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Within-population structure of competition and the dynamics of male-killing *Wolbachia*

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ABSTRACT

Males are evolutionary dead-ends for endosymbiotic bacteria that are transmitted maternally. It has been shown theoretically that male killing will enhance endosymbiont transmission if the fitness of females increases as a result of the death of their male siblings. Despite the intuitive appeal of this explanation for the spread of male-killing bacteria, it has never been examined experimentally. Here we consider how the structure of larval competition within panmictic populations affects the dynamics of a male-killing *Wolbachia* that infects *Drosophila innubila*. In populations with exclusively sib competition for larval resources, the *Wolbachia* infection spreads rapidly to fixation, in contrast to much less deterministic dynamics in populations where competition is unstructured. Sib competition is likely to be important if females lay eggs in clutches on individual breeding sites. *Drosophila innubila*, a mycophagous species, lays eggs in clutches on individual mushrooms in the laboratory, even when several mushrooms are available. The larvae of species that exploit patchy, ephemeral resources typically exhibit aggregated distributions across breeding sites, due at least in part to egg laying in clutches. These species may therefore be particularly susceptible to invasion by male killers.

Keywords: aggregation, clutch size, endosymbionts, intragenomic conflict, larval competition, population structure, sex ratio, sib competition.

INTRODUCTION

A major theoretical generalization to have emerged in the field of evolutionary parasitology is that vertically transmitted parasites, because their fate is closely tied to that of their hosts, should evolve a low level of virulence or even mutualistic associations with their hosts (Fine, 1975; Ewald, 1987; Yamamura, 1993; Bull 1994; Ebert and Herre, 1996; Frank, 1996). However, in sexual species, vertically transmitted symbionts are typically inherited solely from the mother, thus establishing an asymmetry in transmission between nuclear genes and maternally transmitted elements (Cosmides and Tooby, 1981).

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Some of the manifestations of this asymmetry or intragenomic conflict have become evident in the last 10–15 years. In particular, a variety of maternally transmitted endosymbionts have been found to manipulate host reproduction in ways that increase the transmission of the endosymbionts at the expense of host fitness. These mechanisms include parthenogenesis (in which infected females reproduce by thelytoky to produce only female offspring), feminization (in which the endosymbionts cause genetic males to develop into functional females), cytoplasmic incompatibility (in which infected males act as 'suicide bombers' to kill the offspring of uninfected females) and male killing (in which the male offspring of infected females die early in development). These topics have been reviewed in considerable detail recently (Stouthamer *et al.*, 1999; Hurst and Jiggins, 2000; Stevens *et al.*, 2001).

Among the microorganisms that manipulate host reproduction, the most diverse assemblage is associated with male killing (for a review, see Hurst and Jiggins, 2000). Endosymbionts known to cause embryonic male killing in various species of arthropods include members of the α-Proteobacteria (*Wolbachia* and *Rickettsia*), γ-Proteobacteria (*Arsenophonus*), Gram-positive bacteria (*Spiroplasma*) and Flavobacteria. Within some groups, such as *Wolbachia*, male killing has arisen independently several times (Hurst and Jiggins, 2000; K. Dyer and J. Jaenike, submitted). That male killing has evolved numerous times indicates the molecular and developmental mechanisms by which host sex determination and differentiation are controlled can be readily exploited by endosymbiotic bacteria. Although the means by which these bacteria interact with developmental pathways is currently unknown, Starr and Cline (2002) recently showed that *Wolbachia* interact with *Sex-lethal*, which is the master regulator of the sex determination pathway in *Drosophila melanogaster*.

From an evolutionary standpoint, the ecological conditions that favour the spread of male-killing endosymbionts within a host population are as important as the developmental mechanism the bacteria use to manipulate its host. Because males are dead-ends for maternally transmitted endosymbionts, it has been proposed that male-killing bacteria spread because the death of males is beneficial to female siblings, which carry the same bacterial strain (Werren, 1987; Hurst, 1991). The possible benefits include the avoidance of inbreeding in species where sib mating is likely, and the reduction of negative ecological interactions between sibs, the most likely being cannibalism and competition for limited resources. For instance, the common occurrence of male killers in ladybird beetles is thought to be related to the common occurrence of larval cannibalism in these species (Hurst and Jiggins, 2000; Schulenburg *et al.*, 2002). With respect to competition, Hurst (1991) has shown theoretically that the invasion of male-killing endosymbionts depends on increased availability of resources to females – and hence female fitness – resulting from the death of their male siblings.

