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No resistance to male-killing *Wolbachia* after thousands of years of infection

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Abstract

Maternally transmitted male-killing endosymbionts can exert strong and relentless selection pressure on their hosts to evolve resistance to these infections. Surveys of current infection prevalence and mtDNA diversity indicate that *Drosophila innubila* is and has been infected with male-killing *Wolbachia* at moderate frequencies for extended evolutionary periods. Here, we use coalescent simulations to infer the minimum age of the *Wolbachia* infection in this species, and estimate that the infection is at least 15 000 and perhaps over 700 000 years old. We also surveyed this species for genetic variation for resistance to the male-killing effects of infection. Our surveys revealed no evidence for any resistance polymorphism, such that all flies are completely susceptible to male killing. Given the general assumption that *Drosophila* can be selected for anything, the lack of resistance, despite thousands of years of strong selection, is an apparent evolutionary conundrum. We hypothesize that resistance requires a mutation of major effect that acts early in development, and that the adverse pleiotropic consequences of such mutations in both infected and uninfected individuals may exceed the possible benefit to infected flies.

Introduction

The endosymbiotic bacterium *Wolbachia* may infect half or more of all species of insects, spreading and persisting by various means of host reproductive manipulation (Werren *et al.*, 1995; Werren & Windsor, 2000; Kikuchi & Fukatsu, 2003; Kittayapong *et al.*, 2003; Tagami & Miura, 2004; Haine & Cook, 2005; Kyei-Poku *et al.*, 2005; Mateos *et al.*, 2006; reviewed in Werren, 1997; Stouthamer *et al.*, 1999; Hilgenboecker *et al.*, 2008). These reproductive manipulations can result in considerable mortality in the host population. Cytoplasmic incompatibility (CI) is the most widely documented mechanism and results in substantial host mortality during the initial spread of the *Wolbachia* through a population, killing many or all of the offspring produced in matings between infected males and uninfected females (Caspari & Watson, 1959). However, once CI-causing *Wolbachia* reach a high equilibrium prevalence of infection, there is little subsequent *Wolbachia*-induced mortality.

By contrast, male killing, which entails the death of most of the sons of infected females, results in ongoing mortality equal to ~1/2 the prevalence of infection among females. The equilibrium prevalence of infection is determined by the transmission rate and selective effects of the infection (Hurst, 1991). Thus, there can be very strong and persistent selection on the host population to resist the male-killing effects of these infections or prevent their transmission. However, among the species or populations that suffer from male-killing *Wolbachia*, the few that have been studied experimentally show little evidence of harbouring genetic variation for resistance. On the Japanese island Honshu, about 5–7% of the females of *Drosophila bifasciata* are infected with male-killing *Wolbachia*, but there is no evidence for genetic variation among 25 inbred lines for resistance to male killing (Hurst *et al.*, 2001), nor is there resistance in flies

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from the nearby island of Hokkaido, where *D. bifasciata* is not infected with *Wolbachia* (Veneti *et al.*, 2004). Many populations of the African butterfly *Acraea encedon* have a very high prevalence of infection (> 95%) by male-killing *Wolbachia* (Jiggins *et al.*, 2000a, b). Jiggins *et al.* (2002) found that every single infected wild-caught female produced exclusively female offspring and that all males were uninfected, indicating that there is no genetic variation for transmission or resistance to the male-killing effects of these *Wolbachia*.

One possible explanation for the lack of resistance is that the *Wolbachia* present an insoluble problem, and that it is simply not possible for insects to evolve resistance. Such an explanation, however, is contradicted by recent studies on Lepidoptera and *Drosophila*. First, the butterfly *Hypolimnas bolina* exhibits regional variation in resistance to the male-killing effects of *Wolbachia* infection (Hornett *et al.*, 2006). By using reciprocal introgressions, Hornett *et al.* (2006) showed that the butterflies from Southeast Asia are resistant, whereas those from South Pacific islands are susceptible. More recently, a rapid spread of alleles conferring resistance to the male-killing effect of these *Wolbachia* infections has been documented in two island populations of these butterflies (Charlat *et al.*, 2007).

