Pervasive Effects of *Wolbachia* on Host Temperature Preference

Michael T. J. Hague,ª Chelsey N. Caldwell,ª Brandon S. Cooperª

ªDivision of Biological Sciences, University of Montana, Missoula, Montana, USA

ABSTRACT Heritable symbionts can modify a range of ecologically important host traits, including behavior. About half of all insect species are infected with maternally transmitted *Wolbachia*, a bacterial endosymbiont known to alter host reproduction, nutrient acquisition, and virus susceptibility. Here, we broadly test the hypothesis that *Wolbachia* modifies host behavior by assessing the effects of eight different *Wolbachia* strains on the temperature preference of six *Drosophila melanogaster* subgroup species. Four of the seven host genotypes infected with A-group *Wolbachia* strains (*w*Ri in *Drosophila simulans*, *w*Ha in *D. simulans*, *w*Sh in *Drosophila sechellia*, and *w*Tei in *Drosophila teissieri*) prefer significantly cooler temperatures relative to uninfected genotypes. Contrastingly, when infected with divergent B-group *w*Mau, *Drosophila mauritiana* prefers a warmer temperature. For most strains, changes to host temperature preference do not alter *Wolbachia* titer. However, males infected with *w*Sh and *w*Tei tend to experience an increase in titer when shifted to a cooler temperature for 24 h, suggesting that *Wolbachia*-induced changes to host behavior may promote bacterial replication. Our results indicate that *Wolbachia* modifications to host temperature preference are likely widespread, which has important implications for insect thermoregulation and physiology. Understanding the fitness consequences of these *Wolbachia* effects is crucial for predicting evolutionary outcomes of host-symbiont interactions, including how *Wolbachia* spreads to become common.

IMPORTANCE Microbes infect a diversity of species, influencing the performance and fitness of their hosts. Maternally transmitted *Wolbachia* bacteria infect most insects and other arthropods, making these bacteria some of the most common endosymbionts in nature. Despite their global prevalence, it remains mostly unknown how *Wolbachia* influence host physiology and behavior to proliferate. We demonstrate pervasive effects of *Wolbachia* on *Drosophila* temperature preference. Most hosts infected with A-group *Wolbachia* prefer cooler temperatures, whereas the one host species infected with divergent B-group *Wolbachia* prefers warmer temperatures, relative to uninfected genotypes. Changes to host temperature preference generally do not alter *Wolbachia* abundance in host tissues, but for some A-group strains, adult males have increased *Wolbachia* titer when shifted to a cooler temperature. This suggests that *Wolbachia*-induced changes to host behavior may promote bacterial replication. Our results help elucidate the impact of endosymbionts on their hosts amid the global *Wolbachia* pandemic.

KEYWORDS *Drosophila*, host-microbe interaction, symbiosis, thermal adaptation, thermoregulation, *w*Mel

Heritable symbionts have diverse ecological effects on their hosts. In insects, microbial symbionts influence host reproduction (e.g., cytoplasmic incompatibility) (1, 2), acquisition of nutrients (3–5), tolerance of extreme temperatures (6, 7), and susceptibility to viruses (8, 9). Much less is known about symbionts' effects on host behavior and their ecological consequences (10–13). On the one hand, symbionts may

induce behavioral changes that promote the spread of infection through host populations. Because symbiotic relationships can span a continuum from mutualism to parasitism, behavioral modifications that promote infection spread may not necessarily benefit hosts (2, 14). Parasites, for example, can induce behaviors that are detrimental or lethal to hosts, such as altering host locomotor behavior to increase the probability of parasite transmission (15–20). On the other hand, infected hosts may modify their own behavior in ways that mitigate negative aspects of the infection (16, 21–23), such as a "behavioral chill" thermoregulatory response in which hosts seek cool temperatures to increase their survival probability (24). These behavioral effects represent an important component of how symbionts impact host fitness, which ultimately dictates the evolutionary trajectory of host-symbiont interactions.

Maternally transmitted *Wolbachia* bacteria are the most common endosymbionts in nature, infecting the cells of about half of all insect species, as well as other arthropods (2, 25, 26). *Wolbachia* and host phylogenies are often discordant (27–29), and most *Drosophila* hosts have recently acquired *Wolbachia* via introgressive and/or horizontal transfer (30–32). Maternal transmission occurs in the host germ line, but *Wolbachia* also infects a variety of host somatic cells, including metabolic, digestive, and nervous system tissue (33–35). The fitness consequences of *Wolbachia* in host tissues ultimately determine infection spread, and initial spread from low frequencies requires positive *Wolbachia* effects on host fitness (36–38). Exactly how *Wolbachia* alters components of host fitness is poorly understood (39), even though theoretical and population-level analyses indicate pervasive positive effects on host fitness (1, 31, 37, 40–42).

Symbionts are known to influence host thermal tolerance (7, 43–46), and two recent studies found that *Drosophila melanogaster* lines infected with the *w*MelCS or *w*Mel *Wolbachia* strain tend to prefer cooler temperatures than uninfected genotypes (47, 48). Modifications to host temperature preference (T_p) have important implications for insects, because ectothermic performance and fitness explicitly depend on temperature (49–55). Because *Wolbachia* infects most insects (2, 25, 26), it is crucial to understand how infections alter host thermoregulation. Few past analyses of insect behavioral thermoregulation have accounted for *Wolbachia* (51, 55, 56).

Differences in T_p between infected and uninfected flies could arise from conflicting physiological requirements of *Wolbachia* and their hosts. *Wolbachia* titer in host bodies is sensitive to temperature fluctuations (57), such that exceedingly cool (<20°C) and warm (>25°C) temperatures can reduce titer and the efficiency of maternal *Wolbachia* transmission (42, 57–63). *Wolbachia*-induced changes to T_p could provide more favorable thermal conditions for bacterial replication in hosts. Alternatively, host-induced changes to T_p could represent a host behavioral response that reduces *Wolbachia* titer to mitigate negative aspects of infection (e.g., behavioral chill). It is still unknown whether observed changes to T_p increase or decrease *Wolbachia* titer (47, 48).

Here, we broadly test for *Wolbachia* effects on host T_p across the *D. melanogaster* subgroup of flies. Our experiments include seven A-group *Wolbachia*-infected genotypes (*w*Ri in *Drosophila simulans*, *w*Ha in *D. simulans*, *w*MelCS in *D. melanogaster*, *w*Mel in *D. melanogaster*, *w*Sh in *Drosophila sechellia*, *w*Yak in *Drosophila yakuba*, and *w*Tei in *Drosophila teissieri*) and one B-group *Wolbachia*-infected genotype (*w*Mau in *Drosophila mauritiana*), which diverged from A-group strains 6 to 46 million years ago (41). We find that hosts infected with four of the A-group *Wolbachia* strains (*w*Ri, *w*Ha, *w*Sh, and *w*Tei) prefer a significantly cooler T_p than uninfected flies of the same host genotype. In contrast, *D. mauritiana* infected with B-group *w*Mau have a significantly warmer T_p. Unlike previous reports (47, 48), we find no evidence for *w*MelCS or *w*Mel effects on T_p of *D. melanogaster*, indicating host effects on T_p. Shifting infected adults from an intermediate temperature toward their T_p for 24 h generally does not alter *Wolbachia* titer, but in a few instances, reductions in host T_p seem to promote *Wolbachia* replication. Our results motivate future work on the causes and consequences of *Wolbachia* effects on T_p and other host behaviors.

TABLE 1 Analysis of host T_p using generalized linear mixed models (GLMMs) and a Poisson error structure[a]

Explanatory variable	*w*Ri			*w*Ha			*w*MelCS			*w*Mel		
	Coefficient	χ2	P value	Coefficient	χ2	P value	Coefficient	χ2	P value	Coefficient	χ2	P value
Infection status	0.069	6.158	**0.013***	0.063	6.148	**0.013***	−0.017	1.285	0.257	−0.004	0.031	0.86
Sex	−0.06	4.341	**0.037***	−0.07	6.907	**0.009***	−0.007	0.224	0.636	−0.046	3.49	0.062
Age	−0.003	0.016	0.898	−0.001	0.02	0.887	−0.019	11.426	**0.001***	0.012	2.251	0.134
Run order	0.001	0.013	0.909	0.009	1.002	0.317	0.011	4.914	**0.027***	0.005	0.366	0.545
Infection-by-sex	−0.013	0.099	0.754	−0.016	0.186	0.666	0.002	0.005	0.943	0.021	0.368	0.544
Sample size	1,015			857			1,727			1,341		

Explanatory variable	*w*Mau			*w*Sh			*w*Yak			*w*Tei		
	Coefficient	χ2	P value	Coefficient	χ2	P value	Coefficient	χ2	P value	Coefficient	χ2	P value
Infection status	−0.091	7.54	**0.006***	0.042	4.531	**0.033***	0.002	0.007	0.936	0.038	8.36	**0.004***
Sex	−0.052	3.175	0.075	0.009	0.238	0.626	−0.01	0.179	0.673	−0.017	1.622	0.203
Age	−0.018	2.686	0.101	0.003	0.13	0.718	0.026	3.633	0.057	−0.012	2.679	0.102
Run order	0.02	3.968	**0.046***	0.011	3.187	0.074	0.009	1.32	0.251	0.001	0.083	0.773
Infection-by-sex	0.036	0.68	0.409	−0.029	1.151	0.283	0.011	0.108	0.742	0.011	0.348	0.555
Sample size	818			1,087			10,56			2,500		

[a]Statistically significant fixed effects at $P < 0.05$ are shown in bold text with asterisks.

RESULTS

Wolbachia infections modify host temperature preference. We used a thermal gradient apparatus to test whether eight different *Wolbachia* strains alter the temperature preference (T_p) of their *Drosophila* host species (see Fig. S1 and Table S1 in the supplemental material). For each strain, we measured the T_p of *Wolbachia*-infected hosts and uninfected flies of the same genotype. In total, we assayed the T_p of 10,401 flies in 347 replicates on the thermal gradient and analyzed our results using generalized linear mixed models (GLMMs) and a Poisson error structure (Table 1 and Fig. 1). *Wolbachia* infection status had a significant main effect on host T_p for five genotypes: *w*Ri-infected *D. simulans* ($\chi^2 = 6.158$, $P = 0.013$), *w*Ha-infected *D. simulans* ($\chi^2 = 6.148$, $P = 0.013$), *w*Mau-infected *D. mauritiana* ($\chi^2 = 7.540$, $P = 0.006$), *w*Sh-infected *D. sechellia* ($\chi^2 = 4.531$, $P = 0.033$), and *w*Tei-infected *D. teissieri* ($\chi^2 = 8.360$, $P = 0.004$) (Table 1). These results were robust to whether the data were analyzed using GLMMs or linear mixed models (LLMs) (Table S2). Of the five *Wolbachia* strains with a significant effect on T_p, all host genotypes infected with A-group *Wolbachia* preferred a cooler temperature than uninfected flies (Fig. 2): *w*Ri-infected *D. simulans* preferred a least-square (LS) mean temperature of 21.72°C ± 1.02°C (±standard error [SE]) compared to 23.12°C ± 1.02°C for uninfected flies, *w*Ha-infected *D. simulans* preferred an LS mean of 23.56°C ± 1.01°C compared to the uninfected mean of 24.89°C ± 1.01°C, *w*Sh-infected *D. sechellia* preferred an LS mean of 23.32°C ± 1.01°C compared to the uninfected mean of 23.98°C ± 1.01°C, and *w*Tei-infected *D. teissieri* preferred an LS mean of 22.7°C ± 1.01°C compared to the uninfected mean of 23.7°C ± 1.01°C. In contrast, *D. mauritiana* infected with B-group *w*Mau preferred a warmer LS mean temperature of 21.15°C ± 1.01°C compared to the uninfected mean of 19.67°C ± 1.02°C.

