PROCEEDINGS OF THE ROYAL SOCIETY B

Proc. R. Soc. B (2009) **276**, 2805–2811
doi:10.1098/rspb.2009.0287
Published online 6 May 2009

Wolbachia as populations within individual insects: causes and consequences of density variation in natural populations

Robert L. Unckless*, Lisa M. Boelio, Jeremy K. Herren and John Jaenike

Department of Biology, University of Rochester, Rochester, NY 14627, USA

The population-level dynamics of maternally transmitted endosymbionts, including reproductive parasites, depends primarily on the fitness effects and transmission fidelity of these infections. Although experimental laboratory studies have shown that within-host endosymbiont density can affect both of these factors, the existence of such effects in natural populations has not yet been documented. Using quantitative PCR, we survey the density of male-killing *Wolbachia* in natural populations of *Drosophila innubila* females from the Chiricahua Mountains of Arizona. We find that there is substantial (20 000-fold) variation in *Wolbachia* density among wild flies and that within-host *Wolbachia* density is positively correlated with both the efficacy of male killing and maternal transmission fidelity. Mean *Wolbachia* density increases three- to five-fold from early to late in the season. This pattern suggests that *Wolbachia* density declines with fly age, a conclusion corroborated by a laboratory study of *Wolbachia* density as a function of age. Finally, we suggest three alternative hypotheses to account for the approximately lognormal distribution of *Wolbachia* density among wild flies.

Keywords: male killing; transmission fidelity; infection prevalence; lognormal distribution; qPCR

1. INTRODUCTION

The majority of insect species on Earth harbour one or more species of maternally transmitted endosymbionts (Hilgenboecker *et al.* 2008). While primary endosymbionts are absolutely required by the host and thus infect all members of the host populations (Baumann 2005), the effects of secondary symbionts—those the host can live without—range from parasitic to apparently commensal to mutualistic (Stouthamer *et al.* 1999; Haine 2008). The population-level impact of infections by such secondary symbionts depends not only on the fitness effect on individual host insects, but also on the prevalence of infection within the host population.

A variety of models show that the two key variables governing the dynamics of endosymbiont infections are maternal transmission fidelity and the number of female offspring produced by infected versus uninfected females (e.g. Hurst 1991; Turelli 1994). Laboratory studies of *Wolbachia* and other symbionts have shown that both of these variables are often functions of the within-host density of the endosymbionts (reviewed in Jaenike 2008). Thus, key questions for understanding the dynamics of secondary symbiont infection in natural populations of insects include: (i) do endosymbiont densities vary substantially in nature, (ii) if so, do the observed densities encompass the range over which density-dependent effects on transmission and fitness occur, and (iii) what environmental factors influence endosymbiont density. Answers to these questions will shed light on how environmental conditions can influence insect population dynamics by way of their effects on endosymbiont density. Given the potential importance of within-host endosymbiont density on the dynamics and effects of these infections, it is notable that no studies of endosymbiont density among insects from natural populations have yet been published, although one study has examined *Wolbachia* density in the offspring of wild-caught female mosquitoes (Ahantarig *et al.* 2008).

Here we address these questions using *Drosophila innubila*, a mushroom-feeding member of the quinaria species group that lives in the forests and woodlands of the 'sky islands' of southwest North America and that is infected with a male-killing strain of *Wolbachia* (Jaenike *et al.* 2003; Dyer *et al.* 2005). We chose this species for several reasons. First, we already have background information on infection prevalence, transmission fidelity and intensity of male killing in natural populations (Dyer & Jaenike 2004). The mean infection prevalence (35%) is sufficiently high that the approximately 100 per cent male killing represents a substantial selective burden on the population and one that has been expressed for tens of thousands of years (Jaenike & Dyer 2008). Second, experimental manipulation of *Wolbachia* density in *D. innubila* has shown that both the transmission fidelity and the intensity of male killing, a proxy for infected female fitness in standard models (Hurst 1991), vary positively with within-host *Wolbachia* density. Thus, variation in mean *Wolbachia* density within *D. innubila* could have substantial population-level effects, if densities in the wild correspond to those at which male killing and transmission fidelity change with *Wolbachia* density.

Drosophila innubila become active at the onset of the monsoon season, typically in mid to late July in southeast Arizona. The flies feed and breed on the mushrooms brought forth by the summer rains, and they remain active until it becomes too cold in early October, at which point

* Author for correspondence (runckles@mail.rochester.edu).

Received 18 February 2009
Accepted 15 April 2009

the adult flies go into a long dormant state until the next summer. Because monsoon seasons vary dramatically in intensity and duration from one year to the next, the number of *D. innubila* generations probably varies from 1 to 3 per year.

We collected *D. innubila* from the Chiricahua Mountains in southeast Arizona both early and late in the monsoon seasons of 2006 and 2007. We quantified within-host *Wolbachia* density in infected females, maternal transmission fidelity and offspring sex ratio, the latter serving as a measure of the intensity of male killing. We find that *Wolbachia* density varies greatly among wild-caught flies and encompasses the range over which transmission fidelity and intensity of male killing vary as a function of density. Consistent with this, we find that both maternal transmission fidelity and offspring sex ratio are correlated with *Wolbachia* density in these wild females. Finally, the within-host density of *Wolbachia* is substantially lower in early season flies than in flies collected late in the season, consistent with age-related changes in *Wolbachia* density we find experimentally in the laboratory. We cast these results in terms of a general selection model on endosymbiont density in natural populations.

