Density dynamics of diverse *Spiroplasma* strains naturally infecting different species of *Drosophila*

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Facultative heritable bacterial endosymbionts can have dramatic effects on their hosts, ranging from mutualistic to parasitic. Within-host bacterial endosymbiont density plays a critical role in maintenance of a symbiotic relationship, as it can affect levels of vertical transmission and expression of phenotypic effects, both of which influence the infection prevalence in host populations. Species of genus *Drosophila* are infected with *Spiroplasma*, whose characterized phenotypic effects range from that of a male-killing reproductive parasite to beneficial defensive endosymbiont. For many strains of *Spiroplasma* infecting at least 17 species of *Drosophila,* however, the phenotypic effects are obscure. The infection prevalence of these *Spiroplasma* vary within and among *Drosophila* species, and little is known about the within-host density dynamics of these diverse strains. To characterize the patterns of *Spiroplasma* density variation among *Drosophila* we used quantitative PCR to assess bacterial titer at various life stages of three species of *Drosophila* naturally-infected with two different types of *Spiroplasma*. For naturally infected *Drosophila* species we found that non-male-killing infections had consistently lower densities than the male-killing infection. The patterns of *Spiroplasma* titer change during aging varied among *Drosophila* species infected with different *Spiroplasma* strains. Bacterial density varied within and among populations of *Drosophila*, with individuals from the population with the highest prevalence of infection having the highest density. This density variation underscores the complex interaction of *Spiroplasma* strain and host genetic background in determining endosymbiont density.

Introduction

Well over half of all insect species harbor maternally transmitted bacterial endosymbionts that can have dramatic effects on their host.[1,2] Dependent on their host for their own survival, some of these endosymbionts act as mutalists increasing their own fitness by increasing that of the host. Alternatively, as parasites, these bacteria can manipulate their host's reproduction to enhance their own transmission by increasing the proportion of infected females.[3,4] These facultative endosymbionts are not required for host survival, and as such their prevalence, which plays a large role in determining their population level impacts, can vary greatly among host species and populations.[1,2,5] Endosymbiont prevalence is greatly affected by its within-host density dynamics[6] as infection density affects both the fidelity of vertical transmission as well as the strength of expression of fitness effects, 2 key parameters maintaining bacteria in host populations. For many endosymbionts, bacterial strains with higher titers have greater vertical transmission fidelity and stronger phenotypic effects, including both reproductive manipulation phenotypes and fitness benefits.[7-9]

Spiroplasma is 1 of only 2 bacterial endosymbionts found thus far in species of the genus *Drosophila*.[10] It can act as both a reproductive manipulator, causing male-killing in certain species of *Drosophila*,[11] and as a defensive endosymbiont, affording *D. neotestacea* protection against a nematode parasite[12] and conferring resistance to wasp parasitism in *D. hydei*.[13] Although the role of bacterial density in the defensive strains is unclear, it has been hypothesized that a certain bacterial density is necessary for expression of its male-killing phenotype, as studies have shown that male-killing *Spiroplasma* have had a higher density than non-male-killing *Spiroplasma*.[14,15] *Spiroplasma* density also affects the developmental stage at which male-killing occurs, with a higher infection density causing male-killing at an earlier stage.[16]