In addition to *Wolbachia* infection status, we found other significant fixed effects on T_p. Sex had a significant main effect on T_p for both the *w*Ri-infected *D. simulans* ($\chi^2 = 4.341$, $P = 0.037$) and *w*Ha-infected *D. simulans* ($\chi^2 = 6.907$, $P = 0.009$) (Table 1). For both of these *D. simulans* genotypes, females preferred warmer temperatures than males, regardless of infection status (Fig. 1). For the *w*Ri genotype, infected females preferred an LS mean temperature of 22.37°C ± 1.02°C compared to the uninfected female mean of 23.97°C ± 1.02°C. Infected males preferred an LS mean of 21.07°C ± 1.02°C compared to the uninfected male mean of 22.28°C ± 1.02. For the *w*Ha genotype, infected females preferred an LS mean temperature of 24.41°C ± 1.02°C compared to the uninfected female mean of 25.98°C ± 1.02°C. Infected males preferred an LS mean of 22.75°C ± 1.02°C compared to the uninfected male mean of
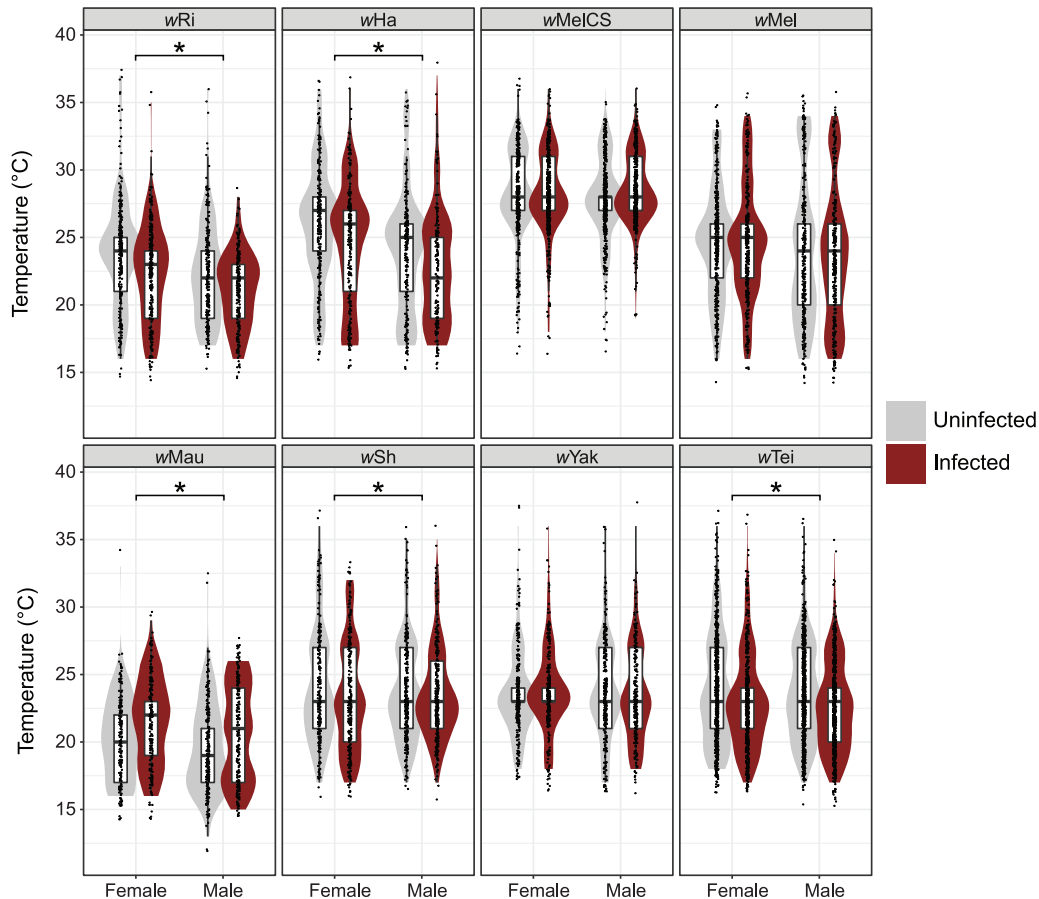
FIG 1 Box plots showing T_p for uninfected and infected flies of each genotype, separated by sex. An asterisk denotes a significant main effect of *Wolbachia* infection on T_p from the GLMMs (Table 1). Individual points are jittered to show overlap. We found a significant main effect of sex on T_p for *w*Ri ($\chi^2 = 4.341$, $P = 0.037$) and *w*Ha ($\chi^2 = 6.907$, $P = 0.009$).

23.83°C ± 1.02°C. The GLMMs also revealed a significant effect of fly age on T_p for *w*MelCS-infected *D. melanogaster* ($\chi^2 = 11.426$, $P = 0.001$), such that older flies tended to prefer cooler temperatures. Finally, we found that the run order each day had a significant effect on T_p for the *w*MelCS-*D. melanogaster* ($\chi^2 = 4.914$, $P = 0.027$) and the *w*Mau-*D. mauritiana* genotypes ($\chi^2 = 3.968$, $P = 0.046$). In both instances, flies assayed earlier in the day tended to prefer cooler temperatures. This is consistent with prior findings that the T_p of *D. melanogaster* increases from morning to evening due to a circadian clock (64). In fact, a substrain of the *Canton Special* fly line (our *w*MelCS-*D. melanogaster* genotype) was specifically shown to have increasing T_p throughout the day (see Materials and Methods for a discussion on *Canton Special* substrains) (64). Circadian clock-dependent temperature preference rhythms help ectotherms maintain homeostasis throughout the day (65). We also detected a main effect of *w*Mau on *D. mauritiana* T_p only after accounting for run order—*w*Mau had only a marginal effect on T_p when we removed run order from the model ($\chi^2 = 3.549$, $P = 0.06$).

Wolbachia effects on T_p may exhibit phylogenetic signal. Notably, hosts infected with A-group *Wolbachia* preferred cooler temperatures, whereas the one species infected with B-group *Wolbachia* preferred a warmer temperature. We conducted a phylogenomic analysis to test whether closely related *Wolbachia* strains exhibit similar effects on host T_p. We generated a *Wolbachia* phylogram and used the change in LS mean T_p of each host genotype to test for phylogenetic signal (Fig. 2). A Pagel's λ value of 1 is consistent with a model of character evolution that entirely agrees with the phylogeny (i.e., *Wolbachia* effects on host T_p exhibit strong phylogenetic signal), whereas a λ value of 0 indicates that character evolution occurs independently of
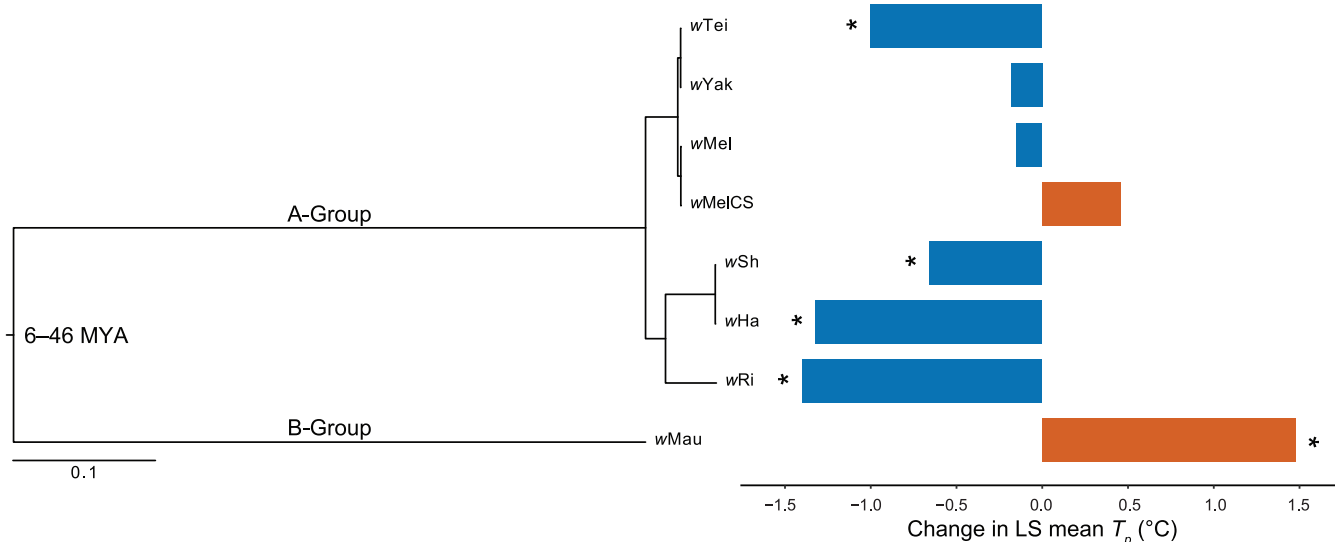
FIG 2 Estimated Bayesian phylogram for A- and B-group *Wolbachia* strains examined in this study. The phylogram was estimated with 214 single-copy genes of identical length in all of the genomes, spanning 181,488 bp. All nodes have Bayesian posterior probabilities of 1. To the right, the change in least-square (LS) mean T_p between uninfected and infected flies is shown for each *Wolbachia* strain. LS means were generated from GLMMs (Table 1), and strains with a significant main effect on T_p are marked with an asterisk. The divergence time estimate (million years ago [MYA]) for A- and B-group *Wolbachia* is from Meany et al. (41).

phylogenetic relationships (66, 67). Our maximum likelihood-fitted λ value was high (λ = 0.778 [0, 0.984]), but not significantly different from a model assuming no phylogenetic signal (likelihood ratio test, $P = 0.203$). Simulations suggest that a much larger number of *Wolbachia* strains are required to statistically distinguish λ ≈ 0.8 from zero (Fig. S2). A simulated $N = 25$ tree had a fitted λ with extremely large confidence intervals (λ = 0.886 [0, 1]), whereas the $N = 50$ tree had a λ estimate that does not overlap with zero (λ = 0.860 [0.376, 0.977]). Unfortunately, far fewer strains exist in laboratory culture, precluding such an analysis. Nevertheless, our finding that most A-group *Wolbachia* decreased host T_p and the one B-group strain increased host T_p hints that divergent *Wolbachia* may have contrasting effects on host behavior.

24-h temperature shifts generally do not alter *Wolbachia* titer. Truitt et al. speculated that the altered T_p of infected flies represents a host-induced behavior to reduce *Wolbachia* titer and ameliorate the negative effects of infection (47). According to this hypothesis, shifting species infected with A-group *Wolbachia* (*w*Ri, *w*Ha, *w*Sh, and *w*Tei) to a cool temperature should reduce *Wolbachia* titer in host bodies (i.e., behavioral chill), whereas shifting *D. mauritiana* infected with *w*Mau to a warm temperature should reduce *Wolbachia* titer (i.e., behavioral fever). We tested whether shifting infected hosts toward their T_p increases or decreases *Wolbachia* titer (Fig. 3). We reared the five infected genotypes mentioned above at an intermediate temperature of 21.5°C and collected female and male virgins for temperature shift experiments. Adults were maintained as virgins, kept at 21.5°C until they were 3 days old, and then shifted to either a cold (18°C) or warm (25°C) incubator for 24 h, after which we measured *Wolbachia* titer.