2. MATERIAL AND METHODS

(a) *Fly collections*

Drosophila innubila were collected in early August 2006, late September 2006, late July 2007 and late September 2007. Thus, for each of the 2 years, we have one early and one late season collection. For each collection except September 2007 (when flies were primarily obtained at the Southwest Research Station), flies were collected from four sites, all located within 5 km of the Southwest Research Station near Portal, Arizona, on the eastern side of the Chiricahua Mountains. We lured flies using commercial mushrooms (*Agaricus bisporus*) as bait, and then used a sweep net to catch them and transfer them to vials with sugar agar. Flies were kept at 17°C at the Southwest Research Station until they were shipped (1–4 days after capture) to Rochester, NY, via overnight mail. Min/max thermometers placed in the shipping containers indicated that the flies experienced temperatures in the range of 12–25°C during shipping. Thus, they did not experience unusual or stressful temperatures subsequent to their capture.

(b) *Fly processing*

Upon arrival at the laboratory, female *D. innubila* were placed individually in vials with food (instant *Drosophila* medium (Carolina Biological Supply, Burlington, NC), approximately 1 g of *A. bisporus* mushroom and a cotton roll) for 5 days to allow them to oviposit. During this time, the flies were kept at 22°C on a 12 L : 12 D light cycle. After the 5-day oviposition period, females were dissected in *Drosophila* Ringer's solution and ovaries were collected. DNA was isolated from the ovaries using the Puregene DNA purification kit (Qiagen Inc., Valencia, CA) with half the suggested reagents for a single *Drosophila melanogaster*. Isolated DNA was stored at 4°C until screening.

(c) Wolbachia *screening*

All females were screened (except those that died before dissection) for *Wolbachia*, using PCR with the *wsp* gene (primers 81f and 691r), as described in Zhou *et al.* (1998). As a control, we also screened for mitochondrial DNA using *COI* (primers 1490 and 2198; Folmer *et al.* 1994) and the same PCR conditions as used for *wsp*. For any ambiguous results, we screened at least three female offspring if available. To assess male infection frequency, 100 wild-caught males were screened from the 2007 collection.

(d) *Real-time/quantitative PCR to assess* Wolbachia *density*

To determine the density of *Wolbachia* in ovaries, we performed real-time/quantitative PCR (qPCR) on all females determined to be infected with *Wolbachia*. Reactions were performed in duplicate using a 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA), as described in Dyer *et al.* (2005), with *wsp* as a target and the single-copy nuclear gene *tpi* as an endogenous control. Although triplicate reactions are recommended for more precise estimates on individual samples, running duplicate reactions enabled us to test 50 per cent more individuals and thus provides greater statistical power for the same total number of reactions. Our criterion for inclusion of a sample in subsequent statistical analyses was that difference between replicate ΔC_T values (as defined below) was less than 0.6, which corresponds approximately to a 50 per cent difference in *Wolbachia* density between replicate estimates. By this criterion, we excluded 34 out of 793 (4.3%) of our samples. We found no correlation between estimated *Wolbachia* density and the difference between replicate ΔC_T values ($r^2 = 0.01$); thus, our exclusion criterion did not bias our sample with respect to *Wolbachia* density. We also quantified *Drosophila* mitochondrial density, using newly designed primers (*COIa*-F, 5′-CAGATGACTTGCAACTTTACATGGAGC; *COIa*-R, 5′-CAGTGGATGAATCCAGCTATAAT) and probe (*COIa*-P, 5′-FAM-AGCTAAAACAACTCCTGTTAACCC TCCAAC-BHQ1) for the *COI* gene.

Relative *Wolbachia* density $[W]$ was estimated as $[W] \propto 2^{\Delta C_T}$, where ΔC_T is the number of PCR cycles required to reach threshold fluorescence for the endogenous control gene (*tpi* or *COI*) minus the number of cycles to reach threshold for *Wolbachia wsp* gene. Because high starting *Wolbachia* densities require fewer cycles for *wsp* to reach threshold, high values of ΔC_T indicate greater within-host densities of *Wolbachia*. Since the number of gene copies potentially doubles every PCR cycle, within-host *Wolbachia* density scales as $2^{\Delta C_T}$, if PCR amplification is 100 per cent efficient. The efficiency of qPCR is often less than 100 per cent, and differences in amplification efficiency between *wsp* and a control gene could bias estimates of *Wolbachia* density (Giuletti *et al.* 2001). We therefore tested PCR amplification efficiency of each gene by performing serial dilutions of a sample, carrying out qPCR and examining the slope of the relationship between threshold cycle number and initial concentration ($\log_2$ transformed).

Because endosymbionts have the potential for exponential growth within their hosts, ΔC_T is more likely to be normally distributed than $2^{\Delta C_T}$, so we use ΔC_T for statistical analysis. To determine which factors influence density, we performed an ANOVA, modelled as ΔC_T = year, season (year) and site (year).

(e) *Offspring sex ratio and transmission efficiency*

The total numbers of male and female offspring were determined for all *Wolbachia*-infected wild-caught females. In 2007, we also counted and sexed all offspring from all

uninfected females. All offspring were stored in 100 per cent ethanol at −20°C. The offspring of a subset of the wild-caught females were screened for *Wolbachia* infection, as described above, in order to determine the relationship between maternal *Wolbachia* density and transmission fidelity.