Despite the intuitive appeal of these arguments and the common occurrence of male-killing endosymbionts in certain groups of insects (Jiggins *et al.*, 2001), there has been very little experimental work done to test these hypotheses. If male killers are favoured because of inbreeding avoidance, it is the offspring of the females whose brothers are killed that experience increased fitness. If the spread of these endosymbionts is due to negative ecological interactions among siblings of the host species, the sisters of the killed males experience a direct increase in fitness. The only relevant data of which we are aware is to be found in Hurst *et al.* (1997), who showed that, for the ladybird beetle *Adalia bipunctata*,

the offspring of infected mothers consume more sibling eggs and survive longer than the offspring of uninfected mothers. The population dynamic consequences of this difference were not examined.

Here we consider *Drosophila innubila*, which was recently discovered to be infected with male-killing *Wolbachia* (K. Dyer and J. Jaenike, submitted). This species inhabits mid- to high-elevation woodlands and forests in the sky islands of the arid southwestern United States. Like most other members of the quinaria group, its principal breeding sites are mushrooms. In the Chiricahua Mountains, where the densest populations of *D. innubila* have been found, approximately 40% of the females are infected with a male-killing strain of *Wolbachia* (K. Dyer and J. Jaenike, submitted). The larvae of mycophagous *Drosophila* often compete for food resources, with competition occurring at the scale of individual mushrooms (Grimaldi and Jaenike, 1984). Furthermore, females within these species often lay clutches of eggs on single mushrooms (Jaenike and Selander, 1979; Jaenike and James, 1991). These conditions suggest that competition among siblings for larval food may be common in mycophagous *Drosophila*. Such conditions are expected to facilitate the spread of male-killing endosymbionts.

In this paper, we ask whether *D. innubila* lays eggs in clutches on individual mushrooms, what the consequences of larval crowding are for fly fitness, and how the population-level structuring of larval competition affects the population dynamics of the male-killing *Wolbachia*. Our results provide the first experimental demonstration that the within-population structure of competition – whether between siblings or unrelated individuals – has a major effect on the dynamics of male-killing *Wolbachia*. These findings, in conjunction with broader surveys showing the prevalence of larval aggregation in many guilds of insects, suggest that many insect species are ecologically susceptible to invasion of male-killing endosymbionts.

MATERIALS AND METHODS

Drosophila strains

The flies used in the clutch size and larval competition experiments were descended from *Drosophila innubila* adults collected by sweep netting over mushroom baits. The flies were collected in August 2000 at the Southwest Research Station (elevation 1600 m), which is near Portal, Arizona in the Chiricahua Mountains. Elsewhere, we have characterized the male-killing phenotype, mode of inheritance, prevalence of infection and the molecular phylogeny of the *Wolbachia* strain responsible for the male killing (K. Dyer and J. Jaenike, submitted). The present experiments included two isofemale strains of *D. innubila*, one that is uninfected by *Wolbachia* (*ST-1*) and one that is infected with the male-killing strain of *Wolbachia* (*mk-3*). Because *Wolbachia* infection causes almost 100% mortality of male offspring, the *mk-3* strain was maintained by mating to infected *ST-1* males each generation. As a result of this introgression procedure, the two strains were similar at nuclear loci, while remaining distinct for cytoplasmic elements, including mitochondrial haplotype and infection with *Wolbachia*.

The flies in the horizontal transmission experiment included two isofemale *Wolbachia* strains, *mk-1* and *mk-3*, both of which were collected in the Chiricahuas in August 2000. In addition, we used two uninfected isofemale strains homozygous for visible eye colour mutations: *CH1-22* and *SR3-2*. These mutations were uncovered by sib mating the

descendents of flies collected in September 2002 in the Chiricahuas (*CH1-22*) and the Santa Rita (*SR3-2*) Mountains of Arizona.

Clutch size

To determine if females lay eggs in clutches on individual mushrooms, 30 *ST-1* females were kept at 22°C in the presence of *ST-1* males for 1 week after the females had emerged as adults. The females were then placed individually, without males, in clear plastic boxes (18 cm long × 12 cm wide × 4 cm high) containing a layer of moist peat moss on the bottom and one *Agaricus bisporus* mushroom, each weighing 2.5 g, in each of the four corners of the box. The boxes were illuminated from directly overhead, to eliminate phototaxis towards one side of the box as a cause of aggregation. After 24 h (12 L : 12 D) the females were removed and the four mushrooms were separated and placed individually in culture jars. The number of offspring emerging from each mushroom was used as a measure of the number of eggs laid by a female.