For *w*Ri-infected *D. simulans*, *Wolbachia* titer did not differ between the 24-h cold and warm temperature treatments for females ($W = 12, P = 1$) or males ($W = 19, P = 0.937$). Similarly, for *w*Ha-infected *D. simulans*, titer did not differ between the temperature treatments for females ($W = 13, P = 0.485$) or males ($W = 18, P = 1$). We also observed no significant difference in titer between temperature treatments for *w*Mau-infected *D. mauritiana* females ($W = 14, P = 0.589$) or males ($W = 14, P = 0.589$). For *w*Sh-infected *D. sechellia*, we detected no difference in *Wolbachia* titer between females from each temperature treatment ($W = 13, P = 0.485$); however, we found that males significantly differed in titer between cold and warm treatments ($W = 32, P = 0.026$).
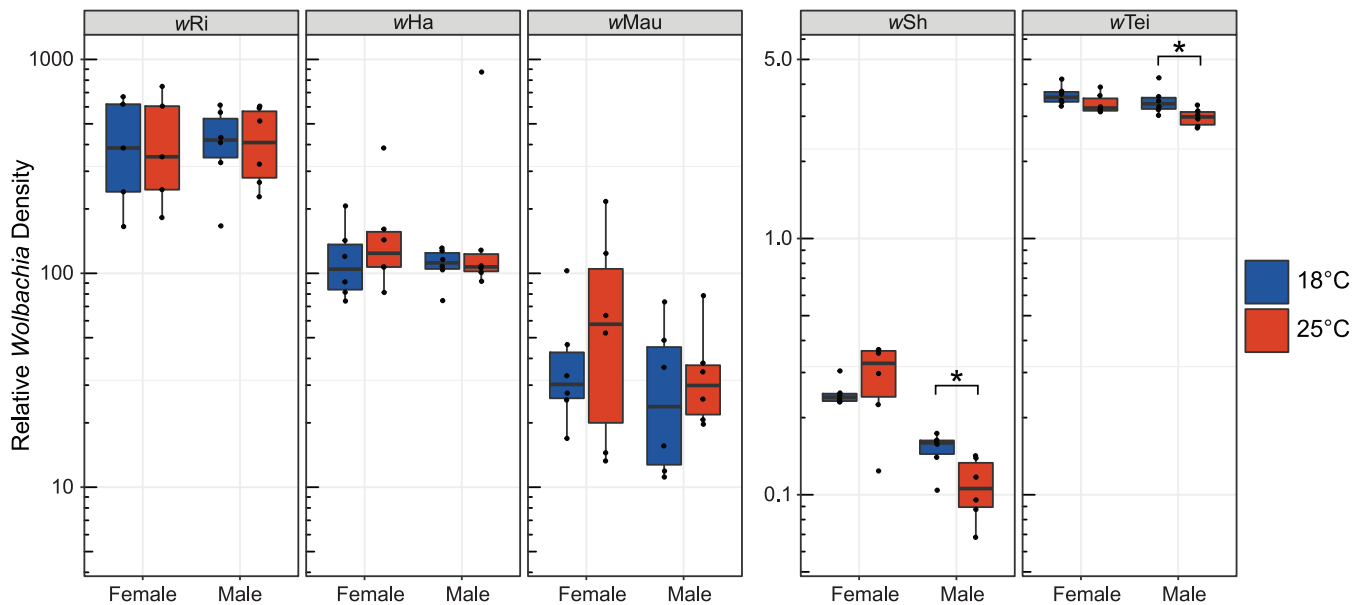
FIG 3 Boxplots of relative *Wolbachia* density from temperature shift experiments for the five *Wolbachia* strains showing main effects on host T_p (Table 1). Relative *Wolbachia* density is shown for virgin females and males shifted to cold (18°C) and warm (25°C) temperatures for 24 h. Graphs are separated into strains with high titer (*w*Ri, *w*Ha, and *w*Mau) and low titer (*w*Sh and *w*Tei). Asterisks denote significant differences in titer between males shifted to 18°C and 25°C based on Wilcoxon rank sum tests at $P < 0.05$.

Male *D. sechellia* shifted to 18°C had a higher median relative *Wolbachia* density (0.16) than males shifted to 25°C (0.11). This pattern suggests that shifting infected males toward their T_p increases *Wolbachia* titer. We found a similar result for *w*Tei-infected *D. teissieri*. While we detected no difference in *Wolbachia* titer between the treatments for females ($W = 28$, $P = 0.132$), males differed significantly in titer between the cold and warm treatments ($W = 31$, $P = 0.041$). As with *D. sechellia*, male *D. teissieri* shifted to 18°C had a higher median relative *Wolbachia* density (3.36) than males shifted to 25°C (2.98). Importantly, the *w*Sh and *w*Tei results suggest that males shifted to a colder temperature experience an increase in titer; however, these titer increases are not significant at a threshold of $P = 0.005$ after a Bonferroni correction for multiple tests.

DISCUSSION

Our analyses suggest that *Wolbachia* may generally influence host thermoregulatory behavior. Five of the eight *Wolbachia* strains we assayed had a significant effect on host T_p: *w*Ri in *D. simulans*, *w*Ha in *D. simulans*, *w*Mau in *D. mauritiana*, *w*Sh in *D. sechellia*, and *w*Tei in *D. teissieri*. In contrast to past reports (47, 48), we found no evidence for *w*MelCS or *w*Mel effects on *D. melanogaster* T_p, which we predict is due to host background effects (see below). Temperature is considered a major ecological factor limiting the distribution of *Drosophila* (55, 56, 68–72) and many other species (73–75). Body temperature is an important determinant of performance and fitness (50, 54, 76–82), and ectotherms depend on thermoregulatory behavior to maintain body temperature within a narrow range (49–53, 55). Given that *Wolbachia* have spread through most insect species and other ectotherms (2, 25, 26), our results motivate additional analyses of *Wolbachia* effects on T_p and thermoregulation of other host taxa.

Interestingly, *Drosophila* species infected with A-group *Wolbachia* generally preferred cooler temperatures, whereas *D. mauritiana* infected with divergent B-group *w*Mau preferred warmer temperatures, suggesting divergent *Wolbachia* effects on host T_p. Our simulations indicate that an unreasonably large number of strains ($N \sim 50$) is required to test whether A- and B-group *Wolbachia* effects on T_p exhibit phylogenetic signal (see Fig. S2 in the supplemental material). Indeed, this number of infected species is not currently available to the research community. Nonetheless, our results specifically motivate analyses of whether other B-group *Wolbachia* increase T_p. The only

other B-group strains that infect hosts in the *D. melanogaster* subgroup (*w*No and *w*Sn) almost always occur as coinfections with other *Wolbachia* (41). *w*No co-occurs with *w*Ha in *D. simulans* (83–86), and *w*Sn co-occurs with *w*Sh in *D. sechellia* (85, 87). *D. simulans* and *D. sechellia* genotypes singly infected with these B-group *Wolbachia* are currently unavailable. While phylogenetic relationships could be an important determinant of *Wolbachia* effects on host T_p, increases or decreases in T_p could also be idiosyncratic from one host genotype to the next.

Our phylogenetic analysis demonstrates that, in some instances, very closely related *Wolbachia* strains may have different effects on hosts. For example, *w*Tei and *w*Yak diverged only about 1,500 years ago and share very high sequence similarity (0.0039% third-position pairwise differences) (32), yet *w*Tei altered the T_p of *D. teissieri* and *w*Yak had no effect on *D. yakuba* (Fig. 2). Similarly, *w*Ha and *w*Sh have high sequence similarity according to our analysis (0.00008% third-position pairwise differences) and likely spread recently via introgression (41, 88), yet our mean estimates of titer for *w*Ha in *D. simulans* (157.1) and *w*Sh in *D. sechellia* (0.2) differ by nearly 3 orders of magnitude (Fig. 3). Host background effects may explain why closely related *Wolbachia* can have variable effects on their hosts. Our results from uninfected flies indicate that T_p varies among host genotypes within species. For *D. simulans*, the T_p of the *Wolbachia*-cleared *w*Ri (mean = 23.13°C) and *w*Ha (24.97°C) genotypes was significantly different (Wilcoxon test, W = 84398, $P < 0.001$). This was also true for the mean T_p of the uninfected *w*MelCS (27.9°C) and *w*Mel (24.3°C) *D. melanogaster* genotypes (Wilcoxon test, W = 429288, $P < 0.001$). Prior work has similarly found that T_p of *D. melanogaster* varies in North America along a latitudinal cline (55). Indeed, host genomes seem to modify *Wolbachia* titer (89), maternal *Wolbachia* transmission (90), components of host fitness (91–93), and the strength of cytoplasmic incompatibility (94–96).

We predict that host background effects also underlie our finding that *Wolbachia* does not influence *D. melanogaster* T_p, in contrast to past reports (47, 48). Arnold et al. (48) found a small, yet statistically significant, reduction in T_p of *w*MelCS-infected *D. melanogaster* (25.06°C versus 25.78°C for uninfected flies), and Truitt and colleagues (47) found that a *w*MelCS variant identical to our own (according to 720 genes totaling 733,923 bp) reduced *D. melanogaster* T_p by nearly 4°C. The effect size reported by Truitt et al. (47) is more than two and a half times greater than the largest effect we document here for any strain, and more than five times larger than the reduction in T_p observed by Arnold and colleagues (48). The *w*MelCS variant assayed in Truitt et al. (47) was introduced into the foreign DrosDel *w*¹¹¹⁸ isogenic background using chromosome replacement (97), while Arnold et al. (48) used a standard *Oregon RC* line that was orginally established in the 1920s (8, 98, 99). Our *w*MelCS-infected genotype is a substrain of the *Canton Special* line that was also established in the 1920s (100, 101), and substrains of *Canton Special* can exhibit phenotypic variation due to founder effects and drift (102). It is also worth considering that experimental differences could contribute to differences among T_p studies; for example, differences in the apparatus used to measure T_p (47, 48), fly mating status (103, 104), or statistical approaches could influence T_p estimates. Our analyses accounted for diurnal variation in T_p and host immobilization in the cold (see Materials and Methods), whereas prior analyses did not (47, 48). Regardless, we expect that future analyses of reciprocally introgressed host and *Wolbachia* genotypes will reveal that host and *Wolbachia* genomes, and their interaction, contribute to the variation in T_p observed here.

Our temperature shift experiments indicate that changes to T_p of infected host genotypes generally do not alter *Wolbachia* titer, but in a few instances, reductions in T_p may increase *Wolbachia* replication within host bodies (Fig. 3). *w*Sh-infected *D. sechellia* and *w*Tei-infected *D. teissieri* preferred cooler temperatures than uninfected flies (Fig. 2), and infected males reared at 21.5°C tended to have higher *Wolbachia* titer when shifted to a cold 18°C treatment for 24 h, compared to a warm 25°C treatment (Fig. 3). Moghadam et al. (105) reported a similar effect of cold temperature on *Wolbachia* titer in male *D. melanogaster*, in which males developed at 13°C had higher

microbial diversity and a higher relative abundance of *Wolbachia* than males developed at 23°C and 31°C (based on 16S rRNA sequencing). Our results are consistent with a hypothesis of parasite manipulation, in which *Wolbachia* alters host behavior to seek environmental conditions that promote *Wolbachia* growth (16, 18–20, 22, 23). Importantly, however, we found no temperature-associated increases in titer for *w*Sh- and *w*Tei-infected females or for any other *Wolbachia* strains we assessed. Future work should explore whether changes to male T_p and *Wolbachia* titer alter traits that determine *Wolbachia* infection spread through host populations. Increased *Wolbachia* titer in males is unlikely to affect rates of maternal *Wolbachia* transmission, but perhaps temperature-associated titer increases could alter the strength of cytoplasmic incompatibility caused by males infected with *w*Sh or *w*Tei (85, 87, 95, 106). Other studies have also reported male-biased effects on *Wolbachia* titer (42, 62, 107); for example, our own work demonstrated that maternal transmission of *w*Yak to sons is more efficient than to daughters when *D. yakuba* mothers are reared in cold 20°C conditions (42).