Logistic regressions were used to model either offspring sex or infection status as functions of their mother's *Wolbachia* density (ΔC_T). These regressions were not done to assess statistical significance, but rather to infer the relative fitness of infected lineages as a function of *Wolbachia* density. In models of male-killing dynamics (e.g. Hurst 1991), the fitness of an infected female increases with the level of male killing experienced by her brothers, i.e. with the proportion females in a sibship. We therefore define the male-killing benefit experienced by the offspring of infected females as s_{MK} = proportion of female offspring in a family—0.5, which can range from 0 to 0.5. For simplicity, we limit our analysis to the simplest case, in which the fitness benefit to females increases linearly with the penetrance of male-killing and with perfect fitness compensation. It is likely that fitness compensation in the wild is less than perfect and that there may be a nonlinear relationship between penetrance of male-killing and the fitness benefit to females. In our model, we also ignore the possibility of benefits conferred by *Wolbachia* that are unrelated to male killing. The infection status of an infected female's daughters provides an estimate of the transmission fidelity of the infection (β). Thus, the fitness of an infected lineage relative to an infected lineage with perfect male killing and transmission is proportional to $2(\beta \times s_{MK})$. Using the measured mean and variance of *Wolbachia* density (ΔC_T), we also fitted a normal distribution to the frequency distribution of *Wolbachia* densities among wild-caught females of *D. innubila*, pooled across all collections. Together, the fitness function and the frequency distribution of *Wolbachia* densities enable a crude estimate of the relative fitnesses of infected lineages in the wild.

(f) *Effect of age and nutrition on* Wolbachia *density*

We mass reared infected *D. innubila* by crossing females from an infected isofemale strain (MSR) to males of an uninfected strain (ST1/ST4), both of which are descended from flies collected in the Chiricahua Mountains. To assess the effect of fly age on *Wolbachia* density, newly emergent flies were placed on instant plus mushroom food (as described above) for 7 days. Subsequently, they were transferred weekly to fresh mushroom–agar medium and held at 8°C. After being aged for periods ranging from 7 to 141 days, the flies ($n=87$ total) were frozen for subsequent quantification of *Wolbachia* density using qPCR, as described above.

To assess the effect of nutrient richness on *Wolbachia* density, newly emergent flies were either placed on sugar agar (nutrient-poor treatment) or instant plus mushroom food (nutrient-rich treatment). After one week, all flies were transferred to fresh instant plus mushroom food. Although this allowed starved flies to feed, this method was necessary to obtain ovaries large enough for dissection and qPCR analysis of *Wolbachia* density. Flies were dissected at two to three weeks of age and subjected to qPCR to estimate *Wolbachia* density.

3. RESULTS

(a) Wolbachia *infection frequency*

The overall infection frequency was 0.344 ($n=1523$) and 0.318 ($n=846$) in 2006 and 2007, respectively. These values are in the middle of the range of infection prevalences found previously in this area (Dyer & Jaenike 2004), indicating that the flies used in our sample were collected from populations experiencing typical *Wolbachia* frequencies. We found significant variation in infection prevalence among sites neither within years ($F=0.60$, d.f. = 6, $p=0.73$), between early and late collections within years ($F=0.06$, d.f. = 2, $p=0.94$) nor between years ($F=0.13$, d.f. = 1, $p=0.72$). The infection frequency of male *D. innubila* was 0.04 and 0.02 in July and September 2007, respectively. Because this is similar to previous estimates of male infection rate (Dyer & Jaenike 2004), this suggests that the *D. innubila* populations in 2006 and 2007 harboured 'typical' *Wolbachia* densities, as the production of infected, viable male offspring depends on *Wolbachia* density (Dyer *et al.* 2005).

(b) *Variation in* Wolbachia *density among wild-caught flies*

The efficiency of qPCR amplification was not significantly different from 1.0 for any gene, so we assume that for each gene the copy number doubles with every cycle. This enables us to estimate relative *Wolbachia* density as $2^{\Delta C_T}$. Using a nuclear gene (*tpi*) as the standard, we find that the relative *Wolbachia* density varied from 0.035 to 734, i.e. approximately a 20 000-fold difference between the most lightly and most heavily infected individuals in our sample of 759 wild-caught females. If we include only the middle 95 per cent of data, relative *Wolbachia* density still varied 70-fold, from 1.35 to 96.8 (figure 1*a*). Using the mtDNA gene *COI* as the standard, relative *Wolbachia* density varied by a factor of approximately 100 000, from 6.63×10^{-5} to 7.06 ($n=701$). The greater density variation using mtDNA as a baseline may be due to the variable numbers of genomes per mitochondrion and mitochondria per cell. Because the *Wolbachia* density estimates are correlated between the sets using nuclear and mitochondrial standards ($r^2=0.27$; $p<0.0001$), we present only the results using the more conservative nuclear DNA (*tpi*) as a baseline.