If females lay eggs singly on a mushroom and then move on, the number of eggs per mushroom is expected to be randomly distributed. In contrast, if they lay clutches of two or more eggs per mushroom, the distribution is expected to be more aggregated. To determine whether aggregation was significantly different from random, we used the standardized Morisita index of dispersion (I_P), which has been found to be one of the best measures of dispersion (Krebs, 1989). A value of $I_P > 0.5$ indicates a distribution pattern significantly more aggregated than random. The use of uninfected *ST-1* females in this experiment facilitates a statistical analysis of egg-laying patterns, as most of the eggs laid by these females do hatch, in contrast to the ~50% hatch rate of eggs laid by infected females (K. Dyer and J. Jaenike, submitted).

Fitness effects of larval competition

This experiment was designed to determine whether resource limitation for larvae affects the probability of egg to adult survival, the size of emergent adults, or both. Because of their rate of egg hatch, we again used the uninfected *ST-1* strain of *D. innubila*. Eggs from reproductively mature females were collected on disks of mushroom–agar medium. Either 10, 20, 40, 80 or 160 eggs were placed on pieces of *A. bisporus* mushroom weighing 1.0 g each. Each mushroom piece was placed in a vial on top of moist absorbent cotton, which prevented desiccation. Four replicate cultures of egg densities 10 through 80 and two of density 160 were set up. Cultures were kept at 22°C and 80% relative humidity. Flies were collected and counted immediately after emergence, dried for 24 h at 60°C, and then weighed on a Cahn microbalance. The flies did not feed as adults before they were dried.

Competition experiment

The aim of this experiment was to establish whether the structure of larval competition within a population affects *Wolbachia* dynamics. The strains used were the infected *mk-3* and the uninfected *ST-1*. Because the *mk-3* strain had been maintained by mating infected females to uninfected *ST-1* males for six generations before the start of the experiment, we expected little nuclear-cytoplasmic disequilibrium in the founding populations of the

experiment. Thus, changes in the frequency of infection should reflect the fitness effects of cytoplasmic elements rather than fitness differences due to associated nuclear genes.

Three replicate populations were set up for each of two treatments. In one treatment, termed 'mass competition', each population was started with a total of 50 *ST-1* and 50 *mk-3* females, all of which had been mated to *ST-1* males. The females were placed in a one-quart (1.136 litres) Mason jar containing 100 g of *A. bisporus* mushrooms. This set-up ensured that competition for food was largely among the offspring of different females. The mushrooms were placed on top of a bed of absorbent cotton, which served as a pupation site for the larvae. When the larvae had pupated, the cotton was removed from the jar and placed in a population cage that contained a dish of Instant Drosophila Medium (Carolina Biological Supply, Burlington, NC) plus *A. bisporus* to feed the emergent adults. One week after the beginning of adult emergence, during which time flies were allowed to mate randomly, 100 female flies were selected at random to start the next generation.

In the second treatment, termed 'sib competition', each population was again started with 50 *ST-1* and 50 *mk-3* females, all of which had been mated to *ST-1* males, but in this case the females were placed individually in culture vials. Each vial contained a 1 g piece of mushroom placed on top a small piece of cotton, in which the larvae pupated. Thus, the quantity of food per female within a population was the same in the two treatments. When the larvae had pupated, the cotton was removed from all 100 culture vials and placed in a population cage identical to that used for the mass competition treatment. As with the mass competition treatment, emergent flies were allowed to mate randomly. One week after the start of emergence, 100 females were selected at random and placed individually in vials.

Every generation, all of the emergent flies in each of the six populations were counted and sexed. Because *Wolbachia* infection results in nearly 100% mortality of male offspring, the population-level sex ratio was used as a proxy for the prevalence of *Wolbachia* infection in these populations. As a check on this, we randomly sampled 50 females per population every three generations. These flies were scored for *Wolbachia* infection by extracting DNA using the squish-prep method of Gloor *et al.* (1993) and amplifying the *wsp* gene using the primers *wsp691R* and *wsp81F* from Zhou *et al.* (1998).