The dynamics of endosymbiont titer are dependent on bacterial strain,[7,17,18] host genotype,[19,20] host age,[17,21] and temperature,[22-24] although the full role of these factors in *Spiroplasma* infections is unclear. *Spiroplasma* density in certain *Drosophila* species increases with age[14,15,25] and decreases at lower temperatures.[26,27] Lower density, either at young ages or lower temperatures, is correlated with either loss of the male-killing phenotype or loss of the *Spiroplasma* altogether.[14,26] *Spiroplasma* density dynamics, however, have been explored in only a few strains
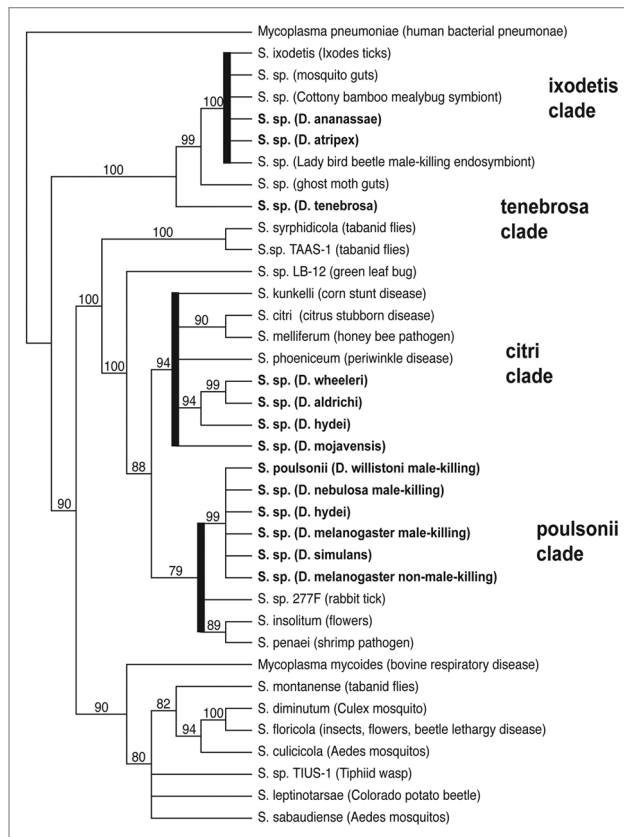
Figure 1. The diversity of *Spiroplasma* infecting *Drosophila*. A Neighbor-Joining cladogram based on partial 16s rDNA sequences illustrating the different strains of *Spiroplasma* that infect *Drosophila* (shown in bold type). One thousand bootstrap replicates were run to assess support on clades.

in a limited number of *Drosophila* species, namely in relation to the male-killing phenotype. These strains are identical or closely related to the first characterized *Spiroplasma* endosymbiont infecting *D. nebulosa*, *S. poulsonii*.[15,28] Furthermore, many of the strains studied were artificial infections; *Spiroplasma* strains from different *Drosophila* species transferred to *D. melanogaster*. While useful for exploring the mechanisms of male-killing, these artificial infections give only limited insight into density variation in natural populations. Furthermore, the phenotypic effects of *Spiroplasma* in *Drosophila*, including male-killing as well as defense against parasites, have been only investigated in the poulsonii-type *Spiroplasma* strains,[13,29,30] a limited subset of the diversity of those *Spiroplasma* now known to infect *Drosophila*.[5,31]

At least 17 species of *Drosophila* are infected with four genetically distinct types of *Spiroplasma* (**Fig. 1**), most of which do not cause male-killing and, for many still, their fitness effects are unknown. Screening of natural populations of *Drosophila* revealed that *Spiroplasma* infection prevalence varies not only among, but within species, with infection prevalence in *D. mojavensis* ranging from 15 to 85% in geographically distinct populations.[5] *Drosophila mojavensis* is infected with one of the newly discovered strains of *Spiroplasma* that is more closely related to *S. citri*, a well-known plant pathogen,[32] than to the poulsonii-type

Spiroplasma. These citri-type *Spiroplasma* infect seven species in the *Drosophila* repleta group, which have some of the highest infection prevalences of *Spiroplasma* screened to date.[5]

Characterizing the density dynamics of various *Spiroplasma* strains among different *Drosophila* species can lend insight into factors driving its distribution and prevalence. In this study we have described the variation in *Spiroplasma* density among different *Spiroplasma* strains infecting three species of *Drosophila*. We asked the following questions: (1) Do the non-male-killing *Spiroplasma* have lower densities than male-killing *Spiroplasma*? We addressed this by measuring the bacterial titers of a natural male-killing infection and a natural non-male-killing infection in *D. melanogaster* and evaluating the consistency of these results with bacterial threshold density hypothesis. (2) How do densities of two different types of *Spiroplasma* differ in naturally occurring infections? Here we examined 2 common *Spiroplasma* types, poulsonii and citri, and assessed whether they exhibit different densities across different *Drosophila* life stages. Variation in bacterial titers among bacterial strains and *Drosophila* species will begin to provide insight into maintenance and effects of these newly discovered and prevalent infections. (3) Do different populations of *Drosophila* that are characterized by contrasting incidence of infection in nature also exhibit different *Spiroplasma* densities? We addressed this question using individuals from two *D. mojavensis* populations with different infection incidences to determine a correlation between bacterial titer and population prevalence.