Our findings do not provide support for the hypothesis proposed by Truitt et al. (47) that modifications to T_p represent an adaptive host response (e.g., behavioral chill) to reduce *Wolbachia* titer and mitigate the negative effects of infection (47). In particular, Truitt et al. (47) speculated that *w*MelCS is costly to the host because the strain has a higher titer and growth rate than *w*Mel (97) and that *w*MelCS-infected *D. melanogaster* prefers colder temperatures to reduce *Wolbachia* titer and limit costly infections. The authors did not measure *w*MelCS titer or estimate host fitness components to test this hypothesis (47), although very recent work has demonstrated that *w*MelCS-infected *D. melanogaster* has reduced *Wolbachia* titer when raised at 18°C compared to 25°C (108). We found no effects of *w*MelCS or *w*Mel on T_p of *D. melanogaster* and no evidence that decreases in T_p reduce *Wolbachia* titer for other infected systems (Fig. 3). Nonetheless, the observation that most *Wolbachia*-infected hosts have altered T_p motivates future analyses of host behaviors that might mitigate negative aspects of infection, especially because *Wolbachia* can have costly effects on hosts (37, 109–111). We found no association between changes to T_p and a decrease in adult *Wolbachia* titer, but perhaps infected females seek oviposition sites that reduce the efficiency of *Wolbachia* maternal transmission (51). *Wolbachia* maternal transmission is reduced in relatively cold temperatures in *Drosophila* (42) and hot temperatures in mosquitoes (60, 61). Future work should evaluate whether reductions in host T_p lead to reduced *Wolbachia* titer and maternal transmission downstream over the course of offspring development. For example, mosquito larvae have reduced *w*AlbB titer when reared at temperatures of <20°C (63). Temperature shifts longer than 24 h may also be required to generate reductions in titer, especially if infected hosts seek their T_p throughout their lifecycles.

Our results add to mounting literature showing that temperature is an important abiotic factor mediating interactions between *Wolbachia* and their hosts (112). *Wolbachia* titer seems to be especially sensitive to temperature (42, 58, 60, 61, 63, 113–116). Our 24-h temperature shift experiments suggest that *Wolbachia* titer can change over very short time periods due to environmental conditions. Lau et al. (63) similarly found that *Wolbachia* titer can change within a single host generation, such that cold temperatures (<20°C) reduce *w*AlbB titer in mosquitoes at the larval stage, but then titer rebounds in adulthood when fourth instar larvae are shifted to warmer conditions (>21°C) (63). Temperature-induced changes to *Wolbachia* titer are likely to have cascading effects, given that titer influences other host phenotypes (57). For example, exposure to heat stress is associated with correlated declines in *Wolbachia* titer and the severity of cytoplasmic incompatibility in *w*Mel-transinfected mosquitoes (60, 61). In *Drosophila* hosts, temperature has been shown to modify the strength of cytoplasmic incompatibility (37, 58, 94, 117), maternal transmission (42, 110), and host fitness effects (118–120). Clearly, more work on how temperature influences *Wolbachia*-host interactions is needed.

Conclusion. We show that A- and B-group *Wolbachia* bacteria induce changes to host T_p and that short shifts in temperature can increase titer in some *Wolbachia*-infected males. Behavioral changes like these are likely to have fundamental consequences for host physiology and thermoregulation. *Wolbachia* also modifies a range of other ecologically important host traits in *Drosophila* species, including reproduction (1, 2), virus blocking (8, 9, 121, 122), nutrient provisioning (123, 124), and activity levels (12, 17). Given that T_p and many other *Drosophila* traits vary clinally (55, 125), future studies should consider the role of *Wolbachia* in classic *Drosophila* clines (72). For example, *w*Mel infection frequencies (120) and the T_p of *D. melanogaster* (55) both vary spatially in eastern North America.

Understanding the impact of *Wolbachia* on host performance and fitness is crucial for predicting evolutionary outcomes of *Wolbachia*-host interactions (39). The initial spread of *Wolbachia* through new host populations is driven by beneficial effects on host fitness that cause infections to deterministically spread from low initial frequencies (36–38). Yet, strong positive host effects have not been directly connected to spread in nature for any *Wolbachia*-infected host species (39, 41, 95, 126), although *w*Ri recently evolved to confer a 10% fecundity advantage to *D. simulans* (111). Few data exist for other components of host fitness, but protection from viruses and nutrient provisioning remain candidates for potential host benefits (8, 9, 121–124, 126, 127). Basic research on how *Wolbachia* modifies different components of host fitness, like the effects on T_p reported here, represents a key step to uncovering how *Wolbachia* benefit hosts and spread to become a global pandemic.

MATERIALS AND METHODS

Fly lines. We evaluated eight different *Wolbachia* strains infecting six different species in the *D. melanogaster* subgroup (see Table S1 in the supplemental material). For two of these host species, we tested multiple *Wolbachia*-infected genotypes: *w*Ri- and *w*Ha-infected *D. simulans* and *w*MelCS- and *w*Mel-infected *D. melanogaster*. With the exception of the *w*MelCS *D. melanogaster* line (*Canton S Berkeley*), all our *Wolbachia*-infected genotypes were naturally sampled to form isofemale lines, such that single gravid females were collected from the field and placed individually in vials. *w*MelCS is found only at low frequency in global populations of *D. melanogaster* (99, 128, 129), because the strain has been largely replaced by a recent sweep of *w*Mel in roughly the last 5,000 years (32, 99, 128, 129). *w*MelCS was originally identified in the common laboratory strain *Canton Special* (99–101), and a substrain (*Canton S Berkeley*) was kindly provided to us by Michael Turelli. All lines were maintained on standard cornmeal medium prior to experiments (Table S3).

We generated *Wolbachia*-uninfected genotypes by treating each infected line with 0.03% tetracycline for four generations. In the fourth generation, we used PCR to confirm that flies were cleared of *Wolbachia*. We amplified both the *Wolbachia* surface protein (*wsp*) and a second set of primers for the arthropod-specific *28S* rDNA that served as a positive control (41, 95). We also used quantitative PCR (qPCR) on 10 females homogenized together as a more sensitive confirmation of *Wolbachia* removal (see qPCR details below). We then reconstituted the gut microbiome of the tetracycline-cleared flies by rearing them on food where infected males of the same genotype had fed and defecated for the prior 48 h. Tetracycline-cleared flies were given at least three more generations before we conducted experiments to avoid detrimental effects of the antibiotic treatment on mitochondrial function (130).

Host temperature preference assays. We assayed the temperature preference (T_p) of each genotype using a thermal gradient apparatus adapted from previous studies (131, 132). The rectangular thermal gradient comprised a $44 \times 13 \times 1$ cm plate of aluminum with a removable Plexiglas lid (see Fig. S1 in the supplemental material). The Plexiglas lid enclosed a 1-cm-high space above the aluminum plate that allows flies to move around on the thermal gradient. We created an air-tight seal between the aluminum plate and the Plexiglas lid using double-sided tape and C-clamps. To keep flies on the temperature-controlled aluminum plate and off the lid, the Plexiglas was coated with Fluon (BioQuip Products), a slick barrier that prevents insects from obtaining a foothold (133, 134). A light-emitting diode (LED) light was placed above the apparatus to ensure that light was evenly distributed across the entire thermal gradient.

All T_p assays were conducted in a cold storage room with a constant temperature of 5°C. A hot plate set at 90°C was placed under one end of the aluminum plate to create a thermal gradient. All experiments began once the apparatus achieved thermal stability after approximately 0.5 h. The aluminum plate was subdivided into seven 10×6 cm sections (Fig. S1), and we recorded the temperature at the center of each section using a thermocouple (Digi-Sense Traceable) prior to the start of each experiment. The temperature decreased linearly along the gradient ($R^2 = 0.92$), ranging from a mean of 34°C at the warmest end (section 1) to 17°C at the coldest end (section 7). Mean temperatures at the center point of each section across all experiments are reported in Table S4.

The following protocol for our assay was adapted from previous experiments (47, 55, 131, 132). Trial runs revealed that a sample size of 50 to 60 flies allowed flies to distribute across the gradient without

overcrowding in preferred temperature ranges, which is consistent with prior studies (47, 131). Flies were reared in a 25°C incubator under a 12-h light:12-h dark light cycle (Pericival model I-36LL) on a standard food diet (Table S3). For each genotype, we collected virgin flies as a batch and separated them into four treatment groups: uninfected females, infected females, uninfected males, and infected males. Flies of each treatment group were separated as virgins in groups of 60 in individual food vials and kept until they were 3 to 5 days old. We selected a single batch each day and ran all four treatment groups separately in a randomized order, such that all flies assayed on a given day were of the same batch and age. All experiments were run between 9 a.m. and 5 p.m. Before each run, we measured the temperature at the center of each section along the gradient and then transferred flies into the apparatus through a small hole located in the middle of the Plexiglas lid where the temperature averaged 22.7°C (Table S4). Flies were allowed to choose their preferred temperatures along the gradient for 30 min (47, 48, 131, 132). At the end of this period, we visually scored the numbers of flies in each section. For our records, we also used a camera mounted above the thermal gradient to take a picture of the distribution of flies in each section. A subset of flies located on the Plexiglas lid were removed from the analysis (132). After each run, the thermal gradient was cleaned with ethanol and allowed to dry. The total number of replicates run for each treatment group ranged from 6 to 21. The final number of flies recorded in each replicate varied due to variation in mortality and the number of flies located on the Plexiglas lid.

For each genotype, we analyzed the T_p data using generalized linear mixed models (GLMMs) and a Poisson error structure in R (135) with the "glmer" function in the *lme4* package (136). We treated the T_p of each fly as the dependent variable and included infection status, sex, an infection-by-sex interaction, fly age (3, 4, or 5 days), and the run order of each replicate over the course of the day (1st, 2nd, 3rd, or 4th) as fixed effects. The replicate identifier (ID) of each run was included as a random effect. We then assessed the significance of fixed effects using an analysis of deviance with chi-squared tests. The T_p data for some genotypes more closely approximated a normal distribution (see Table S2), so we conducted an analogous set of tests using linear mixed models (LMMs) with the "lmer" function in the *lme4* package. Here, we assessed significance of fixed effects using an analysis of variance (ANOVA) with Wald's chi-squared tests. The LMMs produced qualitatively similar results to the GLMMs, so only results from the GLMMs are presented in the main text.

A preliminary analysis of the data revealed that flies seemed to form a bimodal distribution along the thermal gradient, with one cluster of flies located at the cold end of the gradient (section 7) where temperatures averaged about 17°C (Fig. S3). Given that 17°C generally falls below the average T_p of *Drosophila* species reported in previous experiments (47, 48, 55, 131), we hypothesized that flies were becoming immobilized in section 7 due to the cold temperature (51). A similar phenomenon has been identified for *Caenorhabditis elegans* in assays of T_p—the movement speed of *C. elegans* is dependent on temperature, which can leave worms "trapped" in cold sections of a thermal gradient (137). Thus, we removed the putatively immobilized flies in section 7 from each data set and reconducted our analyses. The analyses excluding section 7 are presented in the main text (Table 1); however, including section 7 did not alter our findings of *Wolbachia* effects on T_p (Table S5). We concluded that the data set excluding immobilized flies represents a more biologically accurate measure of T_p for each genotype.