This experiment was started at the University of Arizona in Tucson, but was moved to Rochester, New York after generation 5. The move resulted in a substantial population bottleneck. While in Tucson, all of the mass competition populations remained at high density, while the sib competition populations were declining. These declines were due to the failure of many females to mate, which results from the deficiency of males in the sib competition populations. The consequences of a population bottleneck on *Wolbachia* dynamics could be severe. First, because of maternal inheritance, the effective population size with respect to *Wolbachia* infection is expected to be one-quarter that of autosomal genes. If the bottleneck brings about a random increase in prevalence of *Wolbachia* infection among reproductive females, this could cause a deficiency of males in the next generation, resulting in fewer females being inseminated. Thus, the consequences of a one-generation bottleneck in numbers could be self-sustaining or amplifying. Furthermore, a reduction in the number of inseminated females will result in a lower density of larvae competing for mushrooms, thus reducing the effect of competition on *Wolbachia* dynamics in these populations. Because of these potentially serious effects of a bottleneck, the first five generations of the experiment provide the most informative data on how the population-level structure of competition affects *Wolbachia* dynamics. The effects of the population bottleneck were unplanned and thus not analysed in detail.

Horizontal transmission

Although genetic drift stemming from a population bottleneck may bring about higher frequencies of *Wolbachia* infection, the same result might occur deterministically, one possibility being horizontal (or contagious) transmission of the *Wolbachia* infection. To determine whether horizontal transmission can occur at epidemiologically significant rates, cultures were set up using infected flies that were wild-type in appearance (*mk-1* and *mk-3*) and uninfected flies homozygous for mutations that cause dark eye colour (*CH1-22* and *SR3-2*). Each vial was set up with five infected females (either *mk-1* or *mk-3*) and five uninfected females (either *CH1-22* or *SR3-2*). These females had mated with genotypically similar males, so that the offspring of the infected females would be wild-type in appearance and the offspring of the uninfected females would be identifiable by their eye colour. For each of the four combinations, an average of 10 replicate cultures were set up, and the dark-eyed flies were collected from among the emergent offspring. To determine if horizontal transmission of *Wolbachia* had occurred, dark-eyed flies (i.e. the offspring of the uninfected mutant females) were PCR assayed for *Wolbachia* infection using the *wsp* gene, as described above. Each of the 95 DNA extractions was prepared from two to three dark-eyed flies emerged from a single culture vial. To ensure DNA quality, we also amplified the mitochondrial COI gene, using the primers *TL-2-1460* and *TY-N-3014*, from Simon *et al.* (1994). Thus, approximately 250 dark-eyed offspring were screened for *Wolbachia* infection in this experiment.

The occurrence of horizontal transmission during the competition experiment was assayed directly. The two strains of *D. innubila* used in this experiment (*ST-1* and *mk-3*) carried different mitochondrial haplotypes that differed in their COI sequences in a way that could be distinguished using a single restriction enzyme (data not shown). If the spread of *Wolbachia* in a mass competition population was due to horizontal transmission of the infection from *mk-3* to *ST-1* flies, this would be evident by the existence of *Wolbachia*-infected flies carrying the *ST-1* mtDNA haplotype. The flies in the 'mass 3' population, in which there was a dramatic increase in the prevalence of *Wolbachia* infection, were screened for *Wolbachia* infection and mtDNA haplotype. The flies examined were collected at generation 9, when nearly all flies were infected, and just before this population went extinct due to lack of males. Individual flies were assayed for *Wolbachia*, as described above. To identify mtDNA haplotype, we amplified COI using the primers *TL-2-1460* and *TY-N-3014* from Simon *et al.* (1994), and then digested 10 μl of PCR product with 3 units of the restriction enzyme BclI (New England Biolabs). BclI cuts the *mk-3* COI haplotype once, resulting in two smaller fragments, whereas *ST-1* is not cut and remains as one fragment.

Maturation time

To test the alternative hypothesis that male-killing *Wolbachia* spread because they reduce the adverse effects of inbreeding depression due to sib mating, we determined egg to adult development time and age at which males and females first mate in *D. innubila*. If males develop faster than females and if they can mate with newly emerged females, before the females can disperse, this could allow a significant amount of inbreeding. To measure egg to adult development time, males and females of strain *ST-1* were placed for 7 h in two vials containing Instant Drosophila Medium plus *Agaricus bisporus* mushroom. All eggs laid

during this 7 h period were retained in the culture. Cultures were kept at 22°C. Emergent adults were sexed and counted daily upon emergence.

To determine the age at which individuals are willing to mate, we collected adults that had emerged during the previous 21 h. The emergent flies were separated by sex. At various times after this collection, males or females were placed individually for 2 h with two reproductively mature, 1-week-old individuals of the opposite sex. Flies were observed for 2 h to monitor courtship and mating activity. After the 2 h observation period, the males and females were separated, and the females were placed individually in culture vials to determine if they could produce viable offspring. Twenty males were tested 24 h after the collection. Females were tested 24 h ($n = 20$), 48 h ($n = 20$) and 72 h ($n = 16$) after the initial collection. The flies were kept at 22°C throughout the experiment.

