits acts primarily on the basis of variation in individual fitness, sex ratio is subject to selection acting on multiple levels. Fisherian selection on autosomes favors equal investment in each sex (Fisher 1930). In contrast, maternally inherited endosymbionts are selected on the basis of their effects on the number and fitness of female offspring and thus may be selected to cause a female-biased sex ratio (Stouthamer et al. 1999). Two broad classes of maternally inherited factors spread by distorting the offspring sex ratio of their host: mitochondrial haplotypes that cause cytoplasmic male sterility (CMS) in various plants (reviewed in Budar and Pelletier 2001) and endosymbiotic microbes that cause male-killing, feminization, or parthenogenesis in their arthropod host species (reviewed in Stouthamer et al. 1999).

Male-killing has been documented in numerous groups of endosymbiotic microbes, and the known insect hosts include a variety of beetles, moths, butterflies, and flies (reviewed in Hurst and Jiggins 2000). Both theoretical (Werren 1987; Hurst 1991) and experimental (Jaenike et al. 2003) studies have shown that if the death of host males frees up resources for their female siblings or reduces the likelihood of inbreeding depression, then infected cytoplasmic lineages can leave more descendants than uninfected ones. The success of a cytoplasmic lineage is likely to depend on infection status and, given infection, the severity of the infection. In turn, the severity of the infection is expected to be a function of the intrinsic virulence of the endosymbiont strain and the intrahost bacterial density, as both male-killing penetrance and other components of fitness may respond to the titers of certain bacterial products in a dose-dependent fashion. The density of bacteria within cytoplasmic lineages is likely to depend in part on selection among variants within bacterial populations resident within these lineages. Consequently, selective processes acting below the level of host individuals may influence both endosymbiont-mediated effects on individual host fitness (i.e., the fraction of male offspring that are killed as a result of the mother's infection status) and population-level sex ratios.

The death of sons as well as any other pleiotropic effects on fitness caused by male-killing endosymbionts will impose strong selection on infected hosts to resist their effects. Furthermore, because male-killers cause the host species to have female-biased sex ratios, Fisherian selection imposes additional selection on the host population to evolve resistance to such male-killers. Thus, selection at the individual level is expected to favor suppression of male-killers.

For host species that are geographically distributed as metapopulations, large-scale population dynamics are driven by the extinction-colonization dynamics of local populations (Hanski 1998), which can be influenced by local sex ratios. For instance, gynodioecious populations of plants with a female bias caused by CMS factors may experience more rapid population growth and send out more colonizing seed propagules than populations with more even sex ratios (Couvet et al. 1998). Empirical studies of gynodioecious plants have documented significant variation among local populations in both sex ratio and the frequency of mitochondrial haplotypes associated with cytoplasmic male sterility, consistent with a metapopulation view of these species (e.g., Manicacci et al. 1996; McCauley et al. 2000; Taylor et al. 2001). However, population-level selection might also act against sex-ratio distorters. Specifically, the requirement for both males and females in gonochoristic species can lead to population-level

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selection if sex ratios become so biased that some populations go extinct (Wallace 1968; Hatcher et al. 1999). Thus, selection among populations in a metapopulation context may act for or against the spread of male killers.

Male-killing has evolved in a diversity of endosymbiotic groups; even within groups, such as *Wolbachia*, there have been multiple, independent origins of this trait (Hurst and Jiggins 2000; Jiggins et al. 2002; Dyer and Jaenike 2004). Given the diversity of endosymbionts capable of causing male-killing and of insect host species susceptible to such effects, such male-killing may impose selection at the level of whole communities. This may selectively eliminate species that are developmentally susceptible to manipulation by male killers and ecologically propitious to their spread. Overall, then, the evolutionary dynamics of male-killing endosymbionts may depend on the integration of selective processes occurring below, at, and above the level of host individuals, resulting in dynamics that are more complex than those of adaptive traits governed by host nuclear genes.

In this study we focus on a male-killing factor present in natural populations of *Drosophila innubila*, a mycophagous member of the *quinaria* group within the subgenus *Drosophila*. It inhabits the sky islands, or mid- to high-elevation mesic woodlands and forests that are surrounded by much more xeric habitats in Arizona, New Mexico, and the Mexican Sierra Madre (Patterson and Wagner 1943; Heed et al. 1962). While the geographical distribution of this species may make it susceptible to endosymbiont-mediated colonization-extinction dynamics, in this paper we focus on lower-level processes, first characterizing the mode of inheritance, expression, and causative agent responsible for the male-killing effect. We then describe heritable, quantitative variation in the penetrance of this effect (i.e., the fraction of males killed) and show that variation among flies in bacterial density underlies this variation in penetrance. Our results suggest that multilevel selection is operative in this host-symbiont association.

Materials and Methods

Fly Strains

The *D. innubila* used in these studies were collected in September 2000 at the Southwest Research Station (1660-m elevation) in the Chiricahua Mountains near Portal, Arizona. Flies were collected in riparian habitats by sweep-netting over baits of decaying mushrooms. Laboratory experiments were conducted on four isofemale strains, denoted *ST*-1, *mk*-1, *mk*-2, and *mk*-3. As will be seen below, the *ST*-1 strain produces normal sex ratios, while the three *mk* (male-killer) strains produce almost exclusively female offspring. The three *mk* strains were maintained by crossing *mk* females to *ST*-1 males every generation. As a result of this maintenance procedure, the four strains became increasingly similar at their nuclear loci. All fly cultures were maintained at 22°C on Instant Drosophila Medium (Carolina Biological Supply, Burlington, NC) supplemented with commercial mushroom (*Agaricus bisporus*).

Characterization of Male-Killing in Drosophila innubila

To determine whether the father or mother was the source of the skewed offspring sex ratio, 20 *ST*-1 and 12 *mk*-1 males (i.e., sons of *mk*-1 females, which are occasionally produced) were individually crossed to two *ST*-1 and two *mk*-1 females each, and the offspring sex ratio scored. The fraction of females among the offspring was analyzed as a function of male type, female type, individual male nested within male type, and the interaction of male type by female type, considering only families with at least 20 offspring. The data were arcsine transformed for this and all other analyses involving the proportion of female offspring.

Antibiotic treatment was used to ask whether maternally inherited bacteria were responsible for the male-killing phenotype. For two successive generations, inseminated *ST*-1, *mk*-1, *mk*-2, and *mk*-3 females were allowed to oviposit on *A. bisporus* mushrooms that had been soaked in a 1 mM solution of tetracycline (IBI-Kodak, New Haven, CT). For both the tetracycline-treated and untreated *ST*-1 and *mk* strains, we tested 10 individuals per strain for *Wolbachia* infection using a polymerase chain reaction (PCR) assay. To assay for *Wolbachia* infection in this and all following experiments, we extracted DNA using Gentra's Puregene kit (Gentra Systems, Minneapolis, MN) with their *Drosophila* single fly protocol, and tested for the presence of *Wolbachia* by amplifying the *Wolbachia surface protein* (*wsp*) gene using the primers wsp81F and wsp691R from Zhou et al. (1998). When a fly tested negative for infection, its DNA quality was ensured by amplifying the host autosomal gene *triose phosphate isomerase* (*tpi*) or the mitochondrial DNA *cytochrome oxidase I* (*COI*) gene, as described in Dyer and Jaenike (2004).

Following two generations of antibiotic treatment, 20 inseminated tetracycline-treated and 20 control females from each strain were placed individually in vials with blended mushroom-agar medium for 24 h; 48 h later the numbers of hatched and unhatched eggs were counted. The females were then placed individually in culture for five days and the sex ratio of their offspring determined. This allowed us to determine the relationships between infection with *Wolbachia*, egg hatch rate, and offspring sex ratio.