Results

Male-killing vs. non-male-killing strains

The male-killing *Spiroplasma* infecting *D. melanogaster* (UGA) had the highest density at all life stages ($p < 0.05$), followed by the non-male killing poulsonii-type *Spiroplasma* infecting *D. hydei* ($p < 0.05$). Lower densities were found in the non-male-killing *Spiroplasma* of the citri-type infecting *D. mojavensis* and *D. hydei* (**Fig. 2**). This observation was consistent when calculating *Spiroplasma* density either as number of copies per milligram dry weight or number of *Spiroplasma* copies per *ef1*-α gene (data not shown). The citri-type *Spiroplasma* in both *D. hydei* and *D. mojavensis* had lower densities at all life stages.

The non-male-killing *Spiroplasma* infecting *D. melanogaster* had the lowest titers (**Fig. 2**), undetectable using these quantitative PCR methods at eclosion and at 2 weeks in males. Low titers, around 1000 copies, of *Spiroplasma* were detected only in 1 female at eclosion, and one male at day 6. Infection levels could consistently be detected in one-week-old females (5/6 biological replicates amplified), though titers were quite low. While *Spiroplasma* titer, on average, was higher in 2-week-old females, density measurements were highly variable and infections were not detected in 3 females. Even at the highest titer levels (2.0×10^4 *Spiroplasma* copies per milligram fly), these non-male-killing *D. melanogaster* infections were still several orders of magnitude below that of the male-killing *Spiroplasma* infecting *D. melanogaster* (4.0×10^8 *Spiroplasma* copies per milligram fly). Thus, for all *Spiroplasma* strains measured, the non-male-killing *Spiroplasma* strains had

lower densities, at all life stages, than the male-killing strain.

Bacterial titer change with age

In general, the bacterial titers increased as the flies aged, although not at the same rate among *Drosophila* species or *Spiroplasma* types (**Fig. 2**). The female *D. melanogaster* infected with the male-killing *Spiroplasma* showed a pattern of increasing *Spiroplasma* density from the 3rd instar larval stage (2.8×10^7 *Spiroplasma* copies per mg fly) to 2-week-old females (4.0×10^8 *Spiroplasma* copies per mg fly). A similar trend was seen for the female *D. hydei* infected with the non-male-killing poulsonii *Spiroplasma* strain. Titers in male *D. hydei* infected with this poulsonii-type *Spiroplasma* strain increased from the 3rd instar larval stage to eclosion, but remained the same at the 1-week and 2-week-old stages. In the *D. hydei* females infected with the citri-type *Spiroplasma*, the increase in bacterial titer did not occur until the 2-week-old stage. Similar to the pattern observed for *D. hydei* poulsonii-type *Spiroplasma* infected males, the *D. hydei* citri-type *Spiroplasma* infected males did not increase in bacterial titer with fly age. A different pattern, however, was seen with *D. mojavensis*, infected with the citri-type *Spiroplasma*. This *Spiroplasma* had lower titers and several individuals had no detectable *Spiroplasma* by day 13. In 2-week-old females, 4 out of 6 individuals had no detectable *Spiroplasma,* while in 2-week-old males, 3 out of 6 had no detectable *Spiroplasma*. Individuals with measurable levels of *Spiroplasma* had titers much lower than those at day 6 (D6 females 6.0×10^7 vs. D13 females 6.2×10^6; D6 males 1.6×10^7 vs. D13 males 8.8×10^6). Patterns of *Spiroplasma* titer change during aging varied among *Drosophila* species infected with different *Spiroplasma* strains.

Spiroplasma citri-type density variation

The citri-type *Spiroplasma* infecting *D. hydei* had a consistently lower density than did the poulsonii-type *Spiroplasma* across all life stages ($p < 0.05$) (**Fig. 2**). Within the citri-type *Spiroplasma* infecting *D. mojavensis*, the *Spiroplasma* density also varied among isofemale lines (**Fig. 3**). Three of the 4 isofemale lines of *D. mojavensis* from the Sonoran Desert (OP24, OP27, and OP65) had statistically significant lower densities than those from Catalina

Figure 2. Density of *Spiroplasma* in different *Drosophila* (per mg fly) across life stages. Values for females (**A**) and males (**B**) are an average density of six biological replicates, and the error bars represent standard error. *Drosophila* species measured include *D. mojavensis* infected with citri-type non-male-killing *Spiroplasma*, *D. hydei* infected with citri-type non-male-killing *Spiroplasma*, *D. hydei* infected with non-male-killing poulsonii-type *Spiroplasma*, and *D. melanogaster* infected with poulsonii-type male-killing *Spiroplasma*. The male-killing *Spiroplasma* infecting *D. melanogaster* have the highest densities across all life stages, whereas the citri-type *Spiroplasma* are lower.