RESULTS

Clutch size

Of the 30 females set up, 24 produced viable offspring and our analysis is confined to these. We use the number of emergent flies per mushroom as a measure of how many eggs were laid. For all 24 females, the standardized Morisita index of dispersion was greater than 0.5, indicating statistically significant clustering of eggs across mushrooms. Figure 1 shows that females laid essentially all of their eggs on only one or two of the four mushrooms, typically in clutches of 15–30 eggs per mushroom, assuming 100% egg to adult survival. Given that females lay eggs in clutches when other suitable mushrooms are only centimetres away, it is probable that egg laying in clutches also occurs in the field, where mushrooms are usually much farther apart.

Fitness effects of larval competition

As the density of eggs on a mushroom increases, the probability of egg to adult survival and dry mass of emergent adults both declined (Fig. 2). Using each replicate vial as a data point, the correlation between egg density and probability of egg to adult survival is -0.59 ($P < 0.01$) and that between egg density and mean adult mass is -0.81 ($P < 0.01$). The total mass yield of flies from a culture (number of flies $\times$ adult mass) increased over an egg density range of 10–40, but then plateaued at higher egg densities (Fig. 3). This may have important effects on *Wolbachia* dynamics. Suppose each mushroom receives a large number of eggs, but from only one female. Although *Wolbachia* infection halves the density of larvae by killing males, the total F1 biomass yield of infected and uninfected females may be similar. However, whereas the offspring of uninfected females includes equal numbers of males and females, all of the offspring of infected females are daughters. Thus, if only one female oviposits per mushroom and there is significant larval crowding, an infected female may produce twice the biomass of F1 females as an uninfected female. Because of the complex and variable nature of allometry between body mass and female fitness (Starmer *et al.*, 2003), one cannot equate total female yield with expected transmission of infected or uninfected types in the next generation. Thus, the quantitative effect of reduced larval density on the fitness of an infected female's daughters is unknown.

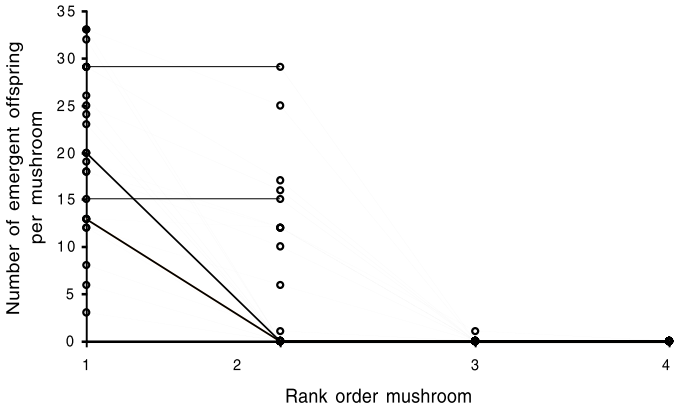

Fig. 1. Distribution of emergent offspring numbers from eggs laid by individual females of *D. innubila* with access to four mushrooms for 24 h. Bold lines denote pairs of females with identical egg distributions. The data show that most females laid all of their eggs on only one or two of the four available mushrooms.

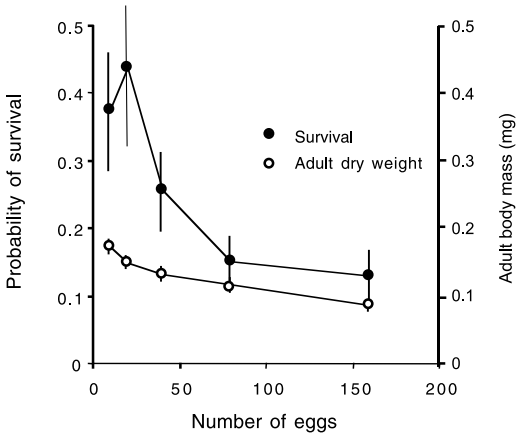

Fig. 2. Effects of egg density on egg to adult survival and adult body mass in cultures containing 1.0 g of *Agaricus bisporus* mushroom.