Wolbachia sequencing and phylogenomic analysis. We conducted a phylogenomic analysis to characterize the evolutionary relationships among *Wolbachia* strains included in this study. Hosts infected with A-group *Wolbachia* (*w*Ri, *w*Ha, *w*Sh, and *w*Tei) preferred cooler temperatures, whereas *D. mauritiana* infected with B-group *w*Mau preferred a warmer temperature. Therefore, we used a *Wolbachia* phylogram to test whether these *Wolbachia* effects on host T_p exhibit phylogenetic signal. We obtained *Wolbachia* sequences from publicly available genome assemblies, which included *w*Ri (138), *w*Ha (139), *w*Mau (41), and *w*Yak and *w*Tei (32). We also obtained raw Illumina reads for a *w*Sh-infected *D. sechellia* individual from a previously published data set (NCBI:SRA accession no. SRX3029362) (140). Importantly, two divergent *Wolbachia* strains may infect *D. sechellia*: A-group *w*Sh and B-group *w*Sn. In nature, *w*Sh singly infects some individuals, but it also occurs as a coinfection with *w*Sn (85). We confirmed that our *D. sechellia* genotype (*Pmuseumbananal*) is singly infected with *w*Sh using qPCR primers described below, which can distinguish between A-group and B-group *Wolbachia*. Finally, we sequenced our *w*MelCS- and *w*Mel-infected *D. melanogaster* genotypes (*Canton S Berkeley* and *PC75*, respectively) to compare the sequence similarity of our variants of these strains to those used in the prior assay of T_p by Truitt et al. (47, 97).

Tissue samples for genomic DNA were extracted using a DNeasy Blood & Tissue kit (Qiagen). DNA quantity was tested on a Nanodrop (Implen), and total DNA was quantified by Qubit fluorometric quantitation (Invitrogen). DNA was cleaned using Agencourt AMPure XP beads (Beckman Coulter, Inc.) following the manufacturer's instructions, and eluted in 50 μl of 1× TE (Tris-EDTA) buffer for shearing. DNA was sheared using a Covaris E220 Focused Ultrasonicator (Covaris Inc.) to a target size of 400 bp. We prepared libraries using NEBNext Ultra II DNA Library Prep with Sample Purification beads (New England BioLabs). Final fragment sizes and concentrations were confirmed using a TapeStation 2200 system (Agilent). We indexed samples using NEBNext Multiplex Oligos for Illumina (Index Primers Set 3 and Index Primers Set 4), and 10 μl of each sample was shipped to Novogene (Sacramento, CA, USA) for sequencing using Illumina HiSeq 4000, generating paired-end 150 bp reads.

Reads were trimmed using Sickle version 1.33 (141) and assembled using ABySS version 2.0.2 (142). K values of 71, 81, and 91 were used, and scaffolds with the best nucleotide BLAST matches to known *Wolbachia* sequences with E values less than 10^{-10} were extracted as the draft *Wolbachia* assemblies. For each genotype, we chose the assembly with the highest N_{50} and the fewest scaffolds (Table S6). The *w*MelCS, *w*Mel, and *w*Sh genomes, along with the five previously published genomes were annotated using Prokka version 1.11, which identifies homologs to known bacterial genes (143). To avoid pseudo-

genes and paralogs, we only used genes present in a single copy with no alignment gaps in all of the genome sequences. Genes were identified as single copy if they uniquely matched a bacterial reference gene identified by Prokka. By requiring all homologs to have identical length in all of the *Wolbachia* genomes, we removed all loci with indels. A total of 214 genes totaling 181,488 bp met these criteria.

We also repeated this analysis to include the *w*MelCS and *w*Mel genomes used in Truitt et al. (47). Here, we restricted our analysis to only *w*MelCS and *w*Mel *Wolbachia*, with the goal of comparing sequence similarity between the variants used in this study to those from Truitt et al. (47). Given that many loci accumulate indels over time, the number of loci included in this analysis of *w*Mel-like *Wolbachia* was relatively high, with a total of 720 genes totaling 733,923 bp that met our criteria. Based on these 720 genes, our *w*MelCS variant infecting the *Canton S Berkeley* genotype was identical to the *w*MelCS variant used in Truitt et al. (47). Our *w*Mel variant infecting the *PC75* genotype was also highly similar to *w*Mel used in Truitt et al. (47), with only 0.000016% third-position pairwise differences (only 4 out of 244,641 third-codon positions).

We estimated a Bayesian phylogram of the 214 genes from the eight different *Wolbachia* strains using RevBayes 1.0.8 under the general tree reversible GTR + Γ model partitioned by codon position (144). Four independent runs were performed for each phylogenetic tree we estimated, and in each instance, all four runs converged on the same topology. All nodes were supported with Bayesian posterior probabilities of 1.

We used the resulting phylogram to test whether *Wolbachia* effects on host T_p exhibit phylogenetic signal. For each genotype, we extracted the least-square (LS) mean T_p for infected and uninfected flies from the GLMMs and then used the change in LS mean T_p as a continuous character to calculate the maximum likelihood value of Pagel's lambda (λ) (67). We used a likelihood ratio test to compare our fitted value of λ to a model assuming no phylogenetic signal ($\lambda = 0$) using the "phylosig" function in the R package *phytools* (145). We also employed a Monte Carlo-based method to generate 95% confidence intervals surrounding our λ estimate using 1,000 bootstrap replicates in the R package *pmc* (146). To evaluate whether larger phylogenies increase the accuracy of λ estimation, we simulated trees with an increasing number of *Wolbachia* strains ($N = 25$, 50, and 100) and our λ estimate of 0.778 using the "sim.bdtree" and "sim.char" functions in the *geiger* R package (147). We then reestimated confidence intervals surrounding λ using the larger simulated trees. See Fig. S2 for an extended description of the simulations.

Host temperature shift experiments. We tested whether shifting infected hosts toward their T_p increases or decreases *Wolbachia* titer. We reared the five infected host genotypes with altered T_p at an intermediate temperature of 21.5°C. We separated female and male virgins, kept them at 21.5°C until they were 3 days old, and then shifted them to either a cold (18°C) or warm (25°C) incubator for 24 h. Flies were separated by sex and maintained in groups of 40 in individual food vials throughout the course of the experiment. Following 24 h of the cold/warm temperature treatment, flies were frozen in a −80°C freezer for subsequent analysis of *Wolbachia* titer.

We used qPCR to compare *Wolbachia* titer in flies shifted to 18°C versus 25°C. Flies from each temperature treatment were homogenized together in groups of 10. The final samples included six biological replicates for each sex and temperature treatment. DNA was extracted using a DNeasy Blood & Tissue kit (Qiagen). Preliminary analyses indicated that our extractions contained DNA quantities that are well within the recommended range for PowerUp SYBR green Master Mix (Thermo Fisher Scientific) used in our qPCRs. We used a Stratagene Mx3000P (Agilent Technologies) to amplify *Drosophila*- and *Wolbachia*-specific loci. In order to quantify the titers of the five different *Wolbachia* strains, we utilized multiple combinations of *Drosophila* and *Wolbachia* qPCR primers (Table S7). Efficiency curves were generated to confirm that each primer pair had adequate efficiency. All qPCRs were amplified using the following cycling conditions: 50°C for 2 min, 95°C for 2 min, and then 40 cycles, with one cycle consisting of 95°C for 15 s, 58°C for 15 s, and 72°C for 1 min. We used the average cycle threshold (*Ct*) value of three technical replicates for each sample. We estimated relative *Wolbachia* density as $2^{\Delta Ct}$, where $\Delta Ct = Ct_{host} - Ct_{Wolbachia}$ (148). We then used a Wilcoxon rank sum test to assess differences in titer between flies shifted to 18°C and 25°C.

Data availability. Genome assemblies are deposited on GenBank (BioProject accession no. PRJNA658309). All other data are available on Dryad (https://doi.org/10.5061/dryad.j9kd51c8r).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, DOCX file, 0.4 MB.
FIG S2, DOCX file, 0.1 MB.
FIG S3, DOCX file, 0.5 MB.
TABLE S1, DOCX file, 0.01 MB.
TABLE S2, DOCX file, 0.01 MB.
TABLE S3, DOCX file, 0.01 MB.
TABLE S4, DOCX file, 0.01 MB.
TABLE S5, DOCX file, 0.02 MB.
TABLE S6, DOCX file, 0.01 MB.
TABLE S7, DOCX file, 0.01 MB.