Island, though there was variation in the flies from Catalina Island as well. Despite this variation, the densities of the *Spiroplasma* from Catalina Island were, on average, higher than that of the Sonoran Desert *Spiroplasma* ($p < 0.05$). Both line and location had a statistically significant effect on density (**Table 3A**), with 48% of variance explained by location and 28% explained by line (**Table 3B**). Thus, even among closely related strains within a particular type of *Spiroplasma* there was large density variation.
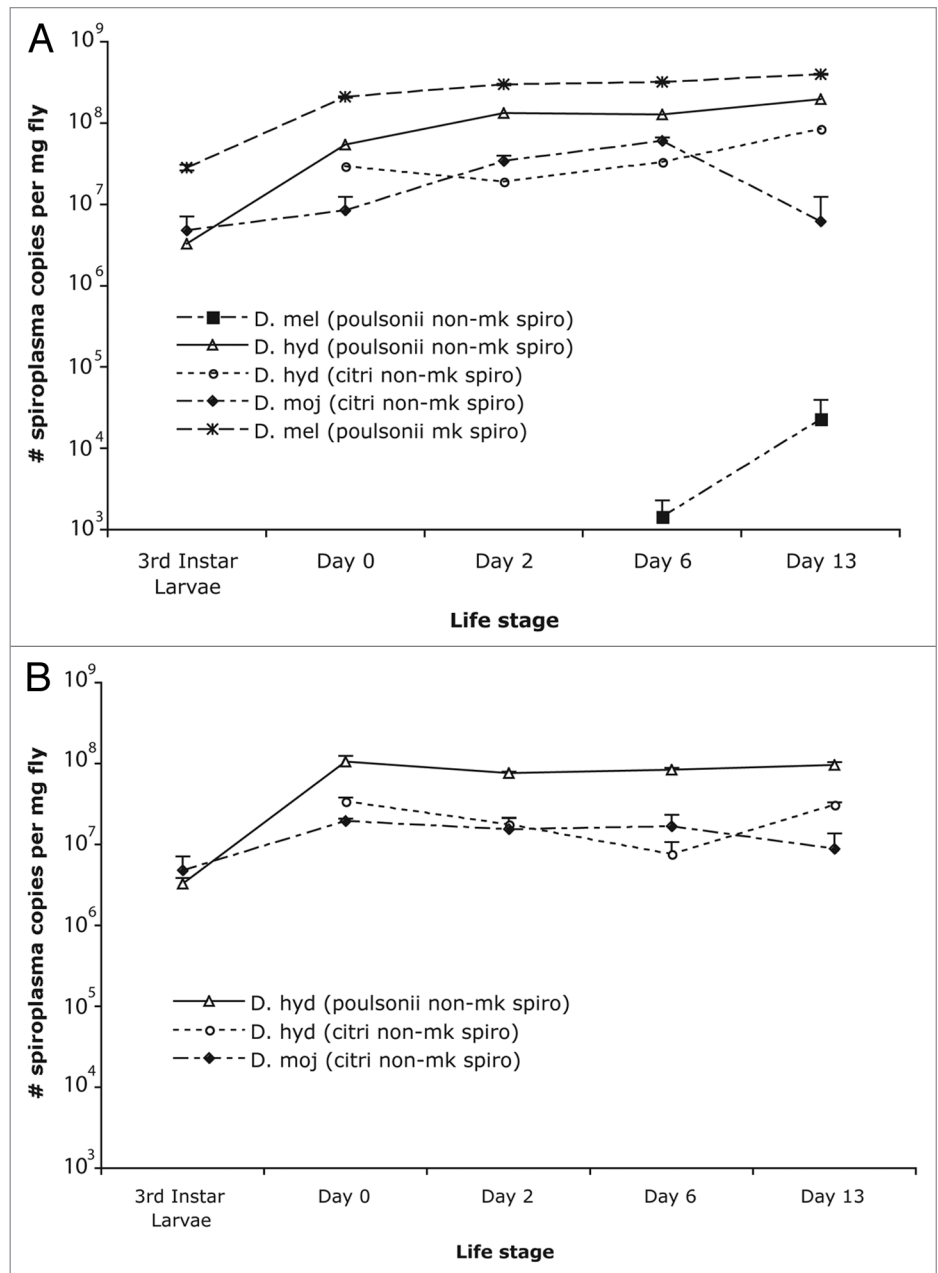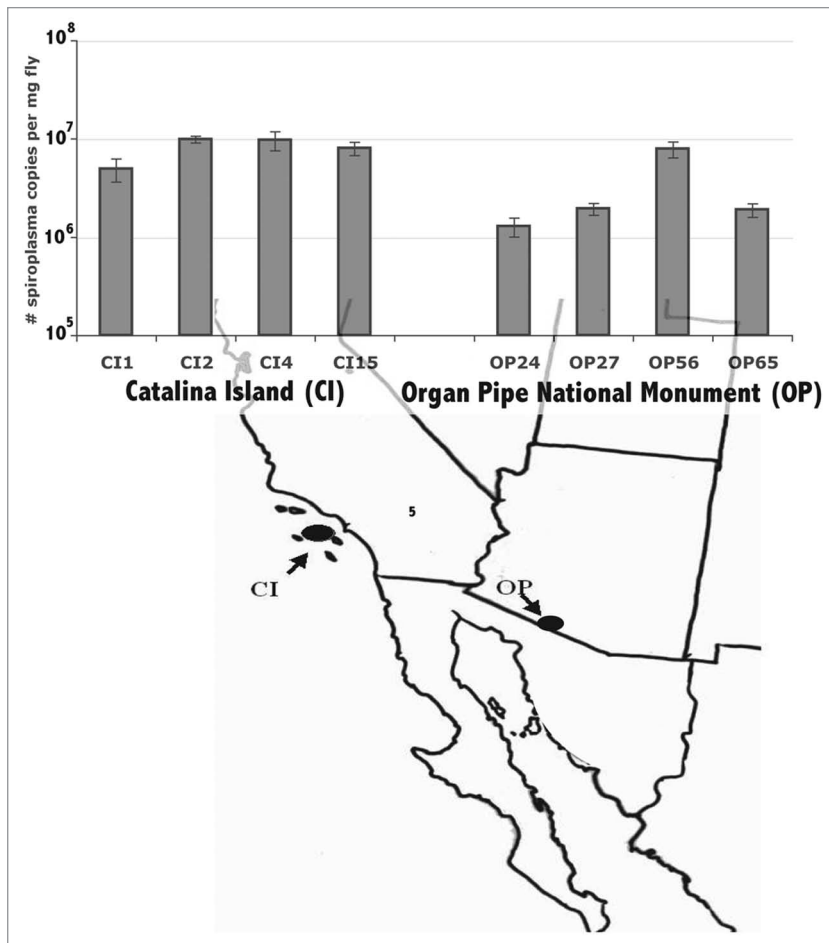
Figure 3. *D. mojavensis Spiroplasma* density variation among 8 fly lines (measured as number of *Spiroplasma* copies per milligram fly) from 2 different populations. Values are an average density of 6 biological replicates, and the error bars represent standard error.