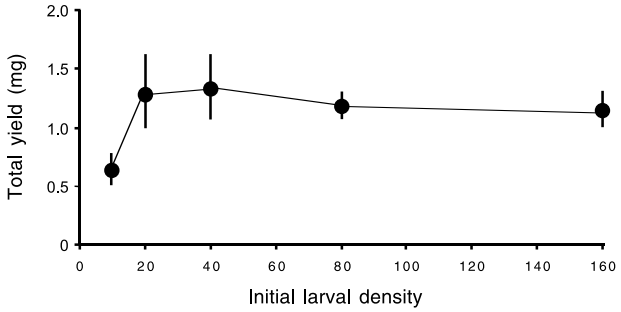

Fig. 3. Total yield of female flies (number of emergent adults × mean body weight) as a function of initial egg density on 1.0 g of *Agaricus bisporus* mushroom.

Competition experiment

The fraction of females among the emergent adults in the experimental populations is shown in Fig. 4. In the early generations of the experiment, before the population bottleneck following generation 5, there was a very noticeable difference between the sib competition and mass competition populations. A repeated-measures analysis of variance reveals a highly significant effect of treatment ($F = 422$, $P < 0.0001$), but no significant difference among replicate populations within treatments ($F = 0.80$, $P = 0.54$). Using the data from all 13 generations, a repeated-measures analysis of variance still yields a significant treatment effect ($F = 13.3$, $P = 0.02$), but a very significant difference among replicates within treatments ($F = 11.4$, $P < 0.001$). We suspect that the difference among replicates in the latter analysis is due to the exaggerated effects of drift following the population bottleneck that occurred as a result of the laboratory move from Tucson to Rochester.

In each of the three sib competition populations, the fraction of the population that was female rapidly increased through time, reaching 100% in generations 3, 6 and 7 in the three replicates (Fig. 4). In each of the three populations, the prevalence of *Wolbachia* infection increased rapidly, reaching 100% in the final generation for each population (Fig. 5). These results are consistent with the hypothesized transmission advantage of male-killing *Wolbachia* in populations that experience significant levels of sib competition for resources.

As the *Wolbachia* infection spread within the sib competition populations, the proportion of females producing viable progeny, and thus which must have mated, declined from one generation to the next, presumably because of a deficiency of males in these populations (Fig. 6). As a consequence, the total number of females used as parents – either 100, the predetermined carrying capacity of breeding females, or the total number of female offspring produced in the population, whichever was greater – also declined. Eventually, the prevalence of *Wolbachia* infection became so high that no females mated, resulting in population extinction (Fig. 6).

The sex ratio dynamics in the mass competition lines were less consistent. Over the first five generations, the three mass competition lines were similar to each other, showing relatively little temporal change in population-level sex ratio. Subsequently, however, the populations diverged in both sex ratio and prevalence of *Wolbachia* infection (Figs 4 and 5).

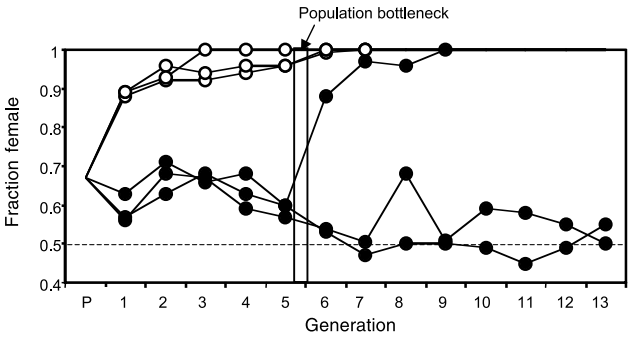

Fig. 4. Fraction of adult population that is female shown for the 13 generations of the competition experiment. A population bottleneck occurred at generation 5 during the experiment. ○, sib competition populations; ●, mass competition populations. The 2/3 proportion female in the parental generation (P) is based on the expected proportion of females in a pooled population of 50% *ST-1* and 50% *mk-3*, assuming all individuals except infected males have equal probabilities of survival.

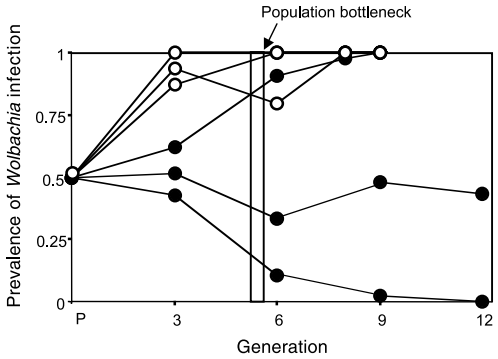

Fig. 5. Prevalence of *Wolbachia* infection among adult females during the competition experiment. Fifty female flies were sampled every three generations. ○, sib competition populations; ●, mass competition populations.