Male killing was correlated with presence of *Wolbachia*, but treatment with antibiotics may also eliminate other bacteria. Therefore, we cloned bacterial 16S sequences and screened for any non-*Wolbachia* inserts. We extracted DNA from four newly eclosed females from each of the *mk*-1, *mk*-2, *ST*-1, and tetracycline-treated *mk*-1 and *mk*-2 strains. Using general eubacterial primers for the 16S gene (fD1 and rP2 from Weisburg et al. 1991), 16S was successfully amplified from all of the *mk*-1 and *mk*-2 flies, one of the four *ST*-1 flies, and none of the tetracycline-treated flies. We TA-cloned (Invitrogen, Carlsbad, CA) the 16S PCR product from two flies each of the *mk*-1 and *mk*-2 strains and from the single *ST*-1 fly, and screened 70 recombinants from each of the five transformations using *Wolbachia* group A–specific primers (16SAf/r) that amplify an internal portion of the 16S gene (Werren et al. 1995). All plasmid inserts consisting of non-*Wolbachia* bacteria as well as one *Wolbachia* insert from the *mk*-1 and *mk*-2 transformations were sequenced using flank-

ing plasmid primers. Sequences from this study have been submitted to Genbank (accession numbers AY876253–AY876256). Homology to other known bacteria 16S sequences was determined from the Ribosomal Database Project II (Cole et al. 2003).

Quantitative Variation in Sex Ratio among Infected Flies

Although *Wolbachia*-infected females typically produce exclusively all female progeny, some viable sons are occasionally produced. For instance, among 110 wild-caught infected females from the Chiricahuas, the average fraction of male offspring was 3.2%; very rarely, infected females produced almost equal numbers of male and female offspring (Dyer and Jaenike 2004). To assess the broad-sense heritability of male production by infected females, we individually crossed 100 *mk*-1 and 35 *mk*-2 females to *ST*-1 males and scored the offspring sex ratios for each female. The crosses were set up after seven generations in laboratory culture, by which time the nuclear genomes of all females were expected to be very similar. These lines are also similar in their cytoplasmic composition, including both *Wolbachia* and mtDNA (Dyer and Jaenike 2004). From the F_1 flies, we selected 16 of the 135 lines to investigate further, including seven lines that produced 100% female offspring, three with a very strong female bias (97–98%), three with a moderate female bias (84–92%), and three with no female bias (~50% female offspring). Lines were chosen nonrandomly to have a broad range in the distribution of offspring sex ratio among infected flies. From each line, we crossed 10–20 F_1 females individually to *ST*-1 males and scored the offspring sex ratio. We estimated the backcross offspring sex ratio for each line by taking the average of these replicates. An analysis of covariance (ANCOVA) was used to determine the degree to which mean backcross sex ratio depended on strain (*mk*-1 vs. *mk*-2) and F_1 sex ratio (within strain).

Production of Males by Infected Females

Drosophila innubila might adapt to the adverse effects of a male-killing *Wolbachia* by parthenogenetic production female offspring or by sexual production of female but asexual production of haploid male offspring, as suggested by Normark (2004). To test these possibilities, we introgressed two different naturally occurring mutations that each conferred dark eye color into two infected strains (*mk*-1 and *mk*-3), yielding four infected lines in which all flies were homozygous for the recessive, autosomal eye color mutations. The females from these four lines were then crossed to uninfected males with wild-type eye color. The occurrence of dark-eyed female offspring would indicate that they had been produced via parthenogenesis (or gynogenesis), while the occurrence of dark-eyed sons and wild-type daughters would indicate asexual production of males but sexual production of females.

We also asked whether the production of viable sons by infected females is due to incomplete transmission of *Wolbachia*, in which case males would develop from eggs lacking *Wolbachia* or from incomplete penetrance of *Wolbachia*'s male-killing effect in infected male embryos. We addressed this question in two ways. First, we screened for *Wolbachia* infection all of the sons produced by 59 wild-caught *Wolbachia*-infected females collected in the Chiricahuas during 2001–2003, which included 50 randomly chosen females plus an additional nine that produced weakly female-biased offspring sex ratios. From these same females we also screened a fraction of the daughters for infection. Second, from the 16 lines described in the previous section we scored F_1 and backcross male and female offspring for *Wolbachia* infection.

Correlation between Wolbachia Density and Offspring Sex Ratio

Bacterial density within flies was experimentally manipulated by treatment with a range of antibiotic concentrations. *Wolbachia*-infected *mk*-3 females were allowed to oviposit on Instant Drosophila Medium prepared with solutions of rifampicin (Sigma Chemical, St. Louis, MO) or tetracycline hydrochloride (IBI-Kodak). We first determined the range of antibiotic concentrations over which offspring sex ratios shifted from 100% to about 50% female: for both antibiotics, this occurred between 0.48 and 4.8 μM. Flies that had developed on food containing lower concentrations produced about 100% female offspring, whereas those reared on food with higher concentrations produced normal, 1:1 offspring sex ratios.

To quantitatively manipulate sex ratio, we crossed infected *mk*-3 strain females to *ST*-1 males and reared the offspring on media containing a range of antibiotic concentrations: 0, 0.48, 1.2, 2.4, 3.6, and 4.8, and 48 μM. Upon emergence, 22 females from each treatment (antibiotic type × concentration) were crossed individually to *ST*-1 males in standard culture vials. After 10 days, the females were removed and frozen at −80°C. The offspring (F_1) of these females were collected within 2 h of emergence, sexed, and immediately frozen in liquid nitrogen prior to being placed at −80°C.

The quantitative relation between antibiotic concentration and offspring sex ratio was similar between the two antibiotic types; therefore, we examined the relationship between the resulting *Wolbachia* density and offspring sex ratio for tetracycline only. We extracted DNA from each of the F_1 and F_2 flies as described above and used real-time quantitative PCR (qPCR) to estimate the density of *Wolbachia* in each fly. From each DNA sample, two single-copy genes were targeted: the *wsp* gene in *Wolbachia* served to estimate the relative density of the bacteria, and the single-copy autosomal gene *triose phosphate isomerase* (*tpi*) of *D. innubila* was used to normalize the DNA samples and control for fly size. For all qPCR analyses we used TaqMan chemistry with TaqMan Universal PCR Master Mix on an ABI Prism 7900HT Sequence Detection System instrument and accompanying software (all by PE Applied Biosystems, Foster City, CA). Primers and probes specific to each gene are detailed in Table 1.

Standard curves were plotted using four 10-fold serial dilutions (from 10^{-1} to 10^{-4}) of TOPO cloning vector (Invitrogen, Carlsbad, CA) containing one copy of either the *tpi* or *wsp* gene fragment amplified and TA cloned from an *mk*-3 infected female. Then the number of copies of both genes in each test sample was estimated by comparison to these standard curves. We standardize samples by dividing the estimated copy number of *wsp* by that of *tpi*, which yields a value that represents the *n*-fold ratio of the number of *wsp*

TABLE 1. DNA sequences of the primers and probes used in quantitative polymerase chain reaction to estimate the relative density of *Wolbachia* in *Drosophila innubila*.

Gene	Direction	Sequence (5′-3′)
wsp	forward	AAATTTGGTTTTGCTGGTCAAGTAA
	reverse	AACGAGCTCCAGCATAAAGTTTG
	probe	*FAM*-CTGGTGTTAGTTATGATGTAACTCCAGAA-*BHQ1*
tpi	forward	GCCGGACAGAATGCCTACAA
	reverse	CCCAATCGGCGCCAAT
	probe	*FAM*-TTCACCGGCGAGATT-*MGBQUENCH*

to *tpi* gene copies within a fly. Each experimental sample was run in triplicate for *wsp* and in duplicate for *tpi*, with the replicates averaged for the analyses. We excluded those individuals from which *tpi* did not amplify, and scored an individual as uninfected if their *wsp*/*tpi* ratio (i.e., relative *Wolbachia* density) was less than 0.1.

Wolbachia Temperature Sensitivity

Stressful temperatures may restore the production of males if they reduce the expression of male-killing, intracellular density, or transmission of *Wolbachia* (e.g., Hurst et al. 2000). In the wild, *D. innubila* occurs in areas where temperatures can exceed or fall below those of standard laboratory conditions. We therefore tested the effect of temperature regime on the transmission and penetrance of the male-killing *Wolbachia* in *D. innubila*. For each of three strains, *ST*-1, *mk*-1 and *mk*-3, we created four to seven replicate populations at three different treatment temperatures: 15°C, 22°C, and 30°C. Each population was initiated with 50 females of the test strain plus 25 uninfected *ST*-1 males. Populations were reared in 1-pint containers on Instant Drosophila Medium and mushroom; after the larvae pupated, the adults were removed and the jar opened and placed in a larger container that also contained fresh medium to feed the emerging adults. One week after the beginning of emergence, the sex ratio of the emergent flies was scored and 50 females were randomly chosen to set up the next generation. We ran the experiment for four generations.