Discussion

This study represents the first characterization of *Spiroplasma* density among several naturally infected *Drosophila* species harboring diverse *Spiroplasma* strains. Much of what is known about *Spiroplasma* density in *Drosophila* is from studies on artificially infected fly strains. While a few naturally infected flies have been measured, the variation in *Spiroplasma* density among *Drosophila* has remained largely obscure.

The density dynamics of *Spiroplasma* in *Drosophila* clearly vary among *Drosophila* species and *Spiroplasma* strains. Non-male-killing *Spiroplasma* strains have lower densities than the male-killing *Spiroplasma* strain in all *Drosophila* species examined in this study. Previous work had only compared an artificial male-killing infection (NSRO: the *D. nebulosa Spiroplasma* transferred to *D. melanogaster*), an artificial non-male-killing infection (NSRO-A: a lab variant of NSRO that lost its male-killing ability), and a single *D. hydei* non-male-killing *Spiroplasma* isolated in Japan.[14,15] Our finding of similar patterns in naturally infected strains of different *Spiroplasma* types in additional *Drosophila* species is consistent with the bacterial threshold density hypothesis

for the expression of the male-killing phenotype. Although, only a single male-killing strain was examined in this study, and the extent of *Spiroplasma* density variation among male-killing strains in different species of *Drosophila* remains to be determined.