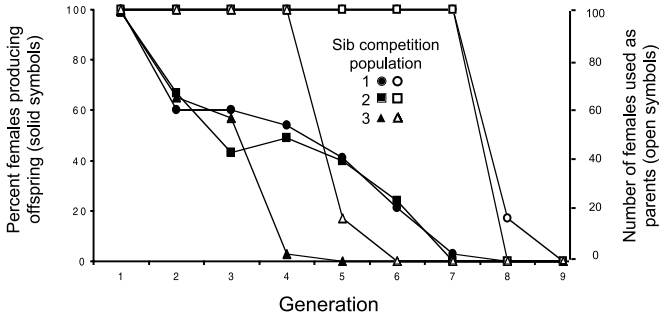

Fig. 6. Proportion of females producing offspring (solid symbols) and number of females used as parents each generation (open symbols) in the three sib competition populations.

In one population (mass 3), the fraction of females increased to 100%, after which it went extinct. In a second replicate, the proportion of females declined to around 50% and the *Wolbachia* infection was lost from the population. The sex ratio in the third population declined somewhat from the starting value but remained significantly greater than 50%, and this population remained polymorphic for *Wolbachia* infection to the end of the experiment. It is important to bear in mind that, as a result of the laboratory move and resulting population bottleneck, the dynamics of these experimental populations after generation 5 were more severely affected by drift.

Horizontal transmission

The dark-eyed flies PCR screened for infection were the offspring of uninfected mothers, and thus a positive signal for *Wolbachia* would indicate that they had become infected via horizontal transmission within the cultures. In fact, none of the ~250 dark-eyed flies were positive for infection, whereas all 12 control flies from the male-killing *mk-3* line were unambiguously positive for *Wolbachia* infection. These results do not rule out the possibility of very rare horizontal transmission events, but they do indicate that the rate of

horizontal transmission, if it occurs at all, is epidemiologically negligible within the context of the competition experiment.

Among the flies present in the mass 3 population at the end of the experiment, all of them tested positive for *Wolbachia* infection ($n = 49$). All of these flies carried the mtDNA haplotype characteristic of the originally infected *mk-3* strain, and none carried the *ST-1* haplotype. Thus, the spread of *Wolbachia* within this population was not due to horizontal transmission from *mk-3* to *ST-1* flies.

Maturation time

An analysis of variance of egg to adult development time showed a significant effect of culture vial ($F = 55.8$, $P < 0.001$), with longer mean development time in the vial with a higher larval density. There was no significant vial × sex effect ($F = 0.89$, $P = 0.35$) and, more importantly, there was no effect of sex ($F = 0.12$, $P = 0.73$). Males and females had essentially identical development times: 11.22 ± 0.06 days for males ($n = 145$) and 11.25 ± 0.06 days for females ($n = 155$) (mean ± standard deviation).

Among the males tested 24 h after collection, 18 of 20 mated within the 2 h observation period, with 12 of them producing viable offspring from these matings. Thus, males of *D. innubila* become reproductively mature in less than 2 days after emergence. Of the females tested 24 h after collection, none of the 20 mated within the 2 h observation period, even though males actively courted them. Of the females tested 48 h after collection, 7 of 20 mated within the observation period, with all 7 producing viable offspring. Of the females tested 72 h after collection, 11 of 16 mated within the observation period, 10 of which produced viable offspring. Thus, females are unreceptive for at least 24 h after emergence.

DISCUSSION

The most widely accepted hypothesis to explain the spread of maternally transmitted male-killing endosymbionts (Hurst, 1991) assumes that an increase in the fitness of infected females results from the death of their brothers. From the endosymbiont's perspective, this is a form of kin selection, as transmission via females is enhanced by the death of males carrying the same endosymbionts. Kin selection-based spread of endosymbionts requires a type of host population structure that brings about negative interactions between relatives. Such a population structure may be manifest at the adult breeding stage of the host, in which case male death reduces inbreeding depression (Werren, 1987), or at the stage where trophic interactions are most important, in which case male death would reduce the adverse effects of cannibalism or competition for resources. Our results on egg to adult development time and age of reproductive maturity provide little support that inbreeding depression could drive the spread of male-killing *Wolbachia* within *D. innubila*. We found that males and females have nearly identical development times and that adult females are unreceptive to courting males for at least 24 h after emergence. It is therefore probable that females will have dispersed away from the vicinity of their larval breeding site before they are willing to mate. Thus, the mating structure of *D. innubila* populations is likely to be panmictic, rather than inbred, at the local level.