Mean *Wolbachia* density did not vary significantly between years ($F=0.07$; d.f. = 1, $p=0.77$) nor among sites within years ($F=0.94$, d.f. = 6, $p=0.47$). However, there were highly significant differences between early and late season samples within years ($F=88.0$, d.f. = 2, $p<0.0001$), with mean *Wolbachia* density being approximately three times greater among flies collected late in the 2006 season than among those collected early, while in 2007 the *Wolbachia* density was approximately five times greater in the late versus early season flies (figure 1*b*). Since there was no difference in density among sites, sites were pooled for subsequent analyses. At any one time, ΔC_T (i.e. *Wolbachia* density on a log scale) exhibited an approximately normal distribution. In other words, *Wolbachia* density itself appeared to be lognormally distributed.

(c) *Offspring sex-ratio and transmission efficiency*

The proportion of female offspring produced by uninfected flies in 2007 was 0.556 ± 0.009 ($n=447$ wild-caught female parents). Among infected flies in 2006 and 2007, the mean proportion of female offspring was 0.971 ± 0.005 ($n=507$ female parents). Although there is a great deal of scatter, there are significant positive correlations between *Wolbachia* density in wild-caught females (ΔC_T) and the

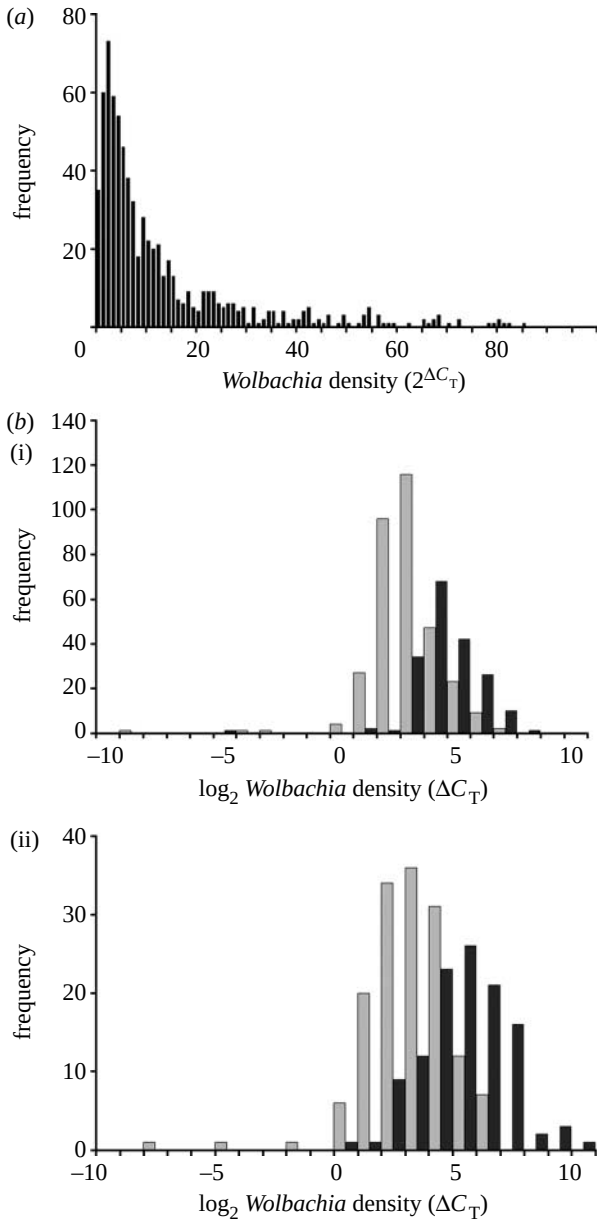

Figure 1. Distribution of *Wolbachia* densities in ovaries from wild-caught *D. innubila* in 2006 and 2007. (*a*) Relative *Wolbachia* densities within infected females, estimated as $2^{\Delta C_T}$. This includes only females within the middle 95% of infection densities for all collections combined. (*b*) Log_2-transformed *Wolbachia* density, ΔC_T, during four collection periods. Grey bars represent flies from early in the monsoon season and black bars represent flies from late in the monsoon season. Data for (i) 2006 and (ii) 2007 plotted separately.

proportion of female offspring (Spearman $\rho = 0.20$; $n = 489$ families, $p < 0.0001$), and between *Wolbachia* density and the proportion of female offspring inheriting the *Wolbachia* infection (Spearman $\rho = 0.21$; $n = 109$ families, $p = 0.040$; figure 2). Because both proportion of female offspring and transmission fidelity are correlated with *Wolbachia* density, we also find a direct correlation between proportion of female offspring and the transmission fidelity among families produced by wild-caught females (Spearman $\rho = 0.52$; $n = 109$ families; $p < 0.0001$).