The other cases involve interspecific transmission of *Wolbachia*, revealing male killing in a novel host species. *Drosophila recens* is ~98% infected with *Wolbachia* that causes CI (Shoemaker *et al.*, 1999). When introgressed into *Drosophila subquinaria*, the sister species of *D. recens*, this same *Wolbachia* strain causes severe male killing in some strains but not others. Although not infected in nature, *D. subquinaria* is polymorphic for resistance to the male-killing effects of these *Wolbachia*. Crosses between *D. recens* and *D. subquinaria* show that resistance to male killing is strongly dominant, suggesting that *Wolbachia* may have initially caused male killing in *D. recens* (Jaenike, 2007a). Similarly, a strain of *Wolbachia* that causes CI in its native host, the moth *Cadra cautella*, causes male killing following transfection into the novel host *Ephestia kuehniella* (Sasaki *et al.*, 2002, 2005), again suggesting that the native host may have evolved resistance to the male-killing capacity of this *Wolbachia* strain.

If resistance is possible at the level of individuals or strains (*D. subquinaria* and *H. bolina*), populations (*H. bolina*) or species (*D. recens* and *C. cautella*), why do other species currently infected with male killers not exhibit genetic variation for resistance? Two explanations seem possible. First, if the prevalence of infection is low, as it is in some species (Hurst & Jiggins, 2000), the resulting weak selective advantage of resistance may be insufficient to outweigh possible costs. Second, there may have been an inadequate pool of favourable mutations during the history of the male-killing infection. This could occur for at least two reasons. The rate of input of new mutations depends on the number of *uninfected* individuals, because mutations that occur in infected individuals are quickly lost from the population, having an expected half-life of just one generation (Engelstädter & Hurst, 2007; Jaenike, 2007b). Paradoxically, therefore, an extremely high prevalence of a male-killing infection might reduce the effective population sufficiently to prevent the evolution of resistance. This paradox applies specifically to resistance alleles that are recessive, as such mutations arising in infected individuals never have a chance to become homozygous and thus express the resistance phenotype.

Alternatively, a male-killing infection may be so recent that there has not been sufficient time for the occurrence and spread of favourable mutations. For instance, individuals of *A. encedon* infected with specific strains of *Wolbachia* exhibit no mtDNA sequence variation, suggesting that these infections – or at least the most recent species-wide sweep of a new variant – occurred in the recent evolutionary past (Jiggins, 2003). In fact, the finding that uninfected females carry different mtDNA haplotypes from those of infected females indicates that this association has not yet reached a theoretically expected transmission–selection equilibrium, assuming that such an equilibrium is maintained by a balance between selection and incomplete transmission (Dyer & Jaenike, 2004).

In this paper, we ask whether *Drosophila innubila* is polymorphic for resistance to the male-killing *Wolbachia* with which it is infected. In this species, *Wolbachia* kills males during the embryonic stage, reducing egg hatch rate of infected females by 50% (Dyer *et al.*, 2005). *Drosophila innubila* is not subject to the limitations on evolution of resistance discussed above. This species experiences an intermediate prevalence of infection (10–40% in different sky island populations), which maintained by a balance between a presumed selective advantage to infected female lineages because of the death of male siblings, which can reduce competition for larval resources, and incomplete maternal transmission of the *Wolbachia* (Dyer & Jaenike, 2004). This prevalence of infection is sufficiently high to impose strong selection for resistance, but low enough for there to be large effective population size of uninfected flies from which to draw favourable mutations.

The strength of selection for resistance can be considered as follows. Assume that an uninfected female lays some number of eggs that results in the production of 100 male and 100 female viable offspring. An infected female laying the same number of eggs will produce about three male offspring, because of incomplete maternal transmission, and about 105 female offspring, because of the estimated ~4–5% selective advantage to infected female lineages (Dyer & Jaenike, 2004). Thus, the net selective advantage of an allele that confers complete resistance to male killing would be proportional to the difference in offspring production because of infection times the prevalence of infection (~0.3) or about 0.15.