Furthermore, the *D. melanogaster* with the non-male-killing *Spiroplasma* strain was an extremely low titer infection, with less than 1000 copies detectable in males and females under 2 weeks of age. This strain is genetically identical to the male-killing strain at the three loci for which it was sequenced;[31] however, undetected genetic variation may exist at other loci. The extremely low titer of this infection could be an inherent property of this bacterial strain, which may have lost either the ability to replicate quickly, or the ability to avoid or suppress the host immune system. Effects of host genetic background, however, cannot be ruled out. This strain could possibly cause male-killing, but may not be able to produce enough effector molecule to have any effect, due to its low titer, in accordance with the bacterial threshold density hypothesis.

The consistently lower density of *Spiroplasma* of the citri-type, along with the lower density of the citri-type *Spiroplasma* compared with the poulsonii-type *Spiroplasma* in *D. hydei* suggest that this may be a general property of the *Spiroplasma* of this type, and that bacterial strain itself plays a role in density regulation. Although, host factors such as immune response also may be involved. Variation in *Spiroplasma* densities among *D. mojavensis* isofemale lines implies the involvement of host genotype in density regulation. There are high levels of genetic variation in both the Sonoran Desert and Catalina Island populations of *D. mojavensis*.[33] Sequencing of these *Spiroplasma* strains at six different loci revealed no genetic variation in the *Spiroplasma* that infect *D. mojavensis*.[31] Though undetected variation in *Spiroplasma* strains cannot be ruled out, genetic variation in host immune response or another host factor that affects *Spiroplasma* density is more likely to be responsible for the variation in bacterial titer across isofemale lines.

The variation in *Spiroplasma* dynamics as the flies age may reflect differences in immune response and/or differences in the age of reproductive maturity. When *Spiroplasma* titer was measured as number of copies per milligram fly, females consistently had higher *Spiroplasma* titers than males from day 2 onwards. This pattern could reflect *Spiroplasma* proliferation in the ovaries, as is necessary for vertical transmission, and the timing may correlate to when flies are most likely to reproduce in the wild. Both *D. hydei* and *D. mojavensis* females mature later (3–5 d of age)[34] than *D. melanogaster* (1–2 d), therefore the lower bacterial titer, particularly before sexual maturity, may not be as detrimental to the ability to be vertically transmitted. In *D. mojavensis*,

Table 1. *Spiroplasma* infected *Drosophila* lines

Fly ID	*Drosophila* species	*Spiroplasma* type	Phenotype (mk = male-killing)	Collection details
D. melUGA	*D. melanogaster*	poulsonii	mk	Africa 2005 (Pool et al. 2005)
D. melSC	*D. melanogaster*	poulsonii	non-mk	San Carlos, Mexico 2008
D. hyd (TEN104–102)	*D. hydei*	poulsonii	non-mk	Mexico 2002
D. moj (OP10)	*D. mojavensis*	citri	non-mk	Organ Pipe National Monument 2007
D. hyd (ABH5)	*D. hydei*	citri	non-mk	Anza Borrego Desert 2009
D. moj (CI1)	*D. mojavensis*	citri	non-mk	Catalina Island 2008
D. moj (CI2)	*D. mojavensis*	citri	non-mk	Catalina Island 2008
D. moj (CI4)	*D. mojavensis*	citri	non-mk	Catalina Island 2008
D. moj (CI15)	*D. mojavensis*	citri	non-mk	Catalina Island 2008
D. moj (OP8)	*D. mojavensis*	citri	non-mk	Organ Pipe National Monument 2007
D. moj (OP27)	*D. mojavensis*	citri	non-mk	Organ Pipe National Monument 2007
D. moj (OP56)	*D. mojavensis*	citri	non-mk	Organ Pipe National Monument2007
D. moj (OP65)	*D. mojavensis*	citri	non-mk	Organ Pipe National Monument 2007

though, the decrease in *Spiroplasma* titer in 2-week-old females as compared with 1-week-old females is striking. Whether this is a general trend of decrease with aging in this species, as bacterial titer decreases with age in males as well, is unclear. In any case, this trend indicates that infection prevalence in natural populations is likely underestimated, depending on the age of the flies that are sampled.