REFERENCES

1. Hoffmann AA, Turelli M. 1997. Cytoplasmic incompatibility in insects, p 42–80. *In* O'Neill SL, Hoffmann AA, Werren JH (ed), Influential passengers: inherited microorganisms and arthropod reproduction. Oxford University Press, Oxford, United Kingdom.
2. Werren JH, Baldo L, Clark ME. 2008. *Wolbachia*: master manipulators of invertebrate biology. Nat Rev Microbiol 6:741–751. https://doi.org/10.1038/nrmicro1969.
3. Baumann P. 2005. Biology of bacteriocyte-associated endosymbionts of plant sap-sucking insects. Annu Rev Microbiol 59:155–189. https://doi.org/10.1146/annurev.micro.59.030804.121041.
4. Moran NA, McCutcheon JP, Nakabachi A. 2008. Genomics and evolution of heritable bacterial symbionts. Annu Rev Genet 42:165–190. https://doi.org/10.1146/annurev.genet.41.110306.130119.
5. Douglas AE. 2009. The microbial dimension in insect nutritional ecology. Funct Ecol 23:38–47. https://doi.org/10.1111/j.1365-2435.2008.01442.x.
6. Brumin M, Kontsedalov S, Ghanim M. 2011. *Rickettsia* influences thermotolerance in the whitefly *Bemisia tabaci* B biotype. Insect Sci 18:57–66. https://doi.org/10.1111/j.1744-7917.2010.01396.x.
7. Mueller UG, Mikheyev AS, Hong E, Sen R, Warren DL, Solomon SE, Ishak HD, Cooper M, Miller JL, Shaffer KA, Juenger TE. 2011. Evolution of cold-tolerant fungal symbionts permits winter fungiculture by leafcutter ants at the northern frontier of a tropical ant-fungus symbiosis. Proc Natl Acad Sci U S A 108:4053–4056. https://doi.org/10.1073/pnas.1015806108.
8. Hedges LM, Brownlie JC, O'Neill SL, Johnson KN. 2008. *Wolbachia* and virus protection in insects. Science 322:702. https://doi.org/10.1126/science.1162418.
9. Teixeira L, Ferreira Á, Ashburner M. 2008. The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster*. PLoS Biol 6:e1000002. https://doi.org/10.1371/journal.pbio.1000002.
10. Feldhaar H. 2011. Bacterial symbionts as mediators of ecologically important traits of insect hosts. Ecol Entomol 36:533–543. https://doi.org/10.1111/j.1365-2311.2011.01318.x.
11. Goodacre SL, Martin OY. 2012. Modification of insect and arachnid behaviours by vertically transmitted endosymbionts: infections as drivers of behavioural change and evolutionary novelty. Insects 3:246–261. https://doi.org/10.3390/insects3010246.
12. Bi J, Wang Y-F. 2020. The effect of the endosymbiont *Wolbachia* on the behavior of insect hosts. Insect Sci 27:846–858. https://doi.org/10.1111/1744-7917.12731.
13. Hosokawa T, Fukatsu T. 2020. Relevance of microbial symbiosis to insect behavior. Curr Opin Insect Sci 39:91–100. https://doi.org/10.1016/j.cois.2020.03.004.
14. Hentschel U, Steinert M, Hacker J. 2000. Common molecular mechanisms of symbiosis and pathogenesis. Trends Microbiol 8:226–231. https://doi.org/10.1016/S0966-842X(00)01758-3.
15. Lefevre T, Thomas F. 2008. Behind the scene, something else is pulling the strings: emphasizing parasitic manipulation in vector-borne diseases. Infect Genet Evol 8:504–519. https://doi.org/10.1016/j.meegid.2007.05.008.
16. Poulin R. 2010. Parasite manipulation of host behavior: an update and frequently asked questions, p 151–186. *In* Macedo R (ed), Advances in the study of behavior, vol 42. Elsevier, New York, NY.
17. van Houte S, Ros VI, van Oers MM. 2013. Walking with insects: molecular mechanisms behind parasitic manipulation of host behaviour. Mol Ecol 22:3458–3475. https://doi.org/10.1111/mec.12307.
18. Heil M. 2016. Host manipulation by parasites: cases, patterns, and remaining doubts. Front Ecol Evol 4:80.
19. Vale PF, Siva-Jothy A, Morrill A, Forbes MR. 2018. The influence of parasites, p 273–291. *In* Cordoba-Aguilar A, Gonzalez-Tokman D, Gonzalez-Santoyo I (ed), Insect behavior: from mechanisms to ecological and evolutionary consequences. Oxford University Press, Oxford, United Kingdom.
20. Weinersmith KL. 2019. What's gotten into you? A review of recent research on parasitoid manipulation of host behavior. Curr Opin Insect Sci 33:37–42. https://doi.org/10.1016/j.cois.2018.11.011.
21. Hart BL. 1988. Biological basis of the behavior of sick animals. Neurosci Biobehav Rev 12:123–137. https://doi.org/10.1016/S0149-7634(88)80004-6.
22. De Roode JC, Lefèvre T. 2012. Behavioral immunity in insects. Insects 3:789–820. https://doi.org/10.3390/insects3030789.
23. Curtis VA. 2014. Infection-avoidance behaviour in humans and other animals. Trends Immunol 35:457–464. https://doi.org/10.1016/j.it.2014.08.006.
24. Fedorka KM, Kutch IC, Collins L, Musto E. 2016. Cold temperature preference in bacterially infected *Drosophila melanogaster* improves survival but is remarkably suboptimal. J Insect Physiol 93-94:36–41. https://doi.org/10.1016/j.jinsphys.2016.08.005.
25. Zug R, Hammerstein P. 2012. Still a host of hosts for *Wolbachia*: analysis of recent data suggests that 40% of terrestrial arthropod species are infected. PLoS One 7:e38544. https://doi.org/10.1371/journal.pone.0038544.
26. Weinert LA, Araujo-Jnr EV, Ahmed MZ, Welch JJ. 2015. The incidence of bacterial endosymbionts in terrestrial arthropods. Proc Biol Sci 282:20150249. https://doi.org/10.1098/rspb.2015.0249.
27. O'Neill SL, Giordano R, Colbert AM, Karr TL, Robertson HM. 1992. 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. Proc Natl Acad Sci U S A 89:2699–2702. https://doi.org/10.1073/pnas.89.7.2699.
28. Raychoudhury R, Baldo L, Oliveira DC, Werren JH. 2009. Modes of acquisition of *Wolbachia*: horizontal transfer, hybrid introgression, and codivergence in the *Nasonia* species complex. Evolution 63:165–183. https://doi.org/10.1111/j.1558-5646.2008.00533.x.
29. Gerth M, Bleidorn C. 2016. Comparative genomics provides a timeframe for *Wolbachia* evolution and exposes a recent biotin synthesis operon transfer. Nat Microbiol 2:16241.
30. Conner WR, Blaxter ML, Anfora G, Ometto L, Rota-Stabelli O, Turelli M. 2017. Genome comparisons indicate recent transfer of *w*Ri-like *Wolbachia* between sister species *Drosophila suzukii* and *D. subpulchrella*. Ecol Evol 7:9391–9404. https://doi.org/10.1002/ece3.3449.
31. Turelli M, Cooper BS, Richardson KM, Ginsberg PS, Peckenpaugh B, Antelope CX, Kim KJ, May MR, Abrieux A, Wilson DA, Bronski MJ, Moore BR, Gao J-J, Eisen MB, Chiu JC, Conner WR, Hoffmann AA. 2018. Rapid global spread of *w*Ri-like *Wolbachia* across multiple *Drosophila*. Curr Biol 28:963–971.e8. https://doi.org/10.1016/j.cub.2018.02.015.

32. Cooper BS, Vanderpool D, Conner WR, Matute DR, Turelli M. 2019. *Wolbachia* acquisition by *Drosophila yakuba*-clade hosts and transfer of incompatibility loci between distantly related *Wolbachia*. Genetics 212: 1399–1419. https://doi.org/10.1534/genetics.119.302349.

33. Dobson SL, Bourtzis K, Braig HR, Jones BF, Zhou W, Rousset F, O'Neill SL. 1999. *Wolbachia* infections are distributed throughout insect somatic and germ line tissues. Insect Biochem Mol Biol 29:153–160. https://doi.org/10.1016/S0965-1748(98)00119-2.

34. Albertson R, Casper-Lindley C, Cao J, Tram U, Sullivan W. 2009. Symmetric and asymmetric mitotic segregation patterns influence *Wolbachia* distribution in host somatic tissue. J Cell Sci 122:4570–4583. https://doi.org/10.1242/jcs.054981.

35. Pietri JE, DeBruhl H, Sullivan W. 2016. The rich somatic life of *Wolbachia*. MicrobiologyOpen 5:923–936. https://doi.org/10.1002/mbo3.390.

36. Caspari E, Watson G. 1959. On the evolutionary importance of cytoplasmic sterility in mosquitoes. Evolution 13:568–570. https://doi.org/10.1111/j.1558-5646.1959.tb03045.x.

37. Hoffmann AA, Turelli M, Harshman LG. 1990. Factors affecting the distribution of cytoplasmic incompatibility in *Drosophila simulans*. Genetics 126:933–948. https://www.genetics.org/content/126/4/933.long.

38. Barton N, Turelli M. 2011. Spatial waves of advance with bistable dynamics: cytoplasmic and genetic analogues of Allee effects. Am Nat 178:E48–E75. https://doi.org/10.1086/661246.

39. Ross PA, Turelli M, Hoffmann AA. 2019. Evolutionary ecology of *Wolbachia* releases for disease control. Annu Rev Genet 53:93–116. https://doi.org/10.1146/annurev-genet-112618-043609.

40. Kriesner P, Hoffmann AA. 2018. Rapid spread of a *Wolbachia* infection that does not affect host reproduction in *Drosophila simulans* cage populations. Evolution 72:1475–1487. https://doi.org/10.1111/evo.13506.

41. Meany MK, Conner WR, Richter SV, Bailey JA, Turelli M, Cooper BS. 2019. Loss of cytoplasmic incompatibility and minimal fecundity effects explain relatively low *Wolbachia* frequencies in *Drosophila mauritiana*. Evolution 73:1278–1295. https://doi.org/10.1111/evo.13745.

42. Hague MT, Mavengere H, Matute DR, Cooper BS. 2020. Environmental and genetic contributions to imperfect *w*Mel-like *Wolbachia* transmission and frequency variation. Genetics 215:1117–1132. https://doi.org/10.1534/genetics.120.303330.

43. Russell JA, Moran NA. 2006. Costs and benefits of symbiont infection in aphids: variation among symbionts and across temperatures. Proc Biol Sci 273:603–610. https://doi.org/10.1098/rspb.2005.3348.

44. Dunbar HE, Wilson AC, Ferguson NR, Moran NA. 2007. Aphid thermal tolerance is governed by a point mutation in bacterial symbionts. PLoS Biol 5:e96. https://doi.org/10.1371/journal.pbio.0050096.

45. Wernegreen JJ. 2012. Mutualism meltdown in insects: bacteria constrain thermal adaptation. Curr Opin Microbiol 15:255–262. https://doi.org/10.1016/j.mib.2012.02.001.

46. Zhang B, Leonard SP, Li Y, Moran NA. 2019. Obligate bacterial endosymbionts limit thermal tolerance of insect host species. Proc Natl Acad Sci U S A 116:24712–24718. https://doi.org/10.1073/pnas.1915307116.

47. Truitt AM, Kapun M, Kaur R, Miller WJ. 2019. *Wolbachia* modifies thermal preference in *Drosophila melanogaster*. Environ Microbiol 21: 3259–3268. https://doi.org/10.1111/1462-2920.14347.

48. Arnold PA, Levin SC, Stevanovic AL, Johnson KN. 2019. *Drosophila melanogaster* infected with *Wolbachia* strain *w*MelCS prefer cooler temperatures. Ecol Entomol 44:287–290. https://doi.org/10.1111/een.12696.

49. Angilletta MJ, Steury TD, Sears MW. 2004. Temperature, growth rate, and body size in ectotherms: fitting pieces of a life-history puzzle. Integr Comp Biol 44:498–509. https://doi.org/10.1093/icb/44.6.498.

50. Martin TL, Huey RB. 2008. Why "suboptimal" is optimal: Jensen's inequality and ectotherm thermal preferences. Am Nat 171:E102–E118. https://doi.org/10.1086/527502.

51. Dillon ME, Wang G, Garrity PA, Huey RB. 2009. Thermal preference in *Drosophila*. J Therm Biol 34:109–119. https://doi.org/10.1016/j.jtherbio.2008.11.007.

52. Garrity PA, Goodman MB, Samuel AD, Sengupta P. 2010. Running hot and cold: behavioral strategies, neural circuits, and the molecular machinery for thermotaxis in *C. elegans* and *Drosophila*. Genes Dev 24:2365–2382. https://doi.org/10.1101/gad.1953710.

53. Hoffmann AA, Sgro CM. 2011. Climate change and evolutionary adaptation. Nature 470:479–485. https://doi.org/10.1038/nature09670.

54. Condon C, Cooper BS, Yeaman S, Angilletta MJ, Jr. 2014. Temporal variation favors the evolution of generalists in experimental populations of *Drosophila melanogaster*. Evolution 68:720–728. https://doi.org/10.1111/evo.12296.

55. Rajpurohit S, Schmidt PS. 2016. Measuring thermal behavior in smaller insects: a case study in *Drosophila melanogaster* demonstrates effects of sex, geographic origin, and rearing temperature on adult behavior. Fly (Austin) 10:149–161. https://doi.org/10.1080/19336934.2016.1194145.

56. Hoffmann A. 2010. Physiological climatic limits in *Drosophila*: patterns and implications. J Exp Biol 213:870–880. https://doi.org/10.1242/jeb.037630.

57. López-Madrigal S, Duarte EH. 2019. Titer regulation in arthropod-*Wolbachia* symbioses. FEMS Microbiol Lett 366:fnz232. https://doi.org/10.1093/femsle/fnz232.