Although there is a significant female-biased sex ratio in the Chiricahuas population of adult flies (Dyer & Jaenike, 2005), almost all wild-caught females have been inseminated (J. Jaenike, unpublished data), indicating that a potential shortage of males as mates does not substantially reduce effective population size of breeding females. Indeed, silent site diversity at nuclear genes is very high in *D. innubila* (mean $\pi = 3.6\%$ across five loci; Dyer & Jaenike, 2004), supporting the conclusion that N_e is large (for comparison with eukaryotes, see Lynch & Conery, 2003). The occurrence of mtDNA sequence variation among infected flies, as well the similar frequencies of different mitochondrial haplotypes in the infected and uninfected components of the population, indicates that the infection is old enough for the prevalence of infection to have reached transmission–selection equilibrium and for there to have occurred several mutations since the initial infection (Dyer & Jaenike, 2004). Below we present coalescent simulations showing that *D. innubila* has probably been infected with *Wolbachia* for tens of thousands of years, if not longer. Thus, there has been intense selection over a prolonged evolutionary period to resist male killing and a large effective population from which to draw mutations conferring resistance. Clearly, *D. innubila* would be expected to manifest some genetic resistance to male-killing *Wolbachia*. We test this prediction below, testing explicitly for resistance that depends on either the maternal or the zygotic genotype of infected flies.

Methods

Age of the infection

We have previously argued that the *Wolbachia* infection in *D. innubila* is evolutionarily old, but had not attempted to estimate the age of the infection (Dyer & Jaenike, 2004). Our approach assumes that the *Wolbachia* in present-day populations of *D. innubila* are descended from a single ancestral infection event in this species. There are two lines of evidence in support of this assumption. First, all of the *Wolbachia* in *D. innubila* that we have examined have the same unique sequence for *wsp*, the most rapidly evolving gene known in *Wolbachia* (Zhou *et al*., 1998). Secondly, the mitochondrial diversity in *D. innubila* is much lower than that in its nearest relative, *Drosophila falleni*, even though the two species have similar levels of nuclear diversity; all of the mitochondria in *D. innubila* are closely related to each other, as would be expected if they were all descended from a single infected individual (Dyer & Jaenike, 2004).

To infer a minimum age of the *Wolbachia* infection in *D. innubila*, we used the mtDNA *Cytochrome Oxidase I* (*COI*) gene of *D. innubila*, because the genealogy of the mtDNA reflects the evolutionary history of the co-transmitted *Wolbachia*. We used 30 individuals from the Chiricahua Mountains (Arizona) population of *D. innubila* that were randomly sampled with respect to *Wolbachia* infection status (GenBank AY541182–AY541211; Dataset I in Dyer & Jaenike, 2004). As described in Dyer & Jaenike (2004), there are seven silent segregating polymorphisms in 1473 bp of coding sequence (of which 348 are silent sites), resulting in silent site diversity estimates of $\pi = 0.00345$ and $\theta = 0.00508$.

We estimated the time to the most recent common ancestor (TMRCA) and the effective population size of the mtDNA ($N_{e\text{-mtDNA}}$) using BEAST version 1.4.6 (Drummond & Rambaut, 2007). We used data at silent sites only, a Hasegawa–Kishino–Yano (HKY) substitution model of sequence evolution with gamma distributed rate variation and allowing for a fraction of invariant sites (to allow for the possibility that some fraction of silent sites are subject to selection, due, for example, to mRNA secondary structure), assumed a constant population size, and enforced a strict molecular clock. For each run, we ran the chain for 10×10^6 iterations, sampling every 1000 steps and excluding the first 1×10^6 steps as burn-in. We ran the chain several times to ensure convergence among chains, and then estimated the posterior density of each parameter, including the mean, median and 95% confidence intervals by combining the log files from the three optimized and independent runs of BEAST. We used the program TRACER version 1.4 (available at http://beast.bio.ed.ac.uk) to analyse the output from BEAST.

To calibrate the molecular clock, we used a mutation rate of $1.5–5.8 \times 10^{-8}$ substitutions/site/year. To arrive at this estimate, we used two previously published rates of nuclear evolution, and then accounted for the ratio of mtDNA : nuclear evolution. For nuclear synonymous sites, a neutral substitution rate of $\sim 1.5 \times 10^{-9}$ substitutions/site/generation has been inferred from divergence data (Andolfatto & Przeworski, 2000; McVean & Viera, 2001) and a nuclear mutation rate of 5.8×10^{-9} substitutions/site/generation that was estimated directly from *Drosophila* mutation accumulation lines (Haag-Liautard *et al*., 2007). This later estimate may be biased towards slightly deleterious mutations, but because the mtDNA is under strong purifying selection this may reflect the type of diversity segregating in the mtDNA. Moriyama & Powell (1997) suggest that synonymous sites in the mtDNA evolve at a rate that is 4.5–9 times faster than nuclear sites; so, to account for faster evolution of the mtDNA compared with nuclear DNA, we conservatively estimated that the mtDNA evolves 10-fold faster than nuclear genes. Finally, we assumed that *D. innubila* has one generation per year, which is thought to be in line with the biology of these flies (W.B. Heed, personal communication).