Spiroplasma infection prevalence varies not only among species, but also among populations within species. In *D. mojavensis* populations, *Spiroplasma* infection prevalence varies dramatically.[5] Populations at Organ Pipe National Monument in the Sonoran Desert have an infection prevalence of ~15%, whereas on Catalina Island the infection prevalence is greater than 85%. Given that within-host symbiont densities are often correlated with factors that affect populations prevalence, namely the fidelity of vertical transmission and the strength of phenotypic effects,[6] we may expect a correlation between infection prevalence and bacterial density. This expectation certainly is observed in the *Spiroplasma* density differences between the Sonoran Desert *D. mojavensis* and the Catalina Island *D. mojavensis*.

The higher densities of the *D. mojavensis Spiroplasma* from Catalina Island compared with those from the Sonoran Desert may reflect either a higher fidelity of vertical transmission, or the expression of a fitness benefit that explains the higher prevalence of infection on Catalina Island. It remains to be seen if the citri-type *Spiroplasma* can act as a defensive endosymbiont, and whether or not the lower density of this *Spiroplasma* would affect the expression of such a phenotype. Of course, environmental factors, such as temperature, also likely play a role in the infection prevalence. The Sonoran Desert population of *D. mojavensis* experiences more temperature extremes, which could lower infection prevalence if the citri-type *Spiroplasma* are effected by temperature in a similar manner as the poulsonii-type *Spiroplasma*.[26,27] Measuring *Spiroplasma* density variation in flies collected directly from the field will lend insight into how environmental variation influences the bacterial density dynamics of

this endosymbiont. Since numerous cactophilic *Drosophila* are infected with *Spiroplasma* from the citri-type, further exploration of the factors affecting *Spiroplasma* density dynamics will lend critical insight into this symbiosis.

Methods

Fly lines and symbionts

Thirteen naturally infected fly lines were measured in this experiment, 5 of which were measured across different life stages. Flies were examined under controlled laboratory conditions to minimize any potential effects of temperature and other environmental factors on bacterial density. The *Drosophila* species and line designation, *Spiroplasma* strain, and origin of isofemale line are shown in **Table 1**.

Fly rearing conditions

Spiroplasma-infected *D. melanogaster* (UGA), *D. melanogaster* (SC), *D. hydei* (TEN104-102), and *D. mojavensis* (OP-10) were reared on standard banana food at room temperature in summer 2008. *D. hydei* (ABH5) and the *D. mojavensis* (CI and OP) isolines were reared in spring 2010. All fly lines were in the laboratory for at least 10 generations prior to collection. Approximately one hundred females were placed in an egg-laying chamber, and allowed to oviposit for three hours to control for the age of offspring. Oviposition plates were replaced multiple times until we obtained a plate with an estimated ~1000 eggs per plate for each species, which resulted in relatively consistent, uncrowded larval densities for each species. Flies were collected at various time points over the course of development, including 3rd instar larvae, day of eclosion (Day 0), 2nd day after eclosion (Day 2), at 1 week (Day 6) and 2 weeks (Day 13). *D. mojavensis* isolines were collected only at 1 week of age. After eclosion, male and female flies were separated and held as virgins for the later time points. At each time point, for each species, 6 flies were frozen at –80°C for quantitative PCR analysis, and another 6 flies were frozen to obtain dry weight