Figure 3 shows the results of logistic regressions of the proportion of female offspring produced by wild-caught females and the proportion of female offspring inheriting the *Wolbachia* infection as functions of maternal *Wolbachia*

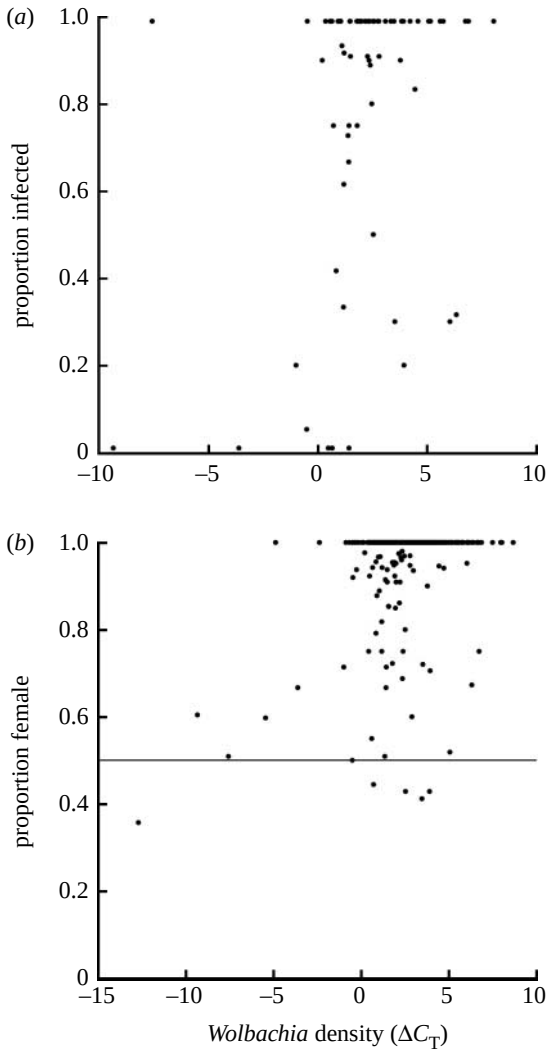

Figure 2. (*a*) Transmission fidelity and (*b*) male-killing penetrance as functions of *Wolbachia* density in wild-caught females. The horizontal line in (*b*) indicates a 1 : 1 sex ratio, as would be expected if males and females had equal chance of survival.

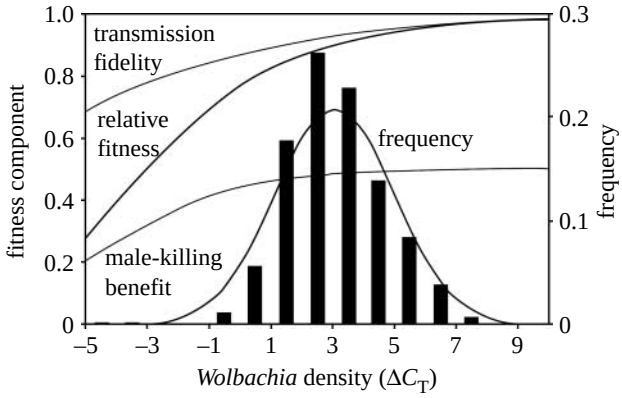

Figure 3. Fitted logistic regressions of transmission fidelity (β) and male-killing benefit (s_{MK}, proportion female offspring in a family—0.5) as functions of maternal *Wolbachia* density. Also shown is $2(\beta \times s_{MK})$ as an indicator of the fitness of an infected cytoplasmic lineage relative to that of an infected lineage with perfect male killing and transmission. A normal distribution has been fitted to the observed frequency distribution (vertical bars) of *Wolbachia* densities measured in wild-caught females of *D. innubila*.

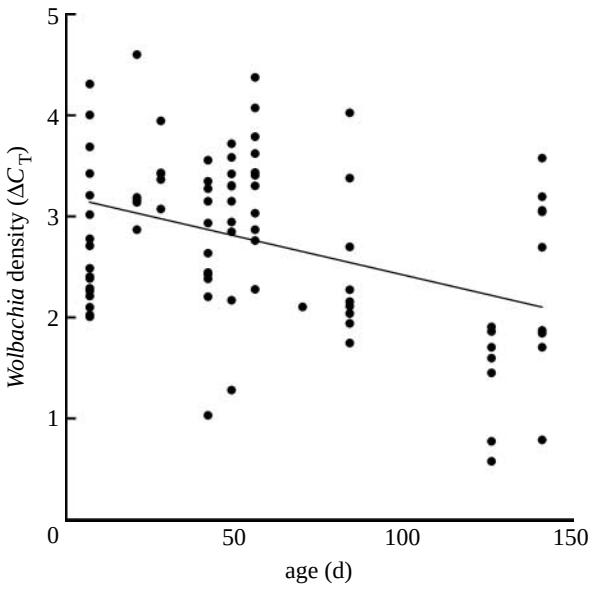

Figure 4. *Wolbachia* density as a function of fly age in laboratory-reared flies.

density. The proportion of female offspring is a measure of the fitness benefit to infected cytoplasmic lineages of male killing (s_{MK}), and the proportion of infected offspring is a measure of transmission fidelity (β). From β and s_{MK}, we estimate the fitness of an infected lineage relative to one that has perfect transmission and male killing as $2(\beta \times s_{MK})$, and this is also plotted in the figure. Finally, figure 3 shows a normal distribution fitted to the overall *Wolbachia* density distribution among wild-caught females, pooled across all collections. The fit of ΔC_T is close to a normal distribution, but does differ significantly. For the middle 95 per cent of data, ΔC_T is much closer to a normal distribution (Wilkes–Shapiro $W = 0.98$) than is $2^{\Delta Ct}$, for which $W = 0.72$; a perfect fit would yield $W = 1$. For the full dataset, $W = 0.97$ and 0.29 for ΔC_T and $2^{\Delta Ct}$, respectively. Note that the peak of the *Wolbachia* density distribution falls just above the densities where β, s_{MK} and relative fitness start to drop off substantially.