Genetic variation for resistance to male killing

Drosophila innubila males were collected in the eastern side of the Chiricahua Mountains in the general vicinity

of Portal, Arizona, in 2004 and 2005. The areas from which they were collected included two areas in 2004 (Southwest Research Station and Turkey Creek) and three areas in 2005 (Southwest Research Station, Cave Creek and Herb Martyr). These areas have the most consistently high densities of *D. innubila* that we have encountered anywhere, and they are the same areas in which we previously estimated infection prevalence and nuclear and mtDNA diversity (Dyer & Jaenike, 2004). The infection prevalence is high (30%), as is nuclear diversity at these sites; thus, these areas are expected to be particularly favourable for the occurrence of resistance polymorphism in *D. innubila*.

Wild-caught males were mated to laboratory-reared, *Wolbachia*-infected, virgin females of strain *mk-3* (Jaenike *et al.*, 2003). Each male was placed with either three or four females for 4 days to allow mating, after which the females were separated and placed individually in culture vials. All emergent offspring were sexed and counted.

If a male carried a dominant autosomal allele conferring resistance to male killing and if resistance were a function of zygotic, rather than maternal, genotype, such a male would sire significant numbers of viable sons with each of the females to which it was mated. Alternatively, the production of sons might occur if a specific female used in one of the crosses carried a low-density *Wolbachia* infection (Dyer *et al.*, 2005). Crossing individual males to several females reduces the risk of falsely concluding that particular males carried zygotically acting resistance factors.

Assaying the offspring of wild males will uncover only paternally derived and zygotically acting dominant genes that contribute to resistance. However, resistance might also depend on the mother's genotype. To test this possibility, the F1 female offspring of the wild-caught males were mated to laboratory-reared males of an uninfected strain (strains *ST-1* in 2004 and *SWRS 2005–50* in 2005, both of which were descended from single uninfected females collected in the Chiricahuas). Because the females used in the original cross were infected, so should be the F1 females. For each of the three to four full-sib families per wild-caught male, we set up four (2004) or two (2005) single pair crosses between the F1 females and laboratory-reared males. The production of F2 males in these crosses would suggest the existence of dominant maternal effect genes that confer resistance to male killing, because the survival of male offspring would depend on their mother's genotype rather than their own. Alternatively, the production of F2 males could result from reduced transmission of *Wolbachia* by the F1 females carrying particular alleles from the wild-caught males.

In the rare cases where some viable F2 males were produced in the crosses, these F2 males were mated to laboratory-reared *Wolbachia*-infected females (strain *mk-3*), and the resulting F3 females were mated to uninfected males in follow-up crosses as above. All of the resulting F3 and F4 offspring were sexed and counted to confirm whether the original male carried alleles conferring resistance to male killing.

Results

Age of the infection

To obtain a minimum age of the *Wolbachia* infection, we used the coalescent time of the *COI* gene in the mtDNA, as the mtDNA genealogy reflects the infection history of the endosymbiont. As summarized in Table 1, the mean TMRCA for COI was about 90 000 years assuming the higher mutation rate of 5.8×10^{-8} substitutions/site/year, and about 350 000 years assuming the lower mutation rate of 1.5×10^{-8} substitutions/site/year. Including both mutation rates, the 95% highest posterior density (HPD; similar to the 95% confidence interval) ranges between 15 000 years at the low end and 730 000 years at the very high end. Note that this analysis yields only the time to the last common ancestor of extant mitochondrial haplotypes. Because the *Wolbachia* infection itself could be much older, this is a minimum age of the infection within *D. innubila*.