RESULTS

Characterization of Male-Killing in Drosophila innubila

The type of female used in a cross had a major effect on sex ratio ($F_{1,102} = 105.4$, $P < 0.0001$), whereas male type ($F_{1,102} = 1.23$, $P = 0.27$), individual male within male type ($F_{1,102} = 0.66$, $P = 0.52$), and male type by female type interaction ($F_{1,102} = 3.21$, $P = 0.08$) did not. This is consistent with maternal determination of offspring sex ratio and is inconsistent with X-chromosome meiotic drive, in which case the male would have a significant effect on offspring sex ratio.

All *ST*-1 and tetracycline-treated *mk*-1, *mk*-2, and *mk*-3 flies were negative for *Wolbachia* infection by the PCR assay, whereas all flies in the untreated *mk*-1, *mk*-2, and *mk*-3 lines were unambiguously positive. Thus, the antibiotic either completely eliminated the *Wolbachia* infection or reduced the bacterial densities to such low levels that they were undetectable by conventional PCR.

Treatment with antibiotics immediately restored the ability of all three *mk* strains to produce an approximate 1:1 offspring sex ratios (Table 2). The strongly female-biased sex ratios in the *mk* strains is due to mortality of male embryos (Table 2), as shown by an egg hatch rate about 50% less among the infected, sex-ratio distorting strains than among the uninfected *ST*-1 and tetracycline-treated strains. There was a highly significant difference in egg hatch rates (arcsine transformed) between infected and uninfected females before tetracycline treatment ($F_{1,52} = 86.0$, $P < 0.001$), but not after treatment ($F_{1,49} = 1.85$, $P = 0.18$). Unhatched eggs in the infected strains were typically brown, indicating that they had been fertilized but died during embryogenesis.

The 16S screening for bacteria other than *Wolbachia* revealed that none of the 70 *ST*-1 transformed colonies contained *Wolbachia* sequence. From the *mk*-1 transformations, 119 the 140 assayed colonies amplified with *Wolbachia*-specific primers and five contained non-*Wolbachia* inserts. From *mk*-2, 130 of 140 of the screened transformed colonies amplified with *Wolbachia*-specific internal primers, and one contained a non-*Wolbachia* insert. Thus, we estimate that the bacterial flora of the *ST*-1, *mk*-1, and *mk*-2 strains are composed of 0%, 96%, and 99% *Wolbachia*, respectively.

TABLE 2. Fraction of female offspring and egg hatch rates of *Wolbachia*-infected (*mk*-1, *mk*-2, and *mk*-3) and uninfected (*ST*-1) strains of *Drosophila innubila*. Means and standard errors were based on arcsine-transformed data, which were then back-transformed into proportions.

	Fraction female offspring				Fraction egg hatch			
	Control		Tetracycline treated		Control		Tetracycline treated	
Strain	Mean (+SE, −SE)	*N*	Mean (+SE, −SE)	*N*	Mean (+SE, −SE)	*N*	Mean (+SE, −SE)	*N*
ST-1	0.506 (0.494, 0.517)	19	0.543 (0.496, 0.589)	21	0.94 (0.90, 0.97)	18	0.83 (0.73, 0.91)	13
mk-1	0.999 (0.999, 1.0)	30	0.539 (0.523, 0.556)	25	0.45 (0.40, 0.49)	13	0.94 (0.91, 0.97)	13
mk-2	0.984 (0.971, 0.993)	30	0.512 (0.496, 0.529)	25	0.49 (0.46, 0.52)	16	0.81 (0.71, 0.88)	15
mk-3	0.999 (0.999, 1.0)	27	0.497 (0.454, 0.540)	20	0.45 (0.40, 0.45)	9	0.98 (0.96, 0.99)	11

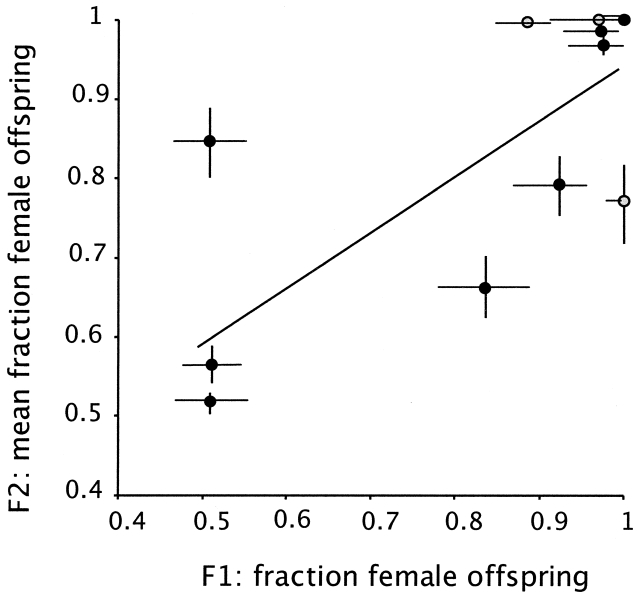

FIG. 1. Heritability of offspring sex ratio among infected *Drosophila innubila*. Each point represents the fraction of females among the F_1 offspring from a single fly and the mean (± SE) fraction of female offspring among the F_2 produced by her daughters. Standard errors were calculated for the F_1 on the basis of a single sample from a binomial distribution and for the F_2 on the basis of variation among F_2 families in sex ratio. The gray points and black points indicate data from strains *mk*-1 and *mk*-2, respectively. The point at (1, 1) represents six different sublines, three each of *mk*-1 and *mk*-2. The line is a least-squares-fitted regression.

We sequenced eight randomly chosen 16S inserts from the *ST*-1 transformants and all of the non-*Wolbachia* inserts from the *mk*-1 and *mk*-2 strains. None of these non-*Wolbachia* 16S inserts show homology to any bacterium known to be a reproductive parasite of arthropods. All eight inserts from the *ST*-1 strain and one of the *mk*-1 inserts were most homologous to the DpLE-like (*Drosophila paulistorum* lepidopteran entomopathogen) bacteria within the family Enterobacteriaceae. These bacteria are thought to be pathogenic to *Drosophila* (Miller et al. 1995; B. Lazzaro, pers. comm.). Three of the other *mk*-1 and one of the *mk*-2 inserts showed strong homology to the genus *Acidovorax* within the Comamonadaceae family of the β-proteobacteria, many of which are decomposers (e.g., Schramm et al. 2003). The remaining *mk*-1 insert was most similar to *Acinetobacter* in the family Moraxellaceae within the γ-proteobacteria. *Acinetobacter* are primarily decomposers but are also known to occur in the gut flora of arthropods (Pidiyar et al. 2004).

Quantitative Variation in Sex Ratio among Infected Flies

The relationship between F_1 and backcross offspring sex ratio among the 16 sublines of infected *D. innubila* is shown in Figure 1. The variation in mean backcross sex ratio was a marginally significant function of fly strain ($F_{1,12} = 3.76$, $P = 0.08$) but was positively correlated with F_1 sex ratio nested within strains ($F_{2,12} = 10.44$, $P = 0.002$). For arcsine-transformed data, the slope of the mother-daughter regression is 0.75. Thus, quantitative variation in offspring sex ratio is heritable in the broad sense, that is, females from less female-biased families themselves produce less female-biased progeny.

Production of Males by Infected Females

We found no evidence for parthenogenetic production of either male of female offspring by infected females. Summing across the four experimental lines, a total of 5377 female and 12 male offspring were produced. Every fly had wild-type eye color, showing that they all resulted from fertilized eggs.

Of the 59 families of wild-caught females, 35 were all female, and 18 of the remaining families produced a total of 53 sons, of which 24 (45%) were infected with *Wolbachia*. Males from families with more female-biased offspring sex ratios were less likely to be infected than males from less female-biased families ($r^2 = 0.405$, $F_{1,16} = 10.88$, $P = 0.0045$). This suggests that the rare males produced in highly female-biased families occur primarily as a result of incomplete transmission. In contrast, males produced in the less female-biased families result both from incomplete maternal transmission and incomplete penetrance of the infection among infected male embryos. Among the same 59 families, there was a positive correlation between the fraction of females among the offspring and the proportion of those females that inherited the *Wolbachia* ($r^2 = 0.386$, $F_{1,57} = 35.83$, $P < 0.0001$), a result that holds up when all-female families are excluded ($r^2 = 0.286$, $F_{1,21} = 8.81$, $P = 0.007$). Thus, the degree of offspring sex ratio bias is positively correlated with *Wolbachia* transmission rate among wild-caught females.