(d) *Effect of age and nutrition on* Wolbachia *density*

In the age experiment, a small fraction of flies (4%) was not infected with *Wolbachia* when screened. However, there was no correlation between fly age when assayed and probability of being uninfected ($r^2 = 0.0029$, $p = 0.75$), suggesting that these flies simply failed to inherit the infection. There was a significant negative correlation between fly age and *Wolbachia* density (slope $= -0.008$ ΔC_T per day, $r^2 = 0.16$, $n = 87$, $p < 0.0001$; figure 4). *Wolbachia* density decreased approximately 50 per cent during the course of experiment. In the nutrient experiment, there was no significant difference in *Wolbachia* density between flies that had fed on nutrient-poor and nutrient-rich food ($t = 0.01$, $p = 0.99$; mean $\pm$ s.e. $\Delta C_T = 2.25 \pm 0.34$ and 2.15 ± 0.77 for low- and high-nutrient conditions, respectively).

4. DISCUSSION

We have attempted to quantify within-host density of *Wolbachia* in natural populations of *D. innubila* and begun to look into the causes and consequences of that variation among flies. Our measure of within-host density used qPCR to estimate the ratio of *Wolbachia* to host gene copies. Using a single-copy host nuclear gene (*tpi*) as a reference, we found that *Wolbachia* density varied approximately 20 000-fold among female *D. innubila* from the wild. Using mtDNA *COI* as a host reference gene suggested an even greater (100 000-fold) range in *Wolbachia* density. Note that this range excludes flies for which there was more than 50 per cent variation between replicate estimates of *Wolbachia* density. Thus, experimental qPCR error probably accounts for only a small fraction of the observed variation among flies.

Within collections, most values of ΔC_T spanned a range of 4–6 units, which corresponds to 16- to 64-fold variation in *Wolbachia* density. The ΔC_T units can be thought of as equivalent to rounds of *Wolbachia* replication within flies (increase ΔC_T by 1) or half-lives of non-replicating *Wolbachia* (decrease ΔC_T by 1). Thus, even if all flies collected at a given time had started out with the same *Wolbachia* density, then the variation in ΔC_T indicates that the *Wolbachia* vary among flies by up to six rounds of replication and/or half-lives. Because emergent adult flies probably vary in the *Wolbachia* densities (Dyer *et al.* 2005), the observed variation in ΔC_T could result from even fewer rounds of replication or half-lives.

A few flies carried extremely low *Wolbachia* densities. Such flies were often characterized by low fidelity of *Wolbachia* transmission and low efficiency of male killing. The *Wolbachia* in such lineages may be living dead, the last vestiges of previously robust infections.

While even our lower estimate of 20 000-fold variation may seem incredible, it is actually less than the 180 000-fold variation in *Wolbachia* density found among *Aedes albopictus* mosquitoes (Ahantarig *et al.* 2008). The mosquitoes studied by Ahantarig *et al.* were actually the laboratory-reared offspring of wild-caught females, meaning that insect age, nutritional status and rearing conditions were unimportant sources of *Wolbachia* density variation. Therefore, the density variation evident in the mosquitoes is probably due in part to variation in density among the wild-caught females. Thus, although only two studies of individuals in the wild (or their F1) have been conducted, both have revealed tremendous variation in within-host *Wolbachia* density. Ours is actually the first estimate of standing variation in *Wolbachia* density for any natural population of insect. Although *Wolbachia* density may be influenced by numerous factors, such as insect age or temperature, it is the actual frequency distribution of densities among insects in the wild that is relevant to understanding the dynamics of these infections.

Previous laboratory studies have uncovered a variety of genetic (both host and *Wolbachia*) and environmental factors that can influence the within-host density of *Wolbachia* (e.g. Breeuwer & Werren 1993; Hurst *et al.* 2000; Goto *et al.* 2006; Mouton *et al.* 2006; reviewed in Jaenike 2008). In our collections of *D. innubila* from natural populations, we found consistent and significant differences in mean *Wolbachia* density between adult flies collected early in the season, when the monsoon rains are just beginning in southeast Arizona, and those collected late in the season, when cooler temperatures and the cessation of the monsoon bring the *Drosophila* season to an end. The mean density of *Wolbachia* ($2^{\Delta C_T}$) was three times greater in late-season flies than early season flies in

2006, and five times greater in 2007. The natural history of *D. innubila* in this region suggests that most or all of the flies collected early in the monsoon season are likely to have been very old, having remained dormant since the previous September or October. By contrast, flies collected in late September are most probably the descendants of the early season flies and are thus likely to have been much younger.

We experimentally tested the effect of fly age on *Wolbachia* density under controlled laboratory conditions. This experiment showed that *Wolbachia* density declines gradually as a function of fly age, being approximately half as great in flies over 100 days old than in those less than 30 days old. Thus, the low *Wolbachia* density in early season flies from natural populations is probably a result of their being very old.

Given that the mean *Wolbachia* density is three to five times lower in the flies collected early in the season and that transmission fidelity and male killing decline with maternal *Wolbachia* density, one may ask why there was no significant seasonal variation in infection prevalence. One possibility is that the late season flies were two or more generations removed from the early season flies, so that infection prevalence had time to recover from a temporary decline. Alternatively, note that the three- to fivefold difference in mean *Wolbachia* density occurs within a part of the density distribution where there is little effect of density on either male killing or transmission fidelity (figure 3). Thus, any changes in prevalence resulting from variation in density in this range would be difficult to detect with our samples of several hundred flies.