We also used BEAST to estimate the effective population size of the mtDNA. Because of the association with *Wolbachia*, $N_{e\text{-mtDNA}}$ is determined primarily by the effective population size of the infected females only, which can be used to infer the long-term effective prevalence of *Wolbachia* infection. The inference of N_e from polymorphism data depends on mutation rate, with a higher mutation rate suggesting a lower N_e for a given level of variation. As shown in Table 1, assuming the higher mutation rate of 5.8×10^{-8} substitutions/site/year yields an inferred $N_{e\text{-mtDNA}}$ of about 55 000, whereas the lower mutation rate of 1.5×10^{-8} substitutions/site/year yields $N_{e\text{-mtDNA}}$ ~213 000. The 95% HPD across both mutation rates ranges from 12 000 to 428 000 infected females.

Based on our previous estimates of the effective population size of $6–10 \times 10^6$ for nuclear genes (Dyer & Jaenike, 2004), we can use the ratio of the overall

Table 1 Estimates of the time (years) to the most recent common ancestor (TMRCA) and effective population size of the mtDNA ($N_{e\text{-mtDNA}}$) based on silent polymorphism in the mtDNA *COI* gene.

	$\mu = 1.5 \times 10^{-8}$ substitutions/site/year		$\mu = 5.8 \times 10^{-8}$ substitutions/site/year	
	TMRCA	$N_{e\text{-mtDNA}}$	TMRCA	$N_{e\text{-mtDNA}}$
Mean	344 200	213 067	89 017	55 103
Median	283 333	190 867	73 276	49 362
95% HPD lower*	61 060	428 400	15 791	110 793
95% HPD upper*	732 667	45 180	189 483	11 684

*Lower and upper bounds of the 95% highest posterior density (HPD) interval.

(nuclear) N_e to the $N_{e\text{-mtDNA}}$ to infer a long-term infection prevalence of 2–14%. This is lower than our previous estimate for the Chiricahua population of *D. innubila* (Dyer & Jaenike, 2004), in which we did not account for the faster mutation rate of the mtDNA compared with nuclear genes. However, the estimated range (2–14%) does encompass the 13% species-wide equilibrium infection prevalence estimated using the ratio of population differentiation of the mtDNA vs. nuclear loci (Dyer & Jaenike, 2005).

Genetic variation for resistance

Pooled across all matings for all males collected and tested in 2004 and 2005, the F1 offspring of the 73 wild-caught males mated to infected females comprised two males and 8706 daughters (Table 2). Thus, we see no evidence for the existence of dominant zygotically acting alleles that confer resistance to male killing.

Among the F2 (produced by crosses between F1 females, which are infected, and uninfected males), pooled across all matings for 2004 and 2005, there were a total of 105 sons and 19 166 daughters. The overall 0.5% fraction of males produced in these crosses tended to be clustered in very small number of specific matings, as indicated in Table 2. While such clustering could result from genetic variation for resistance to male killing, it could also result from variation among the F1 females in the density of their *Wolbachia* infections, which can arise as a result of stochastic variation in the number of *Wolbachia* transmitted to eggs. Elsewhere, we have shown that low-density infections can lead to the production of viable male progeny (Dyer *et al.*, 2005).

Two lines of evidence indicate that the production of F2 males in these crosses is not because of genetic variation for resistance. First, the daughters of the other females to which the wild-caught males were mated did not produce an excess of F2 males (Table 2), as would have been expected if the production of such males were genetically based. There is a combined low probability ($P = 0.5^6 = 0.016$) that these results could have resulted from segregation in a heterozygous male, such that one female in each family inherited the resistant allele and all other tested females ($n = 6$) inherited the susceptible allele. More convincingly, follow-up crosses on these male-yielding crosses resulted in the production of 100% female offspring ($n = 4972$) in all F3 and F4 families (Table 3). Thus, there was no evidence for transmission of alleles conferring resistance to male killing, acting either at the zygotic level or as maternal effects.

Table 2 Offspring sex ratios produced in matings between wild-caught males and *Wolbachia*-infected, laboratory-reared females.

Year	No. males tested	F1		F2	
		Male*	Female	Male†	Female
2004	30	0	4567	68‡	13 637
2005	43	2	4139	37§	5509

*Indicative of resistance based on zygotic genotype.
†Indicative of resistance based on maternal genotype.
‡Twenty three of these males, along with 167 females, were produced by F1 females that had been produced by wild-caught male (S10) × female (D) cross. The F1 females produced by the other three females mated to this male yielded four sons and 589 daughters among the F2.
§Thirteen of these males, along with 37 daughters, were produced from a single wild-caught male (HM-4) × female (1) cross. The other two females mated to this male yielded 0 male and 82 females among the F2. Twelve of the males, along with 142 females, were produced by SWRS-16 × female (2) cross. The other female mated to this male produced 0 male and 146 females among the F2.