Despite its theoretical appeal, the effect of the population-level structure of competitive interactions on the dynamics of male-killing endosymbionts has not previously been tested experimentally. Previous experimental studies of the dynamics of male-killing endo-

symbionts have considered only mass competition situations. For instance, Ikeda (1970) followed the changes in the infection prevalence of a male-killing *Wolbachia* in experimental populations of *Drosophila bifasciata*, with the starting populations containing 1000 female flies. In five of the six experimental populations, the prevalence of infection declined significantly, indicating that infected females had lower fitness in these highly competitive environments. However, Ikeda's results provided no indication about how such male killers could spread within a population.

In the present experiments, we found a dramatic difference in *Wolbachia* dynamics between populations with unstructured larval competition and populations in which all larval competition was between siblings. Thus, our results are the first experimental demonstration of the power of sib competition to favour the spread of maternally transmitted male-killing endosymbionts. However, we do not yet know if the population-level structure of larval competition in natural populations of *D. innubila* favours the spread of these *Wolbachia*.

Findings on *Drosophila falleni*, the closest known relative of *D. innubila* (Perlman *et al.*, 2002), have some bearing on this question. Genetic analyses of *D. falleni* bred from field-collected mushrooms reveal significant population structure at the level of individual mushrooms (Jaenike and Selander, 1979). Thus, the flies emerging from a mushroom are more closely related to each other than flies emerging from different mushrooms at the same site and time. These genetic data indicate that females lay eggs in clutches on individual mushrooms in the field, as we found in our laboratory experiment with *D. innubila*. Thus, the egg-laying behaviour of *D. falleni* meets one requirement for invasion by a male-killing *Wolbachia*. Similar genetic methods have been used to show that *D. melanogaster* females lay multiple eggs on individual breeding sites (Hoffmann and Nielsen, 1985). Interestingly, a male-killing, maternally transmitted spiroplasma has recently been discovered in this species (Montenegro *et al.*, 2000). It is worth noting that multiple mating by females, which may be common in *Drosophila*, can affect certain evolutionary processes and will reduce the degree of relatedness among a female's offspring. However, multiple mating does not affect the degree of relatedness, and hence the kin selection-based population dynamics, of cytoplasmic elements carried by her offspring.

Field experiments have shown that there is often significant larval competition for resources among mycophagous *Drosophila* (Grimaldi and Jaenike, 1984), which could favour the spread of male-killing endosymbionts. However, emergence data from several mushroom collections in different sites indicate that individual mushrooms are often oviposited on by several females of *D. falleni* as well as by females of other *Drosophila* species (Jaenike and James, 1991). Thus, although females of *D. falleni* lay eggs in clutches, the ratio of sib competition to total competition within mushrooms may be unfavourable for the spread of male-killing endosymbionts within this species.

Which species are most susceptible to invasion by male-killing endosymbionts? If all else is equal, species that lay eggs in clutches should be more susceptible than those that lay eggs singly or randomly among suitable substrates. Some closely related species of Lepidoptera differ in this respect (Thompson and Pellmyr, 1991), providing a potential opportunity for independent contrasts of clutch size and infection with male-killing bacteria. Furthermore, Hurst and McVean (1998) have shown theoretically that a species already infected with a male-killing endosymbiont should evolve a larger clutch size, a result implicit in the earlier work of Parker and Courtney (1984). Thus, infection with a male killer imposes selection for a life-history trait (larger clutch size), which, in turn, favours the spread of an existing

male killer or invasion by new ones. Perhaps this sort of evolutionary positive feedback is responsible for the presence of multiple male-killing bacteria within a single host species (e.g. Schulenburg *et al.*, 2002).

Finally, an important development in community ecology is the aggregation model of co-existence of species that use patchy, ephemeral resources, such as fruits, fungi, dung and carrion (Shorrocks *et al.*, 1979; Hartley and Shorrocks, 2002). Intraspecific aggregation across breeding sites, which can result from eggs laid in clutches, contributes to the stable co-existence of multiple species on a single resource type. A recent field study of tropical *Drosophila* communities has shown that the average level of intraspecific aggregation is positively correlated with local species diversity (Krijger and Sevenster, 2001). Consequently, species within diverse communities of insects utilizing such ephemeral resources may be especially vulnerable to invasion by male-killing endosymbionts.

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