Of the F_1 produced in the 16 sublines in the heritability experiment, all females tested were found to be positive for *Wolbachia* infection ($n = 94$), even those in the families in which no male-killing was evident. Of the sons produced, 28 of 39 (72%) were infected with *Wolbachia*. Thus, while the production of viable male offspring is heritable in the broad sense, among both laboratory-reared and field-caught females it can be due to either incomplete maternal transmission of *Wolbachia* or incomplete penetrance of the male-killing effect among infected male embryos.

Correlation between Wolbachia Density and Offspring Sex Ratio

A single generation at a low antibiotic dose is sufficient to restore normal offspring sex ratios (Fig. 2). Using standard pharmacological methods (Welkos and O'Brien 1994), the probit-transformed proportion of female offspring where we have rescaled the proportion of female offspring (p) from zero to one by letting $p' = 2(p - 0.5)$, was regressed against log of the antibiotic concentration. This regression can be used to estimate the 50% effective dose (ED50), defined here as the antibiotic concentration resulting in 75% female offspring. Such analyses yield values of 1.1 μM for tetracycline and 1.8 μM for rifampicin.

Both antibiotics had similar effects on the production on male offspring, thus we limited the qPCR analysis to those exposed to tetracycline. The relative *Wolbachia* density (i.e., abundance of *Wolbachia wsp* gene standardized to the abundance of the fly *tpi* gene) was assayed in 41 parental females and 292 of their offspring (F_1). For the various antibiotic

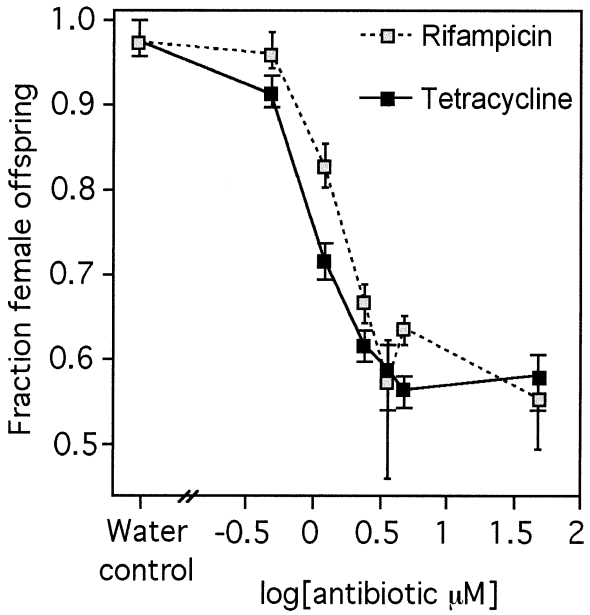

FIG. 2. Mean (± SE) proportion of females among offspring as a function of the antibiotic (tetracycline or rifampicin) concentration at which the mother was reared. For each antibiotic, the number of families per treatment ranges from 13 to 45.

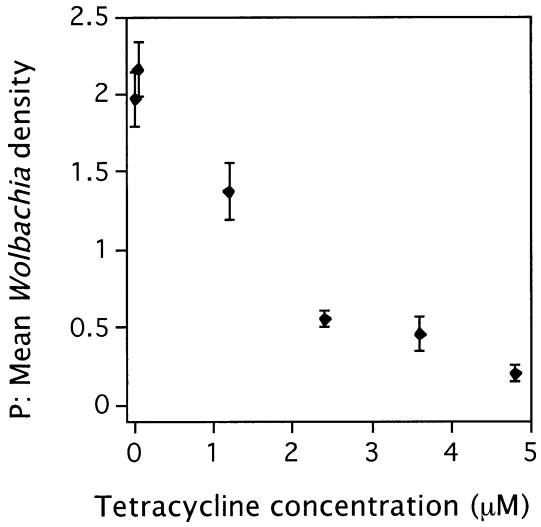

FIG. 3. Mean (± SE) density of *Wolbachia* within individual females as a function of the antibiotic concentration at which they were reared. *Wolbachia* density is measured as the abundance of *wsp* relative to that of the nuclear *tpi* gene (see text for details). These treated females are referred to as the parental generation (P). *Wolbachia* densities are shown as relative number of *wsp*/*tpi* copies (see text for details).

concentrations the numbers of parental, F_1 females, and F_1 males tested were, respectively: antibiotic-free medium (6, 38, 0), 0.48 μM (6, 44, 0), 1.2 μM (6, 41, 0), 2.4 M (7, 32, 22), 3.6 μM (7, 31, 21), 4.8 μM (8, 31, 22). While all parental females were infected with *Wolbachia*, Figure 3 shows that those reared as larvae on higher concentrations of antibiotic harbor lower *Wolbachia* densities as adults ($r^2 = 0.778$, $F_{1,39} = 137.30$, $P < 0.0001$). In turn, females with a lower *Wolbachia* density produced less female biased families ($r^2 = 0.748$, $F_{1,39} = 115.61$, $P < 0.0001$; Fig. 4).

This effect of *Wolbachia* density carries over to the following generation. First, F_1 females from highly female-biased families had higher *Wolbachia* densities than did females from less female-biased families. This holds true whether we include females from all families ($r^2 = 0.473$, $F_{1,179} = 160.44$, $P < 0.0001$) or only infected females from families that were less than 100% female ($r^2 = 0.158$, $F_{1,37} = 6.94$, $P = 0.0123$). Second, there is a positive correlation in *Wolbachia* density between mothers and their daughters ($r^2 = 0.670$, $F_{1,37} = 75.121$, $P < 0.0001$; Fig. 5a), indicating that *Wolbachia* density is an epigenetically heritable trait. This relationship can be broken down into two components: (1) excluding uninfected daughters, there is a positive correlation between the *Wolbachia* density in a mother and that in her daughters ($r^2 = 0.289$, $F_{1,25} = 10.15$, $P = 0.0039$; Fig. 5b); and (2) females with a lower *Wolbachia* density produce a greater fraction of uninfected daughters ($r^2 = 0.729$, $F_{1,37} = 99.41$, $P < 0.0001$; Fig. 6), showing that the maternal transmission rate of *Wolbachia* is a positive function of within-host bacterial density. Note that because mothers and daughters were assayed for *Wolbachia* density at different ages, a comparison of their absolute densities is not meaningful. Finally, among the F_1 males, 62 of 63 were not infected with *Wolbachia*, with the one infected male harboring a very low bacterial density. Thus, our data show that *Wolbachia* density and offspring sex ratio are heritable from mother to daughter, with the production of sons a direct result of a low bacterial density.

Wolbachia Temperature Sensitivity

Figure 7 shows the population-level sex ratio for each strain averaged by temperature treatment for each of the four generations of the experiment. All the *ST*-1 control populations remained at a roughly 1:1 population sex ratio and all the infected strains remained between 90% and 100% female population sex ratio for the duration of the experiment, re-

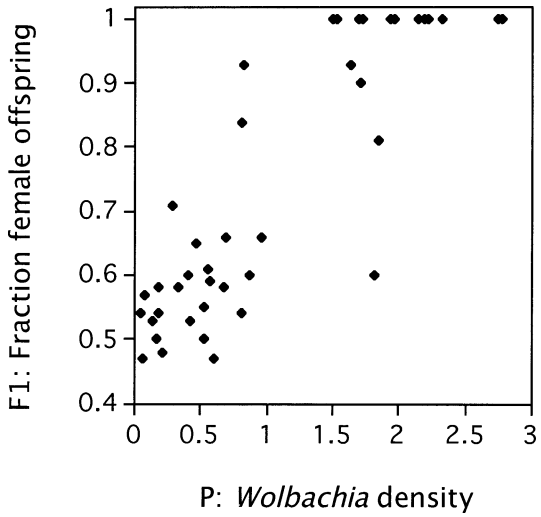

FIG. 4. Fraction of females among the F_1 offspring as a function of *Wolbachia* density in parental (P) females. Each point represents one family.