58. Clancy DJ, Hoffmann AA. 1998. Environmental effects on cytoplasmic incompatibility and bacterial load in *Wolbachia*-infected *Drosophila simulans*. Entomol Exp Applic 86:13–24. https://doi.org/10.1046/j.1570-7458.1998.00261.x.

59. Ulrich JN, Beier JC, Devine GJ, Hugo LE. 2016. Heat sensitivity of *w*Mel *Wolbachia* during *Aedes aegypti* development. PLoS Negl Trop Dis 10:e0004873. https://doi.org/10.1371/journal.pntd.0004873.

60. Ross PA, Wiwatanaratanabutr I, Axford JK, White VL, Endersby-Harshman NM, Hoffmann AA. 2017. *Wolbachia* infections in *Aedes aegypti* differ markedly in their response to cyclical heat stress. PLoS Pathog 13:e1006006. https://doi.org/10.1371/journal.ppat.1006006.

61. Ross PA, Ritchie SA, Axford JK, Hoffmann AA. 2019. Loss of cytoplasmic incompatibility in *Wolbachia*-infected *Aedes aegypti* under field conditions. PLoS Negl Trop Dis 13:e0007357. https://doi.org/10.1371/journal.pntd.0007357.

62. Foo IJ-H, Hoffmann AA, Ross PA. 2019. Cross-generational effects of heat stress on fitness and *Wolbachia* density in *Aedes aegypti* mosquitoes. Trop Med Infect Dis 4:13. https://doi.org/10.3390/tropicalmed4010013.

63. Lau M-J, Ross PA, Endersby-Harshman NM, Hoffmann AA. 2020. Impacts of low temperatures on *Wolbachia* (Rickettsiales: Rickettsiaceae)-infected *Aedes aegypti* (Diptera: Culicidae). J Med Entomol. https://doi.org/10.1093/jme/tjaa074.

64. Kaneko H, Head LM, Ling J, Tang X, Liu Y, Hardin PE, Emery P, Hamada FN. 2012. Circadian rhythm of temperature preference and its neural control in *Drosophila*. Curr Biol 22:1851–1857. https://doi.org/10.1016/j.cub.2012.08.006.

65. Refinetti R, Menaker M. 1992. The circadian rhythm of body temperature. Physiol Behav 51:613–637. https://doi.org/10.1016/0031-9384(92)90188-8.

66. Freckleton RP, Harvey PH, Pagel M. 2002. Phylogenetic analysis and comparative data: a test and review of evidence. Am Nat 160:712–726. https://doi.org/10.1086/343873.

67. Pagel M. 1999. Inferring the historical patterns of biological evolution. Nature 401:877–884. https://doi.org/10.1038/44766.

68. Hoffmann AA, Anderson A, Hallas R. 2002. Opposing clines for high and low temperature resistance in *Drosophila melanogaster*. Ecol Lett 5:614–618. https://doi.org/10.1046/j.1461-0248.2002.00367.x.

69. Umina PA, Weeks AR, Kearney MR, McKechnie SW, Hoffmann AA. 2005. A rapid shift in a classic clinal pattern in *Drosophila* reflecting climate change. Science 308:691–693. https://doi.org/10.1126/science.1109523.

70. Kellermann V, van Heerwaarden B, Sgrò CM, Hoffmann AA. 2009. Fundamental evolutionary limits in ecological traits drive *Drosophila* species distributions. Science 325:1244–1246. https://doi.org/10.1126/science.1175443.

71. Kellermann V, Loeschcke V, Hoffmann AA, Kristensen TN, Fløjgaard C, David JR, Svenning J-C, Overgaard J. 2012. Phylogenetic constraints in key functional traits behind species' climate niches: patterns of desiccation and cold resistance across 95 *Drosophila* species. Evolution 66:3377–3389. https://doi.org/10.1111/j.1558-5646.2012.01685.x.

72. Adrion JR, Hahn MW, Cooper BS. 2015. Revisiting classic clines in *Drosophila melanogaster* in the age of genomics. Trends Genet 31:434–444. https://doi.org/10.1016/j.tig.2015.05.006.

73. Crisp MD, Arroyo MT, Cook LG, Gandolfo MA, Jordan GJ, McGlone MS, Weston PH, Westoby M, Wilf P, Linder HP. 2009. Phylogenetic biome conservatism on a global scale. Nature 458:754–756. https://doi.org/10.1038/nature07764.

74. Tittensor DP, Mora C, Jetz W, Lotze HK, Ricard D, Berghe EV, Worm B. 2010. Global patterns and predictors of marine biodiversity across taxa. Nature 466:1098–1101. https://doi.org/10.1038/nature09329.

75. Quintero I, Wiens JJ. 2013. Rates of projected climate change dramat-

ically exceed past rates of climatic niche evolution among vertebrate species. Ecol Lett 16:1095–1103. https://doi.org/10.1111/ele.12144.

76. Siddiqui W, Barlow C. 1972. Population growth of *Drosophila melanogaster* (Diptera: Drosophilidae) at constant and alternating temperatures. Ann Entomol Soc Am 65:993–1001. https://doi.org/10.1093/aesa/65.5.993.

77. Huey RB, Berrigan D. 2001. Temperature, demography, and ectotherm fitness. Am Nat 158:204–210. https://doi.org/10.1086/321314.

78. Chown SL, Nicolson S. 2004. Insect physiological ecology: mechanisms and patterns. Oxford University Press, Oxford, United Kingdom.

79. Angilletta MJ. 2009. Thermal adaptation: a theoretical and empirical synthesis. Oxford University Press, Oxford, United Kingdom.

80. Cooper BS, Hammad LA, Fisher NP, Karty JA, Montooth KL. 2012. In a variable thermal environment selection favors greater plasticity of cell membranes in *Drosophila melanogaster*. Evolution 66:1976–1984. https://doi.org/10.1111/j.1558-5646.2011.01566.x.

81. Huey RB, Kearney MR, Krockenberger A, Holtum JA, Jess M, Williams SE. 2012. Predicting organismal vulnerability to climate warming: roles of behaviour, physiology and adaptation. Philos Trans R Soc Lond B Biol Sci 367:1665–1679. https://doi.org/10.1098/rstb.2012.0005.

82. Hoekstra LA, Siddiq MA, Montooth KL. 2013. Pleiotropic effects of a mitochondrial-nuclear incompatibility depend upon the accelerating effect of temperature in *Drosophila*. Genetics 195:1129–1139. https://doi.org/10.1534/genetics.113.154914.

83. O'Neill SL, Karr TL. 1990. Bidirectional incompatibility between conspecific populations of *Drosophila simulans*. Nature 348:178–180. https://doi.org/10.1038/348178a0.

84. Mercot H, Llorente B, Jacques M, Atlan A, Montchamp-Moreau C. 1995. Variability within the Seychelles cytoplasmic incompatibility system in *Drosophila simulans*. Genetics 141:1015–1023.

85. Rousset F, Solignac M. 1995. Evolution of single and double *Wolbachia* symbioses during speciation in the *Drosophila simulans* complex. Proc Natl Acad Sci U S A 92:6389–6393. https://doi.org/10.1073/pnas.92.14.6389.

86. James A, Dean M, McMahon M, Ballard J. 2002. Dynamics of double and single *Wolbachia* infections in *Drosophila simulans* from New Caledonia. Heredity (Edinb) 88:182–189. https://doi.org/10.1038/sj.hdy.6800025.

87. Giordano R, O'Neill SL, Robertson HM. 1995. *Wolbachia* infections and the expression of cytoplasmic incompatibility in *Drosophila sechellia* and *D. mauritiana*. Genetics 140:1307–1317.

88. Ballard JWO. 2000. Comparative genomics of mitochondrial DNA in members of the *Drosophila melanogaster* subgroup. J Mol Evol 51:48–63. https://doi.org/10.1007/s002390010066.

89. Funkhouser-Jones LJ, van Opstal EJ, Sharma A, Bordenstein SR. 2018. The maternal effect gene Wds controls *Wolbachia* titer in *Nasonia*. Curr Biol 28:1692–1702. https://doi.org/10.1016/j.cub.2018.04.010.

90. Serbus LR, Sullivan W. 2007. A cellular basis for *Wolbachia* recruitment to the host germline. PLoS Pathog 3:e190. https://doi.org/10.1371/journal.ppat.0030190.

91. Fry A, Palmer M, Rand D. 2004. Variable fitness effects of *Wolbachia* infection in *Drosophila melanogaster*. Heredity (Edinb) 93:379–389. https://doi.org/10.1038/sj.hdy.6800514.

92. Dean MD. 2006. A *Wolbachia*-associated fitness benefit depends on genetic background in *Drosophila simulans*. Proc Biol Sci 273:1415–1420. https://doi.org/10.1098/rspb.2005.3453.

93. Gruntenko NE, Karpova EK, Adonyeva NV, Andreenkova OV, Burdina EV, Ilinsky YY, Bykov RA, Menshanov PN, Rauschenbach IY. 2019. *Drosophila* female fertility and juvenile hormone metabolism depends on the type of *Wolbachia* infection. J Exp Biol 222:jeb195347. https://doi.org/10.1242/jeb.195347.

94. Reynolds KT, Hoffmann AA. 2002. Male age, host effects and the weak expression or non-expression of cytoplasmic incompatibility in *Drosophila* strains infected by maternally transmitted *Wolbachia*. Genet Res 80:79–87. https://doi.org/10.1017/S0016672302005827.

95. Cooper BS, Ginsberg PS, Turelli M, Matute DR. 2017. *Wolbachia* in the *Drosophila yakuba* complex: pervasive frequency variation and weak cytoplasmic incompatibility, but no apparent effect on reproductive isolation. Genetics 205:333–351. https://doi.org/10.1534/genetics.116.196238.

96. Cattel J, Nikolouli K, Andrieux T, Martinez J, Jiggins F, Charlat S, Vavre F, Lejon D, Gibert P, Mouton L. 2018. Back and forth *Wolbachia* transfers reveal efficient strains to control spotted wing *Drosophila* populations. J Appl Ecol 55:2408–2418. https://doi.org/10.1111/1365-2664.13101.

97. Chrostek E, Marialva MSP, Esteves SS, Weinert LA, Martinez J, Jiggins

FM, Teixeira L. 2013. *Wolbachia* variants induce differential protection to viruses in *Drosophila melanogaster*: a phenotypic and phylogenomic analysis. PLoS Genet 9:e1003896. https://doi.org/10.1371/journal.pgen.1003896.

98. Hartl D, Jungen H. 1979. Estimation of average fitness of populations of *Drosophila melanogaster* and the evolution of fitness in experimental populations. Evolution 33:371–380. https://doi.org/10.1111/j.1558-5646.1979.tb04690.x.

99. Riegler M, Sidhu M, Miller WJ, O'Neill SL. 2005. Evidence for a global *Wolbachia* replacement in *Drosophila melanogaster*. Curr Biol 15:1428–1433. https://doi.org/10.1016/j.cub.2005.06.069.

100. Stern C. 1943. Genic action as studied by means of the effects of different doses and combinations of alleles. Genetics 28:441–475.

101. Stern C, Schaeffer EW. 1943. On primary attributes of alleles in *Drosophila melanogaster*. Proc Natl Acad Sci U S A 29:351–361. https://doi.org/10.1073/pnas.29.11.351.

102. Colomb J, Brembs B. 2014. Sub-strains of *Drosophila* Canton-S differ markedly in their locomotor behavior. F1000Res 3:176. https://doi.org/10.12688/f1000research.4263.1.