The two key variables governing the dynamics of endosymbiont infections are the maternal transmission fidelity (β) and the cytoplasmic selection coefficient (s) associated with the infection. Elsewhere, we have shown theoretically how variation in β and s can influence the equilibrium prevalence of an endosymbiont infection (Jaenike 2008). For male-killing infections, the selection coefficient is likely to be a consequence of the male killing itself, whether this comes about through reduced larval competition (Hurst 1991) or reduced sib-mating and inbreeding depression (Werren 1987). In the following, we use the term s_{MK} to refer to the fitness benefit derived specifically from male killing. (The more general term s would incorporate other costs or benefits of the infection, which we have not studied.) We found that both transmission fidelity (β) and offspring sex ratio (and thus s_{MK}) are positively correlated with *Wolbachia* density among *D. innubila* collected from natural populations. Thus, the within-host *Wolbachia* density in natural populations of *D. innubila* encompasses a range over which β and s_{MK} do change with density. This was not a preordained result: it is entirely possible that even the lowest densities in the wild would be sufficient for perfect transmission and male killing (e.g. Duron *et al.* 2006; Mouton *et al.* 2006). Interestingly, the range of *Wolbachia* densities in wild *D. innubila* includes the range over which β and s_{MK} change as a function of experimentally manipulated *Wolbachia* density in the laboratory (Dyer *et al.* 2005).

The observation that some *Wolbachia* densities in wild *D. innubila* are insufficient to bring about perfect transmission and male killing raises the question of how the frequency distribution of densities is determined. We suggest three possibilities: stabilizing selection on density, density drift and a balance between natural selection and density drift. Elsewhere, we have found that, even within a single strain of infected *D. innubila*, there is a positive correlation in *Wolbachia* density between mothers and their female offspring (Dyer *et al.* 2005), indicating that *Wolbachia* density can be heritable as an epigenetic trait.

With respect to stabilizing selection, natural selection for maximum transmission fidelity and penetrance of a particular phenotype (e.g. male killing or cytoplasmic incompatibility) should favour lineages with the greatest endosymbiont densities. By contrast, if the endosymbiont has any density-dependent adverse effects on the female host, selection will favour less heavily infected host lineages. Depending on the shapes of these functions, cytoplasmic lineage fitness may be the greatest at an intermediate density. Furthermore, because these *Wolbachia* are male killers, the direction of selection on *Wolbachia* density is likely to be in opposite directions between host nuclear genes and those of the endosymbiont. In this context, *Wolbachia* density is considered to be an organismal trait subject to selection.

For density drift, the *Wolbachia* within a host are considered as a population. Imagine a population of *Drosophila* flies, each of which experiences or creates conditions that influence r_t, the *per capita* rate of population increase (or decrease) in the resident *Wolbachia* at time t within a host fly. Thus, the *Wolbachia* density at time T can be expressed as $\ln(N_T) = \ln(N_0) + \int_{t=0}^{T} r_t \mathrm{d}t$ (MacArthur 1960; May 1975). If the values of r_t vary randomly through time and among flies, then the integral $\int_{t=0}^{T} r_t \mathrm{d}t$, which is a sum of random variables, will be normally distributed among flies. Thus, if *Wolbachia* densities in wild-caught flies are determined primarily by cumulative growth rates, $\int_{t=0}^{T} r_t \mathrm{d}t$, rather than by initial densities or carrying capacities, then *Wolbachia* densities should be approximately lognormally distributed among flies.

Although the accumulated effects of numerous multiplicatively acting factors could account for the lognormal shape of the *Wolbachia* density distribution, such a process says nothing about where the peak of this distribution should be situated. The observed peak occurs at a relative *Wolbachia* density of ΔC_T values of 3–5 (figure 1). Below this range, transmission fidelity and male-killing penetrance decline notably, both in laboratory-reared flies (Dyer *et al.* 2005) and among the wild-caught *D. innubila* examined in the present study (figure 4). Therefore, density drift, as discussed above, may act in conjunction with selection against cytoplasmic lineages in which *Wolbachia* densities have dropped to levels at which transmission and male killing are incomplete. Such selection could set a lower limit to the position of the overall *Wolbachia* density distribution.

Regardless of the mechanism responsible for the density distribution, our data show that there is a great deal of variation in *Wolbachia* density among female *D. innubila* in natural populations and that this variation is correlated with both transmission fidelity and male killing. We have shown that fly age explains some, but by no means all, of the variation in *Wolbachia* density. The identification of other factors influencing within-host endosymbiont densities will provide a fuller understanding of the dynamics of infection prevalence in the wild.

We would like to thank Dawn Wilson and the staff of the Southwest Research Station for providing an exceptional base of operations for our fieldwork. We also thank members of the Jaenike lab, including Alex Papastrat, Deby Philbrick, Viral Patel, Emily Myers, Meghan Jacobs and Mark Loria for their assistance and comments, and two anonymous referees for very helpful suggestions. This research was supported by an NSF grant DEB-0542094 to J.J. and a Robert and Mary Sproull Fellowship from the University of Rochester to R.L.U.