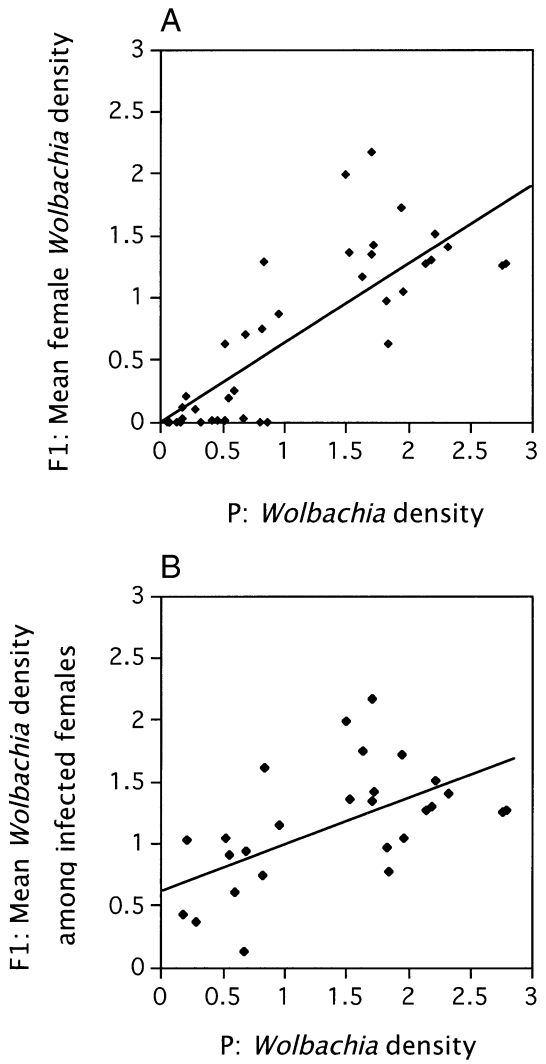

FIG. 5. Correlation between *Wolbachia* density in parental (P) females and their F_1 daughters, including (A) both infected and uninfected daughters (slope = 0.66) or (B) infected daughters only (slope = 0.33). The lines are least squares fitted regressions.

gardless of the temperature treatment. Thus, high and low temperatures have a negligible effect on the penetrance and transmission of the male-killing *Wolbachia* in *D. innubila*.

DISCUSSION

Wolbachia-infected females of *D. innubila* almost invariably produce very strongly female-biased progeny. The association between *Wolbachia* infection and offspring sex ratio holds both in laboratory culture (this study) and among wild-caught flies (Dyer and Jaenike 2004). Three lines of evidence show that the sex ratio bias is due to *Wolbachia*: (1) the maternal but not paternal effect on offspring sex ratio, showing that the effect is not due to X-chromosome drive, which occurs in many other species of *Drosophila* (reviewed in Jaenike 2001); (2) the restoration of normal offspring sex ratios following antibiotic treatment, showing that the effect is due to a maternally transmitted bacterium; and (3) the demonstration that *Wolbachia* are by far the numerically dominant bacterial symbiont in the sex-ratio distorting lines of *D. innubila*. Our data on egg hatch rates show that the sex ratio distortion is brought about by the death of male embryos, although the molecular and developmental mechanisms remain unknown.

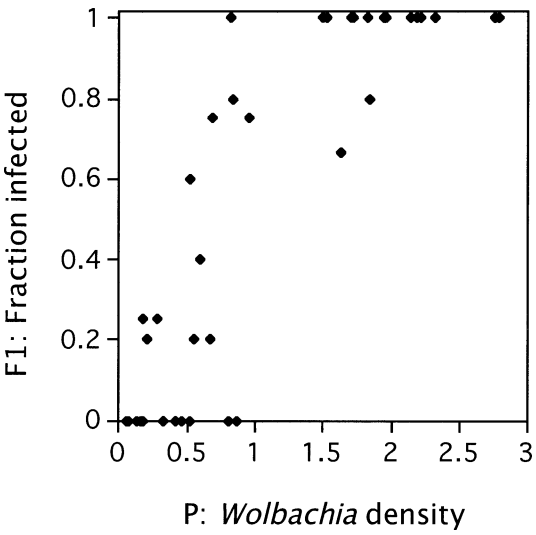

FIG. 6. *Wolbachia* transmission rate as measured by fraction of F_1 females as a function of *Wolbachia* density in parental (P) female. Each point represents one family.

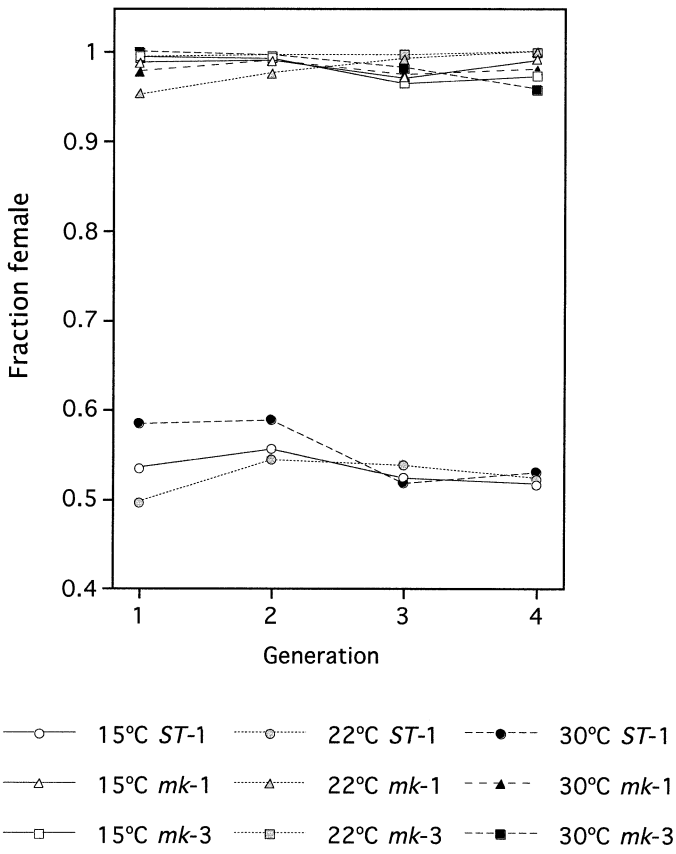

FIG. 7. Effect of temperature on *Wolbachia* male-killing expression. Each point represents the mean of the four to seven population treatments for each strain and temperature.

Wolbachia-infected females of *D. innubila* do occasionally produce some viable sons. We investigated three possible mechanisms by which such sons might be produced: parthenogenetic production of males from unfertilized eggs, incomplete maternal transmission of *Wolbachia*, and incomplete penetrance of the male-killing effect among males that do inherit the infection. Normark (2004) suggested that parthenogenetic production of male offspring might represent an adaptive response to infection with male-killing endosymbionts and perhaps ultimately lead to the evolution of haplodiploid sex determination. Our genetic data revealed that all of the viable male offspring produced by infected female *D. innubila* develop from fertilized eggs, thus providing no indication that *D. innubila* has embarked along this evolutionary pathway. However, our PCR screen for *Wolbachia* infection among the viable sons of infected females revealed both infected and uninfected individuals, showing that both incomplete maternal transmission and incomplete penetrance are operating and potentially important.

If an infected female developed in a family in which some males survived, she was more likely to produce some viable sons. Thus, within the infected class of females, we found broad-sense heritability for offspring sex ratio. Because the *Wolbachia*-infected strains used in this experiment were maintained in a way that resulted in introgression of the same *ST*-1 nuclear genes into all infected flies, the correlation between mothers and daughters in their offspring sex ratios is likely the result of heritable, epigenetic variation among flies.