Table 2. Quantitative PCR primers

Primer name	Locus	*Drosophila* species	Direction	Sequence
EF23F	*ef1 α*	*D. melanogaster*	Forward	TTAACATTGT GGTCATTGGC CA
EF123R			Reverse	CTTCTCAATC GTACGCTTGT CG
EF1hydF	*ef1 α*	*D. hydei*	Forward	TTAACATCGT TGTTATTGGC CA
EF1hydR			Reverse	CTTCTCAATC GTACGCTTAT CG
EF1mojF	*ef1 α*	*D. mojavensis*	Forward	TTAACATCGT TGTGATTGGC CA
EF1mojR			Reverse	CTTCTCAATT GTACGCTTAT CG
		***Spiroplasma* type**		
109F	*dna-A*	poulsonii	Forward	CCGATTTTGA AACTGCTCTT AA
246R			Reverse	TGAAAAAAAC AAACAAATTG TTATTACTTC
270MJF	*dna-A*	citri	Forward	TGAAAAAAAT AAACAAATTG TAATTACTTC
407MJR			Reverse	TTAAGTGCTG TTTCAAAATC TGG
SRdnaAF1	*dna-A*	all	Forward	GGAGAYTCTG GAYTAGGAAA
SRdnaAR1			Reverse	CCYTCTAWYT TTCTRACATC A

Table 3. Variation among lines and between populations of *D. mojavensis*

A. Analysis of variance

Source	DF Num	Sum of Sqrs	F Ratio	Prob > F
Model	7	274015555	12.7047	< 0.0001
location	1	1.34E+08	7.49	0.0334
line[location]	6	1.11E+08	6.0145	0.0002

B. Variance component estimates

Component	Var comp est	% of Total
location	5436760	48.151
line[location]	2773153	24.561
Residual	3081143	27.288
Total	11291057	100

measurements. Flies were dried at 55°C for 72 h and weighed individually to obtain average dry weights.

DNA extraction

Each biological replicate for each species at each time point was extracted individually using a Qiagen DNA extraction kit. DNA was eluted in 100–200 µl of buffer AE, and quantified using a Nanodrop. DNA samples were diluted to 25 ng/µl for quantitative PCR analysis.

Quantitative PCR standard curve construction

We measured bacterial density using quantitative real-time PCR with the bacterial *dnaA* gene. To estimate *Spiroplasma* titer, absolute *dnaA* copy number was determined using a standard curve. To generate this standard curve, a 500 base pair region containing the quantitative PCR DNA amplicon was amplified using primers SRdnaAF1 and SRdnaAR1[14] (**Table 2**) from *Spiroplasma* from each *Drosophila* species, sequenced, and cloned using Invitrogen's Topo-TA pCR 2.1 topo vector cloning kit. Quantitative PCR primers were verified and redesigned as necessary for the different *Spiroplasma* strains (**Table 2**). Plasmids containing the larger cloned fragment were used to construct two separate standard curves, one for poulsonii-type

Spiroplasma and one for citri-type *Spiroplasma*, using dilutions of 10^9, 10^8, 10^7, 10^6, 10^5, 10^4, 10^3, and 10^2 *dnaA* copies per four microliters. An internal DNA standard, designed in the *Drosophila* single copy nuclear gene elongation factor 1 α (*ef1-α*), was constructed for each *Drosophila* species in a similar manner. Specific *ef1-α* primers for each *Drosophila* species are listed in **Table 2**.

Quantitative PCR

Quantitative PCR was performed using Aplied Biosystems (ABI) Power Sybrgreen PCR mix on an ABI 7000 machine. Twenty-five microliter PCR reactions, using 4 µl of DNA, were run on a program of 95°C for 10 min, then 95°C for 15 sec, 55°C for 45 sec, 60°C for 45 sec for 45 cycles. Primer concentrations were 300 nm each per reaction. Technical replicates were performed for each biological replicate, and if there was a discrepancy of greater than 0.5 amplification cycles, then the sample was run again. Otherwise, the 2 amplification cycle values were averaged and used for copy number calculations. Dissociation curves were constructed for verification of the target amplicon.

Statistical analyses

Spiroplasma titer was calculated at all *Drosophila* life stages as *dnaA Spiroplasma* copy equivalents per milligram fly weight. For a subset of life stages (D0, D6, and D13), the internal standard *ef1-α* was also amplified, and *Spiroplasma* titer was calculated as number of *dnaA Spiroplasma* copy equivalents per number of *ef1-α* copies. DnaA copy numbers were square root transformed to fit a normal distribution. All statistical analyses were performed using JMP v.6 (SAS). *T*-tests and a one-way analysis of variance were used to compare densities among species at different life stages. For comparisons of *Spiroplasma* density among *D. mojavensis* lines from Catalina Island (CI) and Organ Pipe National Monument (OP) a fully nested analysis of variance was performed with line nested within location, with location set as a random factor.

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