Table 3 Offspring sex ratios in follow-up crosses to test whether males that sired sons or grandsons in the original set of crosses carried alleles conferring resistance to male killing.

Year	Ancestral wild-caught male	No. of F2 males tested	F3		F4	
			Male*	Female	Male†	Female
2004	S10	45	0	1251	0	1317
2005	SWRS-16	2	0	116	0	448
	HM-4	12	0	495	0	1345

*Indicative of resistance based on zygotic genotype.
†Indicative of resistance based on maternal genotype.

Discussion

Despite very strong and evolutionarily prolonged selection on *D. innubila* to evolve resistance to the male-killing effects of *Wolbachia* infection, our crosses failed to uncover any genetic variation for resistance, whether expressed through the maternal or zygotic genotype. This lack of variation stands in striking contrast to the generally accepted notion that genetic variation is ubiquitous and that almost any trait can be selected (e.g. Lynch & Walsh, 1998; Barton & Partridge, 2000). In a general discussion of genetic variation in *Drosophila*, Lewontin (1974, p. 92) stated, 'There appears to be no character – morphogenetic, behavioural, physiological, or cytological – that cannot be selected in Drosophila… The suggestion is very strong, from the extraordinary variety of selection responses, that genetic variation relevant to all aspects of the organism's development and physiology exists in natural populations'. However, Blows & Hoffmann (2005) have recently questioned the presumed ubiquity of genetic variation, suggesting that the prevailing view may have been skewed by the use of a small number of generalist model species, such as *Drosophila melanogaster*.

What can explain the apparent lack of resistance to male killing in *D. innubila*? One possibility is that our experimental assay would not detect resistant alleles that are recessive. This is because the infected females used in our crosses to wild males were from a strain in which

Wolbachia causes ~100% male killing; these females therefore are presumably homozygous for susceptible alleles. However, a newly arisen resistance allele that is recessive is not likely to spread within a *Wolbachia*-infected population, because, being very rare initially, it would occur almost exclusively in heterozygotes. Upon passage into the infected portion of the population, such a recessive allele would not express resistance and thus would be quickly eliminated (Engelstädter & Hurst, 2007). This theoretical expectation is supported by both of the known cases of male-killing suppressors (*H. bolina* and *D. recens – D. subquinaria*), in which resistance is dominant, although there is one possible example of recessive resistance to an unknown maternally transmitted male killer in *Drosophila prosaltans* (Cavalcanti *et al.*, 1958).

It is also possible that *D. innubila* is polymorphic for resistance, but that resistance alleles are so rare that we did not obtain any in our sample. Our 2-year sample included 73 males, or 146 haploid genomes. Therefore, for any potential autosomal resistance locus, we found 0/146 resistant alleles, with a 95% confidence limit of 0–2%. Thus, if *D. innubila* is polymorphic for resistance, then the likely frequency of alleles conferring resistance is less than 2% at every locus that might harbour relevant variation.

Several studies besides ours have found little or no genetic variation for resistance to male-killing *Wolbachia* (Hurst *et al.*, 2001; Jiggins *et al.*, 2002; Veneti *et al.*, 2004). Among species naturally infected with potentially male-killing *Wolbachia*, resistance to male killing has been found in both Lepidoptera (Sasaki *et al.*, 2002, 2005 Hornett *et al.*, 2006; Charlat *et al.*, 2007) and *Drosophila* (Jaenike, 2007b), but in these cases the resistance characterizes an entire species or population, or it has spread very rapidly within a population.