Our results show that such epigenetic variation arises, at least in part, from variation among flies in their *Wolbachia* density. Specifically, we found a positive correlation between a female's *Wolbachia* density and the proportion of females among her progeny, indicating that the production of viable males is associated with low-density *Wolbachia* infections. In other insects, intracellular density of endosymbionts has been implicated to affect the intensity of male killing (Anbutsu and Fukatsu 2003), cytoplasmic incompatibility (O'Neill and Karr 1990; Boyle et al. 1993; Breeuwer and Werren 1993; Bourtzis et al. 1996; Clancy and Hoffmann 1998), and parthenogenesis induction (Zchori-Fein et al. 2000). The correlation between a female's *Wolbachia* density and offspring sex ratio may arise via two mechanisms. First, if stochastic processes affect the segregation of *Wolbachia* into germ cells, then a female with a low-density *Wolbachia* infection will produce more uninfected eggs than a female with a high-density infection. Such uninfected eggs can develop into viable male offspring. Alternatively, if the male-killing phenotype depends on the titers of certain bacterial products, then incomplete penetrance might occur if females with low-density *Wolbachia* infections transmit fewer bacteria to their eggs. Our data on the maternal transmission rate as a function of *Wolbachia* density (Fig. 6) and the parent-offspring correlation in *Wolbachia* density among infected flies (Fig. 5b) provide support for both of these possibilities.

The production of a few viable male offspring by some infected females has several potentially important implications. First, in three years of collecting *D. innubila* from the Chiricahua Mountains of Arizona, we found that the mean annual prevalence of *Wolbachia* infection among females ranged from 25% to 45% (Dyer and Jaenike 2004). Thus, prevalence levels can change rapidly, perhaps in response to year-to-year variation in ecological conditions. Even if the *Wolbachia* prevalence temporarily reached 100% in a population, the production of a few males by the infected females could serve to delay extinction, perhaps long enough for environmental conditions to allow the infection to return to more moderate frequencies. Second, the incomplete penetrance of the male-killing effect associated with low-density *Wolbachia* infections may uncover genetic variation for resistance to the male-killing effect. Specifically, alleles that are effective only against low-density infections would be visible to selection in such flies and thus increase in frequency. An iteration of this process might eventually lead to the evolution of resistance against higher-density infections, along the lines outlined in Wallace's (1968, pp. 450–451) model of genetic assimilation. Despite this possibility, we have found very little evidence for such resistance within the Chiricahua population of *D. innubila* (Dyer and Jaenike 2004; J. Jaenike, unpubl. data).

The experiments reported here show that not only is offspring sex ratio heritable among infected females, but so is *Wolbachia* density. This suggests that *Wolbachia* density itself may be subject to selection. The heritability of *Wolbachia* density could arise if cytoplasmic lineages varied in *Wolbachia* population growth rate (r) or carrying capacity (K), perhaps due to differences in host nuclear genes, cytoplasmic elements such as mtDNA, or to the *Wolbachia* strains themselves. Evidence for variation in K among *Wolbachia* strains within a single host has been documented for *Callosobruchus* beetles and *Drosophila* (Clark and Karr 2002; Ijichi et al. 2002; McGraw et al. 2002). However, our experimental test revealed significant heritability in both sex ratio and bacterial density among flies carrying exactly the same *Wolbachia* strain and essentially the same mtDNA and nuclear genes. Thus, genetic differences unlikely account for our observations. Alternatively, the population dynamics of *Wolbachia* within hosts may be relatively slow. Should the within-host density be knocked well below K, it may take the bacteria more than one fly generation to return to equilibrium. In such nonequilibrium conditions, selection may favor *Wolbachia* variants with the greatest intrinsic rate of increase, whereas more stable populations will be selected to maximize K (Roughgarden 1971). In either case, selection among bacterial variants within host lineages may affect both the penetrance of the male-killing phenotype and the maternal transmission rate of a lineage's *Wolbachia*.

The existence of a dose-response relationship between *Wolbachia* density and male-killing is reminiscent of the parasite-density-dependent host mortality documented in other host-parasite associations (Tompkins and Begon 1999). Consequently, the fitness rewards of male killing are likely to increase with intracellular *Wolbachia* density, as higher *Wolbachia* densities result in the death of more male offspring. Furthermore, higher *Wolbachia* densities are associated with greater transmission rates to female offspring. All else being equal, lineages with the greatest *Wolbachia* densities would be favored by interlineage selection. However, if exceptionally high intracellular densities of *Wolbachia* adversely affect the viability of female progeny, then an intermediate density of *Wolbachia* may maximize the production of viable, in-

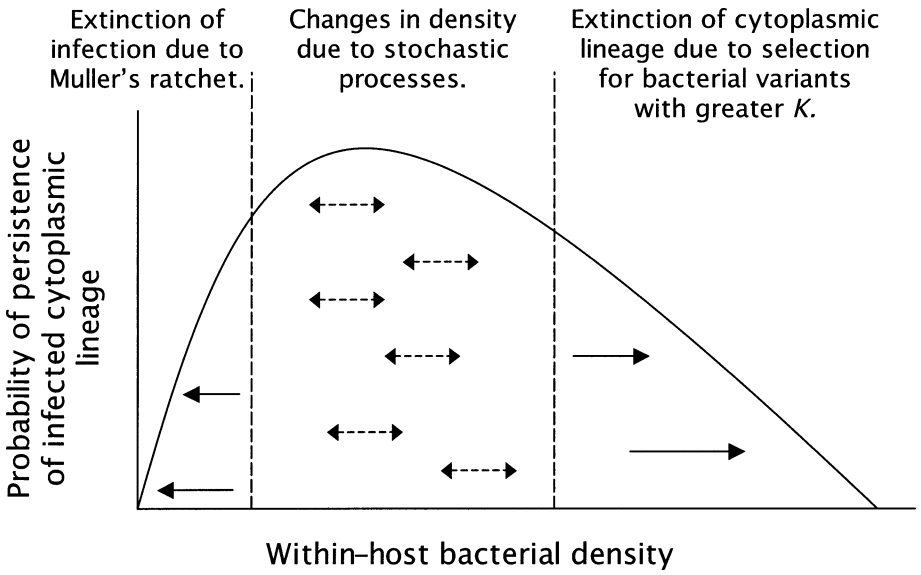

FIG. 8. Model of *Wolbachia* transmission and fitness. At low bacterial effective population size (N_e), the accumulation of slightly deleterious mutations and Muller's rachet may result in symbiont mutational meltdown, whereas at high N_e, the symbionts may evolve to be too virulent to be maintained in the host population. Thus, selection may favor an intermediate bacterial density that is a balance between transmission rate, male-killing, and the adverse fitness effects to the host. Solid-lined single-headed arrows indicate directional processes, and dashed double-headed arrows indicate stochastic changes in bacterial density.

fected female offspring. In the egg hatch experiment, we observed egg mortality as high as 80% for some infected females. Although we have no data on the heritability of this effect or its relation to intracellular *Wolbachia* density, one possibility is that these females carried supra-optimal *Wolbachia* densities. Similar to *D. innubila*, some *Spiroplasma*-infected ladybird beetles (*Harmonia axyridis*) show egg hatch rates higher than 50%, and egg hatch can vary in this species during the lifetime of an infected female (Majerus 2003). It is also worth noting in many species infection with a male-killer can have direct negative effects on female fitness (reviewed in Majerus 2003), for example through decreased female fecundity (Ikeda 1970; Hurst et al. 1994). This suggests the possibility of a bacterial density–host fecundity trade-off, which would strengthen selection on females for an optimal intermediate bacterial density.

While selection among cytoplasmic lineages is likely to favor an intermediate density of *Wolbachia*, selection within lineages can, in theory, favor bacterial variants with the greatest equilibrium density (Roughgarden 1971). How will such a levels-of-selection conflict be resolved? Elsewhere, we estimated that the effective population size of *D. innubila* in the Chiricahuas is on the order of $6–10 \times 10^6$ (Dyer and Jaenike 2004). Under neutrality, the effective population size (N_e) of mtDNA is 1/4 that of autosomal nuclear genes (Hartl and Clark 1989). However, because 25–45% of the females in *D. innubila* are infected with *Wolbachia*, this leads to an effective population size of infected cytoplasmic lineages of $(1/4)(1/3)(8 \times 10^6) \approx 7 \times 10^5$. Such an effective population size should still be more than sufficient for stabilizing selection on bacterial density to overcome stochastic, driftlike effects on within-host bacterial density.