REFERENCES

Ahantarig, A., Trinachartvanit, W. & Kittayapong, P. 2008 Relative *Wolbachia* density of field-collected *Aedes albopictus* mosquitoes in Thailand. *J. Vector Ecol.* **33**, 173–177. (doi:10.3376/1081-1710(2008)33[173:RWDOFA]2.0.CO;2)

Baumann, P. 2005 Biology of bacteriocyte-associated endosymbionts of plant sap-sucking insects. *Annu. Rev. Microbiol.* **59**, 155–189. (doi:10.1146/annurev.micro.59.030804.121041)

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

Duron, O., Labbe, P., Berticat, C., Rousset, F., Guillot, S., Raymond, M. & Weill, M. 2006 High *Wolbachia* density correlates with cost of infection for insecticide resistant *Culex pipiens* mosquitoes. *Evolution* **60**, 303–314.

Dyer, K. A. & Jaenike, J. 2004 Evolutionary stable infection by a male-killing endosymbiont in *Drosophila innubila*: molecular evidence from the host and parasite genome. *Genetics* **168**, 1443–1455. (doi:10.1534/genetics.104.027854)

Dyer, K. A., Minhas, M. S. & Jaenike, J. 2005 Expression and modulation of embryonic male-killing in *Drosophila innubila*: opportunities for multilevel selection. *Evolution* **59**, 838–848.

Folmer, O., Black, M., Hoeh, W., Lutz, R. & Vrijenhoek, R. 1994 DNA primers for amplification of mitochondrial cytochrome *c* oxidase subunit I from diverse metazoan invertebrates. *Mol. Mar. Biol. Biotechnol.* **3**, 294–299.

Giuletti, A., Overbergh, L., Valckx, D., Decallonne, B., Bouillon, R. & Mathieu, C. 2001 An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. *Methods* **25**, 386–401. (doi:10.1006/meth.2001.1261)

Goto, S., Anbutsu, H. & Fukatsu, T. 2006 Asymmetrical interactions between *Wolbachia* and *Spiroplasma* endosymbionts coexisting in the same insect host. *Appl. Environ. Microbiol.* **72**, 4805–4810. (doi:10.1128/AEM.00416-06)

Haine, E. R. 2008 Symbiont-mediated protection. *Proc. R. Soc. B* **275**, 353–361. (doi:10.1098/rspb.2007.1211)

Hilgenboecker, K., Hammerstein, P., Schlattmann, P., Telschow, A. & Werren, J. H. 2008 How many species are infected with *Wolbachia*—a statistical analysis of current data. *FEMS Microbiol. Lett.* **281**, 215–220. (doi:10.1111/j.1574-6968.2008.01110.x)

Hurst, L. D. 1991 The incidences and evolution of cytoplasmic male-killers. *Proc. R. Soc. Lond. B* **244**, 91–99. (doi:10.1098/rspb.1991.0056)

Hurst, G. D. D., Johnson, A. P., Von der Schulenburg, J. H. G. & Fuyama, Y. 2000 Male-killing *Wolbachia* in *Drosophila*: a temperature-sensitive trait with a threshold bacterial density. *Genetics* **156**, 699–709.

Jaenike, J. 2008 Coupled population dynamics of endosymbionts within and between hosts. *Oikos* (*Early View*). **118**, 353–362. (doi:101111/j1600-0706.2008.17110.x)

Jaenike, J. & Dyer, K. A. 2008 No resistance to male-killing after thousands of years of infection. *J. Evol. Biol.* **21**, 1570–1577. (doi:10.1111/j.1420-9101.2008.01607.x)

Jaenike, J., Dyer, K. A. & Reed, L. K. 2003 Within-population structure of competition and the dynamics of male-killing *Wolbachia*. *Evol. Ecol. Res.* **5**, 1023–1036.

MacArthur, R. H. 1960 On the relative abundance of species. *Am. Nat.* **94**, 25–36. (doi:10.1086/282106)

May, R. M. 1975 Patterns of species abundance and diversity. In *Ecology and evolution of communities* (eds M. L. Cody & J. M. Diamond), pp. 81–120. Cambridge, MA: Belknap Press.

Mouton, L., Henri, H., Bouletreau, M. & Vavre, F. 2006 Effect of temperature on *Wolbachia* density and impact on cytoplasmic incompatibility. *Parasitology* **132**, 49–56. (doi:10.1017/S0031182005008723)

Stouthamer, R., Breeuwer, J. A. J. & Hurst, G. D. D. 1999 *Wolbachia pipientis*: microbial manipulators of arthropod reproduction. *Annu. Rev. Microbiol.* **53**, 71–102. (doi:10.1146/annurev.micro.53.1.71)

Turelli, M. 1994 Evolution of incompatibility-inducing microbes and their hosts. *Evolution* **48**, 1500–1513. (doi:10.2307/2410244)

Werren, J. H. 1987 The coevolution of autosomal and cytoplasmic sex ratio factors. *J. Theor. Biol.* **124**, 317–334. (doi:10.1016/S0022-5193(87)80119-4)

Zhou, W. G., Rousset, F. & O'Neill, S. 1998 Phylogeny and PCR-based classification of *Wolbachia* strains using *wsp* sequences. *Proc. R. Soc. Lond. B* **265**, 509–515. (doi:10.1098/rspb.1998.0324)