103. Isaac RE, Li C, Leedale AE, Shirras AD. 2010. *Drosophila* male sex peptide inhibits siesta sleep and promotes locomotor activity in the post-mated female. Proc Biol Sci 277:65–70. https://doi.org/10.1098/rspb.2009.1236.

104. Avila FW, Sirot LK, LaFlamme BA, Rubinstein CD, Wolfner MF. 2011. Insect seminal fluid proteins: identification and function. Annu Rev Entomol 56:21–40. https://doi.org/10.1146/annurev-ento-120709-144823.

105. Moghadam NN, Thorshauge PM, Kristensen TN, de Jonge N, Bahrndorff S, Kjeldal H, Nielsen JL. 2018. Strong responses of *Drosophila melanogaster* microbiota to developmental temperature. Fly (Austin) 12:1–12. https://doi.org/10.1080/19336934.2017.1394558.

106. Charlat S, Nirgianaki A, Bourtzis K, Merçot H. 2002. Evolution of *Wolbachia*-induced cytoplasmic incompatibility in *Drosophila simulans* and *D. sechellia*. Evolution 56:1735–1742.2.0.CO;2]. https://doi.org/10.1111/j.0014-3820.2002.tb00187.x.

107. Correa CC, Ballard JWO. 2012. *Wolbachia* gonadal density in female and male *Drosophila* vary with laboratory adaptation and respond differently to physiological and environmental challenges. J Invertebr Pathol 111:197–204. https://doi.org/10.1016/j.jip.2012.08.003.

108. Chrostek E, Martins N, Marialva MS, Teixeira L. 2020. *Wolbachia*-conferred antiviral protection is determined by developmental temperature. bioRxiv https://doi.org/10.1101/2020.06.24.169169.

109. Stouthamer R, Luck R. 1993. Influence of microbe-associated parthenogenesis on the fecundity of *Trichogramma deion* and *T. pretiosum*. Entomol Exp Applic 67:183–192. https://doi.org/10.1111/j.1570-7458.1993.tb01667.x.

110. Turelli M, Hoffmann AA. 1995. Cytoplasmic incompatibility in *Drosophila simulans*: dynamics and parameter estimates from natural populations. Genetics 140:1319–1338.

111. Weeks AR, Turelli M, Harcombe WR, Reynolds KT, Hoffmann AA. 2007. From parasite to mutualist: rapid evolution of *Wolbachia* in natural populations of *Drosophila*. PLoS Biol 5:e114. https://doi.org/10.1371/journal.pbio.0050114.

112. Charlesworth J, Weinert LA, Araujo E, Jr, Welch JJ. 2019. *Wolbachia*, *Cardinium* and climate: an analysis of global data. Biol Lett 15:20190273. https://doi.org/10.1098/rsbl.2019.0273.

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

114. Mouton L, Henri H, Charif D, Boulétreau M, Vavre F. 2007. Interaction between host genotype and environmental conditions affects bacterial density in *Wolbachia* symbiosis. Biol Lett 3:210–213. https://doi.org/10.1098/rsbl.2006.0590.

115. Bordenstein SR, Bordenstein SR. 2011. Temperature affects the tripartite interactions between bacteriophage WO, *Wolbachia*, and cytoplasmic incompatibility. PLoS One 6:e29106. https://doi.org/10.1371/journal.pone.0029106.

116. Sumi T, Miura K, Miyatake T. 2017. *Wolbachia* density changes seasonally amongst populations of the pale grass blue butterfly, *Zizeeria maha* (Lepidoptera: Lycaenidae). PLoS One 12:e0175373. https://doi.org/10.1371/journal.pone.0175373.

117. Hoffmann AA, Turelli M, Simmons GM. 1986. Unidirectional incompatibility between populations of *Drosophila simulans*. Evolution 40:692–701. https://doi.org/10.1111/j.1558-5646.1986.tb00531.x.

118. Olsen K, Reynolds KT, Hoffmann AA. 2001. A field cage test of the

effects of the endosymbiont *Wolbachia* on *Drosophila melanogaster*. Heredity (Edinb) 86:731–737. https://doi.org/10.1046/j.1365-2540.2001.00892.x.

119. Versace E, Nolte V, Pandey RV, Tobler R, Schlötterer C. 2014. Experimental evolution reveals habitat-specific fitness dynamics among *Wolbachia* clades in *Drosophila melanogaster*. Mol Ecol 23:802–814. https://doi.org/10.1111/mec.12643.

120. Kriesner P, Conner WR, Weeks AR, Turelli M, Hoffmann AA. 2016. Persistence of a *Wolbachia* infection frequency cline in *Drosophila melanogaster* and the possible role of reproductive dormancy. Evolution 70:979–997. https://doi.org/10.1111/evo.12923.

121. Osborne SE, Leong YS, O'Neill SL, Johnson KN. 2009. Variation in antiviral protection mediated by different *Wolbachia* strains in *Drosophila simulans*. PLoS Pathog 5:e1000656. https://doi.org/10.1371/journal.ppat.1000656.

122. Martinez J, Longdon B, Bauer S, Chan Y-S, Miller WJ, Bourtzis K, Teixeira L, Jiggins FM. 2014. Symbionts commonly provide broad spectrum resistance to viruses in insects: a comparative analysis of *Wolbachia* strains. PLoS Pathog 10:e1004369. https://doi.org/10.1371/journal.ppat.1004369.

123. Brownlie JC, Cass BN, Riegler M, Witsenburg JJ, Iturbe-Ormaetxe I, McGraw EA, O'Neill SL. 2009. Evidence for metabolic provisioning by a common invertebrate endosymbiont, *Wolbachia pipientis*, during periods of nutritional stress. PLoS Pathog 5:e1000368. https://doi.org/10.1371/journal.ppat.1000368.

124. Newton ILG, Rice DW. 2019. The Jekyll and Hyde symbiont: could *Wolbachia* be a nutritional mutualist? J Bacteriol 202:e00589-19. https://doi.org/10.1128/JB.00589-19.

125. Hoffmann AA, Weeks AR. 2007. Climatic selection on genes and traits after a 100 year-old invasion: a critical look at the temperate-tropical clines in *Drosophila melanogaster* from eastern Australia. Genetica 129:133–147. https://doi.org/10.1007/s10709-006-9010-z.

126. Shi M, White VL, Schlub T, Eden J-S, Hoffmann AA, Holmes EC. 2018. No detectable effect of *Wolbachia* wMel on the prevalence and abundance of the RNA virome of *Drosophila melanogaster*. Proc Biol Soc 285:20181165.

127. Nikoh N, Hosokawa T, Moriyama M, Oshima K, Hattori M, Fukatsu T. 2014. Evolutionary origin of insect-*Wolbachia* nutritional mutualism. Proc Natl Acad Sci U S A 111:10257–10262. https://doi.org/10.1073/pnas.1409284111.

128. Nunes MDS, Nolte V, Schlötterer C. 2008. Nonrandom *Wolbachia* infection status of *Drosophila melanogaster* strains with different mtDNA haplotypes. Mol Biol Evol 25:2493–2498. https://doi.org/10.1093/molbev/msn199.

129. Richardson MF, Weinert LA, Welch JJ, Linheiro RS, Magwire MM, Jiggins FM, Bergman CM. 2012. Population genomics of the *Wolbachia* endosymbiont in *Drosophila melanogaster*. PLoS Genet 8:e1003129. https://doi.org/10.1371/journal.pgen.1003129.

130. Ballard J, Melvin R. 2007. Tetracycline treatment influences mitochondrial metabolism and mtDNA density two generations after treatment in *Drosophila*. Insect Mol Biol 16:799–802. https://doi.org/10.1111/j.1365-2583.2007.00760.x.

131. Matute DR, Novak CJ, Coyne JA. 2009. Temperature-based extrinsic reproductive isolation in two species of *Drosophila*. Evolution 63:595–612. https://doi.org/10.1111/j.1558-5646.2008.00588.x.

132. Goda T, Leslie JR, Hamada FN. 2014. Design and analysis of temperature preference behavior and its circadian rhythm in *Drosophila*. J Vis Exp 83:e51097.

133. Dierick HA. 2007. A method for quantifying aggression in male *Drosophila melanogaster*. Nat Protoc 2:2712–2718. https://doi.org/10.1038/nprot.2007.404.

134. Dankert H, Wang L, Hoopfer ED, Anderson DJ, Perona P. 2009. Automated monitoring and analysis of social behavior in *Drosophila*. Nat Methods 6:297–303. https://doi.org/10.1038/nmeth.1310.

135. R Core Team. 2018. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

136. Bates D, Mächler M, Bolker B, Walker S. 2015. Fitting linear mixed-effects models using lme4. J Stat Soft 67:1–48.

137. Anderson JL, Albergotti L, Proulx S, Peden C, Huey RB, Phillips PC. 2007. Thermal preference of *Caenorhabditis elegans*: a null model and empirical tests. J Exp Biol 210:3107–3116. https://doi.org/10.1242/jeb.007351.

138. Klasson L, Westberg J, Sapountzis P, Näslund K, Lutnaes Y, Darby AC, Veneti Z, Chen L, Braig HR, Garrett R, Bourtzis K, Andersson SGE. 2009. The mosaic genome structure of the *Wolbachia* wRi strain infecting *Drosophila simulans*. Proc Natl Acad Sci U S A 106:5725–5730. https://doi.org/10.1073/pnas.0810753106.

139. Ellegaard KM, Klasson L, Näslund K, Bourtzis K, Andersson SG. 2013. Comparative genomics of *Wolbachia* and the bacterial species concept. PLoS Genet 9:e1003381. https://doi.org/10.1371/journal.pgen.1003381.

140. Schrider DR, Ayroles J, Matute DR, Kern AD. 2018. Supervised machine learning reveals introgressed loci in the genomes of *Drosophila simulans* and *D. sechellia*. PLoS Genet 14:e1007341. https://doi.org/10.1371/journal.pgen.1007341.

141. Joshi N, Fass J. 2011. Sickle: a sliding-window, adaptive, quality-based trimming tool for FastQ files.

142. Jackman SD, Vandervalk BP, Mohamadi H, Chu J, Yeo S, Hammond SA, Jahesh G, Khan H, Coombe L, Warren RL, Birol I. 2017. ABySS 2.0: resource-efficient assembly of large genomes using a Bloom filter. Genome Res 27:768–777. https://doi.org/10.1101/gr.214346.116.

143. Seemann T. 2014. Prokka: rapid prokaryotic genome annotation. Bioinformatics 30:2068–2069. https://doi.org/10.1093/bioinformatics/btu153.

144. Höhna S, Landis MJ, Heath TA, Boussau B, Lartillot N, Moore BR, Huelsenbeck JP, Ronquist F. 2016. RevBayes: Bayesian phylogenetic inference using graphical models and an interactive model-specification language. Syst Biol 65:726–736. https://doi.org/10.1093/sysbio/syw021.

145. Revell LJ. 2012. phytools: an R package for phylogenetic comparative biology (and other things). Methods Ecol Evol 3:217–223. https://doi.org/10.1111/j.2041-210X.2011.00169.x.

146. Boettiger C, Coop G, Ralph P. 2012. Is your phylogeny informative? Measuring the power of comparative methods. Evolution 66:2240–2251. https://doi.org/10.1111/j.1558-5646.2011.01574.x.

147. Harmon LJ, Weir JT, Brock CD, Glor RE, Challenger W. 2008. GEIGER: investigating evolutionary radiations. Bioinformatics 24:129–131. https://doi.org/10.1093/bioinformatics/btm538.

148. Pfaffl MW. 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29:e45. https://doi.org/10.1093/nar/29.9.e45.