Is this population size adequate to overcome the expected within-lineage directional selection for greater equilibrium density or growth rate of *Wolbachia*? The effectiveness of such selection will depend on bacterial $N_e s$ within cytoplasmic lineages, where s represents the strength of selection for greater *Wolbachia* density or growth rate. Although there have been no estimates of N_e for *Wolbachia*, the observation that endosymbiotic bacteria in general show a genome-level imprint of Muller's ratchet indicates that the effective population size in these species is small (Wernegreen 2002). Because N_e depends on the harmonic mean of N_e over time, a reasonable approximation of the within-lineage N_e of these endosymbionts is the number of *Wolbachia* that enter the primordial germ cell. While we have not estimated this for *D. innubila*, *Callosobruchus* beetles and *Drosophila simulans* have been estimated to have 100–400,000 and 3000–20,000 *Wolbachia* per egg, respectively (Clark and Karr 2002; Ijichi et al. 2002). The lower bounds of these estimates are likely to be closer to the number in the primordial germ cell, and thus a better estimate of within-lineage *Wolbachia* N_e. Given such a low within-lineage N_e compared to the population-level N_e, we suggest that interlineage selection for an intermediate bacterial density will overcome intralineage selection for greater density, even if such selection is strong.

For maternally inherited endosymbionts, patterns of within-lineage evolution may affect the long-term persistence and success of infected lineages. First, bacterial populations within lineages characterized by infections perpetually at low density are susceptible to the deleterious effects of Muller's rachet (Rispe and Moran 2000). Because the accumulation of deleterious mutations may adversely affect within-host density, such bacterial populations may spiral downward to extinction, although the cytoplasmic lineages themselves would survive. Second, high-density bacterial populations within lineages are expected to generate more genetic variation on which selection can act. Furthermore, any beneficial

variants that arise within such populations will be more visible to selection than in lineages with a lower N_e (Ohta 1973). Thus, although deterministic life-history theory predicts selection for increased bacterial density within lineages (Roughgarden 1971), it is likely that those lineages that already have a high N_e would be most capable of such an evolutionary response. Because excessively high bacterial densities are likely to have adverse effects on their insect hosts, evolution in such high-density infections could lead to upward spirals in density, ultimately causing the extinction of the host cytoplasmic lineage along with its resident endosymbionts. These scenarios are illustrated in Figure 8. Rispe and Moran (2000) more formally explored the interplay of within- and between-lineage selection for mutualistic endosymbionts.

A variety of factors may affect within-host densities of *Wolbachia*, including the antibiotic activity of larval or adult food sources, genetically based host defensive responses, host age, within-host interactions among the bacteria, and the environmental conditions to which flies are exposed. In addition, bacterial densities will be affected by stochastic variations in bacterial birth and death rates and partitioning among a female's germ cells. Because *D. innubila* shows no evidence for host suppression of male-killing (this study; Dyer and Jaenike 2004; J. Jaenike, unpubl. data) and low *Wolbachia* densities render male-killing less effective, *D. innubila* likely harbors little genetic variation to suppress within-host *Wolbachia* densities. Thus, variation in intracellular *Wolbachia* densities in *D. innubila* is more likely to stem from various environmental sources.

In the Chiricahua Mountains of Arizona, *D. innubila* occupies a broad elevational range, from 1500 to 2700 m, over which there are major changes in vegetation type and mushroom species, *Drosophila* community composition, precipitation, and temperature (Lowe 1964; F. Nishida, pers. comm.; J. Jaenike and K. Dyer, unpubl. data). Our laboratory studies revealed no effect of temperatures from 15°C to 30°C on *Wolbachia* transmission and penetrance. Thus, our results suggest that temperature is unlikely to play a major role in governing the dynamics of *Wolbachia* in natural populations of *D. innubila*. The only other male-killing strain of *Wolbachia* known in *Drosophila* occurs in *D. bifasciata*, a Palearctic member of the obscura group (Hurst et al. 2000). Although the *D. bifasciata* strain of *Wolbachia* is heat sensitive, the effect of elevated temperature (25–26°C) on offspring sex ratio becomes evident only after prolonged exposure (Hurst et al. 2000, 2001).

In contrast to its insensitivity to temperature, the male-killing *Wolbachia* strain in *D. innubila* is extraordinarily susceptible to both tetracycline (which binds to bacterial ribosomes to inhibit protein synthesis) and rifampicin (which binds to bacterial RNA polymerase and thus inhibits transcription). Like other mycophagous members of the *quinaria* group, *D. innubila* breeds on a variety of fleshy mushrooms. Although few mushroom species have been screened for possible antimicrobial activity, some are known to produce antibiotics (e.g., Hirasawa et al. 1999; Suay et al. 2000; Rosa et al. 2003). Thus, it is possible that breeding on a variety of mushroom species could generate variation in *Wolbachia* density among flies, allowing male production even in populations harboring a high *Wolbachia* prevalence. Similarly, Stevens and Wicklow (1992) suggested that antibiotics produced by fungal growth on flour may be the underlying cause of reduced *Wolbachia* transmission rates in *Tribolium* flour beetles.

Regardless of the proximate causes of variation among flies in the density of *Wolbachia* they carry, the existence of such variation and its effects on maternal transmission and male-killing penetrance establishes the condition for conflicting selection on bacterial density within and among cytoplasmic lineages. This levels-of-selection conflict within *Wolbachia* is coupled to the conflict between *Drosophila* and *Wolbachia* over the optimal within-population sex ratio and to the potential for male-killing endosymbionts to affect the persistence of host populations or species. Consequently, these associations are arenas for extraordinarily rich and complex evolutionary interactions.

Acknowledgments

We thank L. Ascroft and A. Brooks for assistance with qPCR; the Southwest Research Station (Portal, AZ) for opportunities to conduct fieldwork in the Chiricahuas; and A. Betancourt, M. Clark, J. Werren, and two anonymous reviewers for valuable discussion and/or comments on the manuscript. This work was supported by National Science Foundation, Caspari, and American Association of University Women fellowships to KAD and National Science Foundation grants DEB-0074141 and DEB-0315521 to JJ.

Literature Cited

Anbutsu, H., and T. Fukatsu. 2003. Population dynamics of male-killing and non-male-killing spiroplasmas in *Drosophila melanogaster*. Appl. Environ. Micro. 69:1428–1434.

Bourtzis, K., A. Nirgianaki, G. Markakis, and C. Savakis. 1996. *Wolbachia* infection and cytoplasmic incompatibility in *Drosophila* species. Genetics 144:1063–1073.

Boyle, L., S. L. O'Neill, H. M. Robertson, and T. L. Karr. 1993. Interspecific and intraspecific horizontal transfer of *Wolbachia* in *Drosophila*. Science 260:1796–1799.

Breeuwer, J. A. J., and J. H. Werren. 1993. Cytoplasmic incompatibility and bacterial density in *Nasonia vitripennis*. Genetics 135:565–574.

Budar, F., and G. Pelletier. 2001. Male sterility in plants: occurrence, determinism, significance and use. CR. Acad. Sci. Ser. III Sci. Vie 324:543–550.

Clancy, D. J., and A. A. Hoffmann. 1998. Environmental effects on cytoplasmic incompatibility and bacterial load in *Wolbachia*-infected *Drosophila simulans*. Entomol. Exp. Appl. 86:13–24.

Clark, M. E., and T. L. Karr. 2002. Distribution of *Wolbachia* within *Drosophila* reproductive tissue: implications for the expression of cytoplasmic incompatibility. Integr. Comp. Biol. 42:332–339.

Cole, J. R., B. Chai, T. L. Marsh, R. J. Farris, Q. Wang, S. A. Kulam, S. Chandra, D. M. McGarrell, T. M. Schmidt, G. M. Garrity, and J. M. Tiedje. 2003. The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. Nucleic Acids Res. 31:442–443.

Couvet, D., O. Ronce, and C. Gliddon. 1998. The maintenance of nucleocytoplasmic polymorphism in a metapopulation: the case of gynodioecy. Am. Nat. 152:59–70.

Dyer, K. A., and J. Jaenike. 2004. Evolutionarily stable infection by a male-killing endosymbiont in *Drosophila innubila*: molecular evidence from the host and parasite genomes. Genetics 168: 1443–1455.