Nevertheless, there are reasons to expect that resistance, should it arise, may be polymorphic within an infected population. One can imagine resistance to male-killing *Wolbachia* acting in several ways: (i) reducing the transmission fidelity of the infection, so that fewer male offspring inherit *Wolbachia* from their infected mothers; (ii) reducing the density of *Wolbachia* and thus the probability of mortality in infected males (Anbutsu & Fukatsu, 2003; Dyer *et al.*, 2005); and (iii) being resistant to the male-killing effects of a normal-density infection. In theory, the equilibrium prevalence of a male-killing *Wolbachia* infection depends on the fitness advantage conferred by male killing (the infected sisters benefit because of the death of their brothers) and the transmission fidelity of the infection (Hurst, 1991; Dyer & Jaenike, 2004). The resistance mechanisms mentioned above reduce either the transmission rate (i) or selective benefit (ii and iii) of the infection, thus lowering the equilibrium prevalence of infection. A decline in the prevalence of infection reduces the intensity of selection for resistance. Thus, the prevalence of *Wolbachia* infection and the frequency of resistance alleles should interact in a manner that maintains both infection and resistance polymorphisms via negative frequency-dependent mechanisms, as in other host–pathogen interactions (e.g. Haldane, 1949; Clay & Kover, 1996; Dybdahl & Lively, 1998; Brunet & Mundt, 2000).

Among *Wolbachia*-infected individuals in *D. innubila*, male killing occurs in flies of all mtDNA haplotypes (Dyer & Jaenike, 2004). Thus, all *Wolbachia* variants that may be circulating in the population cause male killing, as *Wolbachia* and mitochondria are co-transmitted maternally. Because all *Wolbachia* variants cause male killing, susceptibility to male killing in *D. innubila* very likely goes back to most recent common ancestor of these *Wolbachia* strains and the mitochondria with which they are co-transmitted. We previously inferred an effective population size of $6–10 \times 10^6$ for *D. innubila* (Dyer & Jaenike, 2004) based on silent site diversity at nuclear markers. If the long-term prevalence is about 2–14% (derived above) over 90 000 generations, we estimate that 3×10^{10} or more males of *D. innubila* have been killed as a result of *Wolbachia* infection. Over this time span, apparently not one single resistant mutation has arisen and then spread to appreciable frequency. Because the *Wolbachia* associated with all mtDNA haplotypes in *D. innubila* (and thus all *Wolbachia* variants) cause male killing (Dyer & Jaenike, 2004, 2005), this renders unlikely a scenario in which resistance to male killing spreads within *D. innubila* followed by evolution of a new *Wolbachia* variant that is not susceptible to the resistance. It is remarkable, and extremely puzzling, that such large, genetically diverse species like *D. innubila* should remain completely susceptible to these male-killing *Wolbachia*.

We offer one possible explanation for the lack of resistance in *D. innubila*. In general, male killing causes the infected portion of the population to act as a genetic sink or black hole (Engelstädter & Hurst, 2007; Jaenike, 2007b). Consider a hypothetical resistance allele that reduces the severity of *Wolbachia* infection in males. For such an allele to escape from the genetic black hole, infected males must survive to reproductive maturity; anything less, and the resistance alleles will be lost. Thus, resistance to male killing cannot evolve by gradual delay of the developmental stage at which *Wolbachia*-induced male killing occurs. Suitable mutations, therefore, must have a major effect on resistance to male killing, yet not have sufficiently deleterious side effects – in both infected and uninfected flies – to prevent their spread.

Because male-killing occurs in the embryonic stage, a potential resistance allele must be expressed very early in development. Furthermore, the peculiar population genetics associated with male killing means that a recessive resistance allele is likely to be lost before it ever reaches a sufficiently high frequency to occur in homozygous condition and thus manifest resistance. Because recessive mutations typically involve a loss of function, this suggests that resistance is unlikely to

evolve via loss of target sites for the male killers. On the other hand, dominant mutations for resistance can be expressed phenotypically – and thus selected for – as soon as they arise. But dominant mutations often involve a gain of function.

Thus, we hypothesize that resistance to male killing will involve early-acting, major effect, gain of function mutations. However, such mutations are very likely to have significant pleiotropic effects. In complex organisms like *Drosophila*, the universe of suitable mutations may be very small (Orr, 2000). In support of this idea, it has recently been found in *Drosophila* that genes encoding proteins expressed during embryogenesis – and especially those expressed in mid- to late embryonic stages – evolve much more slowly than genes expressed either later or very early in development (Davis *et al.*, 2005; Levine *et al.*, 2007; Rodriguez *et al.*, 2007). This indicates that such genes are under severe selective constraint and, therefore, that mutations in these genes for purposes of resisting embryonic male killing are likely to entail substantial deleterious pleiotropic consequences. In general, this finding also suggests that male killers that act during the mid- to late embryonic stages might be most invulnerable the evolution of host resistance and thus be the ones most likely to persist over extended evolutionary periods.

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