Fisher, R. A. 1930. The genetical theory of natural selection. Oxford Univ. Press, Oxford, U.K.
Hanski, I. 1998. Metapopulation dynamics. Nature 396:41–49.
Hartl, D. L., and A. G. Clark. 1989. Principles of population genetics. 2nd ed. Sinauer, Sunderland, MA.
Hatcher, M. J., D. E. Taneyhill, A. M. Dunn, and C. Tofts. 1999. Population dynamics under parasitic sex ratio distortion. Theor. Popul. Biol. 56:11–18.
Heed, W., J. Russell, and D. Harrington. 1962. Diversity and density of *Drosophila* in the immediate vicinity of Tucson with special reference to *D. pseudoobscura*. Dros. Inf. Serv. 36:73–74.
Hirasawa, M., N. Shouji, T. Neta, K. Fukushima, and K. Takada. 1999. Three kinds of antibacterial substances from *Lentinus edodes* (Berk.) Sing. (shiitake, an edible mushroom). International Journal of Antimicrobial Agents 11:151–157.
Hurst, L. D. 1991. The incidences and evolution of cytoplasmic male killers. Proc. R. Soc. Lond. B 244:91–99.
Hurst, G. D. D., and F. M. Jiggins. 2000. Male-killing bacteria in insects: mechanisms, incidence, and implications. Emer. Inf. Dis. 6:329–336.
Hurst, G. D. D., E. L. Purvis, J. J. Slogett, and M. E. N. Majerus. 1994. The effect of infection with male-killing *Rickettsia* on the demography of female *Adalia bipunctata* L. (two-spot ladybird). Heredity 73:309–316.
Hurst, G. D. D., A. P. Johnson, J. H. G. von der Schulenburg, and Y. Fuyama. 2000. Male-killing *Wolbachia* in *Drosophila*: a temperature-sensitive trait with a threshold bacterial density. Genetics 156:699–709.
Hurst, G. D. D., F. M. Jiggins, and S. J. W. Robinson. 2001. What causes inefficient transmission of male-killing *Wolbachia* in *Drosophila*? Heredity 87:220–226.
Ijichi, N., N. Kondo, R. Matsumoto, M. Shimada, H. Ishikawa, and T. Fukatsu. 2002. Internal spatiotemporal population dynamics of infection with three *Wolbachia* strains in the adzuki bean beetle, *Callosobruchus chinensis* (Coleoptera: Bruchidae). Appl. Env. Micro. 68:4074–4080.
Ikeda, H. 1970. The cytoplasmically-inherited 'sex-ratio' condition in natural and experimental populations of *Drosophila bifasciata*. Genetics 65:311–333.
Jaenike, J. 2001. Sex chromosome meiotic drive. Annu. Rev. Ecol. Syst. 32:25–49.
Jaenike, J., K. A. Dyer, and L. K. Reed. 2003. Within-population structure of competition and the dynamics of male-killing *Wolbachia*. Evol. Ecol. Res. 5:1023–1036.
Jiggins, F. M., J. K. Bentley, M. E. N. Majerus, and G. D. D. Hurst. 2002. Recent changes in phenotype and patterns of host specializaton in *Wolbachia* bacteria. Mol. Ecol. 11:1275–1283.
Lowe, C. H. 1964. Arizona's natural environment: landscapes and habitats. Univ. of Arizona Press, Tucson.
Majerus, M. E. N. 2003. Sex wars: genes, bacteria, and biased sex ratios. Princeton Univ. Press, Princeton, NJ.
Manicacci, D., D. Couvet, E. Belhassen, P. H. Gouyon, and A. Atlan. 1996. Founder effects and sex ratio in the gynodioecious *Thymus vulgaris*L. Mol. Ecol. 5:63–72.
McCauley, D. E., M. S. Olson, S. N. Emery, and D. R. Taylor. 2000. Population structure influences sex ratio evolution in a gynodioecious plant. Am. Nat. 155:814–819.
McGraw, E. A., D. J. Merritt, J. N. Droller, and S. L. O'Neill. 2002. *Wolbachia* density and virulence attenuation after transfer into a novel host. Proc. Natl. Acad. Sci. USA 99:2918–2923.
Miller, S. G., B. C. Campbell, J. Becnel, and L. Ehrman. 1995. Bacterial entomopathogens from the *Drosophila paulistorum* species complex. J. Invert. Pathol. 65:125–131.
Normark, B. B. 2004. Haplodiploidy as an outcome of coevolution between male-killing cytoplasmic elements and their hosts. Evolution 58:790–798.
Ohta, T. 1973. Slightly deleterious mutant substitutions in evolution. Nature 246:96–98.
O'Neill, S. L., and T. L. Karr. 1990. Bidirectional incompatibility between conspecific populations of *Drosophila simulans*. Nature 348:178–180.
Patterson, J. T., and R. R. Wagner. 1943. Geographical distribution of species of the genus *Drosophila* in the United States and Mexico. University of Texas Publications 4313:217–281.
Pidiyar, V. J., K. Jangid, M. S. Patole, and Y. S. Shouche. 2004. Studies on cultured and uncultured microbiota of wild *Culex quinquefasciatus* midgut based on 16S ribosomal gene analysis. Am. J. Trip. Med. Hyg. 70:597–603.
Rispe, C., and N. A. Moran. 2000. Accumulation of deleterious mutations in endosymbionts: Muller's rachet with two levels of selection. Am. Nat. 156:425–441.
Rosa, L. H., K. M. G. Machado, C. G. Jacob, M. Capelari, C. A. Rosa, and C. L. Zani. 2003. Screening of Brazilian basidiomycetes for antimicrobial activity. Mem. Inst. Oswaldo Cruz 98: 967–974.
Roughgarden, J. 1971. Density-dependent natural selection. Ecology 52:453–468.
Schramm, A., S. K. Davidson, J. A. Dodsworth, H. L. Drake, D. A. Stahl, and N. Dubilier. 2003. Acidovorax-like symbionts in the nephridia of earthworms. Environ. Micro. 5:804–809.
Stevens, L., and D. T. Wicklow. 1992. Multispecies interactions affect cytoplasmic incompatibility in *Tribolium* flour beetles. Am Nat 140:642–653.
Stouthamer, R., J. A. J. Breeuwer, and G. D. D. Hurst. 1999. *Wolbachia pipientis*: microbial manipulator of arthropod reproduction. Annu. Rev. Microbiol. 53:71–102.
Suay, I., F. Arenal, F. J. Asensio, A. Basilio, M. C. Cabello, M. T. Diez, J. B. Garcia, A. G. del Val, J. Gorrochategui, P. Hernandez, F. Pelaez, M. Francisca Vicente. 2000. Screening of basidiomycetes for antimicrobial activities. Antonie Leeuwenkoek Int. J. Gen. Mol. Microbiol. 78:129–139.
Taylor, D. R., M. S. Olson, and D. E. McCauley. 2001. A quantitative genetic analysis of nuclear-cytoplasmic male sterility in structured populations of *Silene vulgaris*. Genetics 158:833–841.
Tompkins, D. M., and M. Begon. 1999. Parasites can regulate wildlife populations. Parasitol. Today 15:311–313.
Wallace, B. 1968. Topics in population genetics. Norton, New York.
Weisburg, W. W., S. M. Barns, D. A. Pelletier, and D. J. Lane. 1991. 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. 173:697–703.
Welkos, S., and A. O'Brien. 1994. Determination of median lethal and infectious doses in animal-model systems. Method Enzymol. 235:29–39.
Werren, J. H. 1987. The coevolution of autosomal and cytoplasmic sex ratio factors. J. Theor. Biol. 124:317–334.
Wernegreen, J. J. 2002. Genome evolution in bacterial endosymbionts of insects. Nat. Rev. Genet. 3:850–861.
Zchori-Fein, E., Y. Gottlieb, and M. Coll. 2000. *Wolbachia* density and host fitness components in *Muscidifurax uniraptor* (Hymenoptera: Pteromalidae). J. Invert. Pathol. 75:267–272.
Zhou, W., F. Rousset, and S. O'Neill. 1998. Phylogeny and PCR-based classification of *Wolbachia* strains using *wsp* gene sequences. Proc. R. Soc. Lond. B 265:509–515.

Corresponding Editor: T. Day