Pervasive Effects of *Wolbachia* on Host Temperature Preference

Michael T. J. Hague, Chelsey N. Caldwell, Brandon S. Cooper

**Division 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 (*wRi* in *Drosophila simulans*, *wHa* in *D. simulans*, *wSh* in *Drosophila sechellia*, and *wTei* in *Drosophila teissieri*) prefer significantly cooler temperatures relative to uninfected genotypes. Contrastingly, when infected with divergent B-group *wMau*, *Drosophila mauritiana* prefers a warmer temperature. For most strains, changes to host temperature preference do not alter *Wolbachia* titer. However, males infected with *wSh* and *wTei* 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, wMel
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 wMelCS or wMel Wolbachia strain tend to prefer cooler temperatures than uninfected genotypes (47, 48). Modifications to host temperature preference (Tp) 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 Tp 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 Tp could provide more favorable thermal conditions for bacterial replication in hosts. Alternatively, host-induced changes to Tp 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 Tp increase or decrease Wolbachia titer (47, 48).

Here, we broadly test for Wolbachia effects on host Tp across the D. melanogaster subgroup of flies. Our experiments include seven A-group Wolbachia-infected genotypes (wRi in Drosophila simulans, wHa in D. simulans, wMelCS in D. melanogaster, wMel in D. melanogaster, wSh in Drosophila sechellia, wYak in Drosophila yakuba, and wTei in Drosophila teissieri) and one B-group Wolbachia-infected genotype (wMau 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 (wRi, wHa, wSh, and wTei) prefer a significantly cooler Tp than uninfected flies of the same host genotype. In contrast, D. mauritiana infected with B-group wMau have a significantly warmer Tp. Unlike previous reports (47, 48), we find no evidence for wMelCS or wMel effects on Tp of D. melanogaster, indicating host effects on Tp. Shifting infected adults from an intermediate temperature toward their Tp for 24 h generally does not alter Wolbachia titer, but in a few instances, reductions in host Tp seem to promote Wolbachia replication. Our results motivate future work on the causes and consequences of Wolbachia effects on Tp and other host behaviors.
**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: wRi-infected *D. simulans* (\(\chi^2 = 6.158, P = 0.013\)), wHa-infected *D. simulans* (\(\chi^2 = 6.148, P = 0.013\)), wMau-infected *D. mauritiana* (\(\chi^2 = 7.540, P = 0.006\)), wSh-infected *D. sechellia* (\(\chi^2 = 4.531, P = 0.033\)), and wTei-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): wRi-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, wHa-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, wSh-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 wTei-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 wMau 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 wRi-infected *D. simulans* (\(\chi^2 = 4.341, P = 0.037\)) and wHa-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 wRi 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 wHa 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

### Table 1: Analysis of host \(T_p\) using generalized linear mixed models (GLMMs) and a Poisson error structure

| Explanatory variable | wRi | wHa | wMe | wMel |
|----------------------|-----|-----|-----|------|
|                      | Coefficient | \(\chi^2\) | \(P\) value | Coefficient | \(\chi^2\) | \(P\) value | Coefficient | \(\chi^2\) | \(P\) value | Coefficient | \(\chi^2\) | \(P\) value |
| Infection status     | 0.069 | 21.15°C | 0.013* | 0.063 | 6.148 | 0.013* | 0.017 | 1.285 | 0.257 | 0.004 | 0.257 | 0.003 | 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: 818 1,087 1,727 2,500

*Statistically significant fixed effects at \(P < 0.05\) are shown in bold text with asterisks.
The GLMMs also revealed a significant effect of fly age on \( T_p \) for \( w_{\text{MelCS}} \)-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 \( w_{\text{MelCS}} \)-\( D. \ melanogaster \) \((\chi^2 = 4.914, P = 0.027)\) and the \( w_{\text{Mau}} \)-\( D. \ mauritiana \) 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 \( w_{\text{MelCS}} \)-\( D. \ melanogaster \) 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 \( w_{\text{Mau}} \) on \( D. \ mauritiana \) \( T_p \) only after accounting for run order—\( w_{\text{Mau}} \) 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 \( \lambda \) 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 \( \lambda \) value of 0 indicates that character evolution occurs independently of
phylogenetic relationships (66, 67). Our maximum likelihood-fitted λ value was high (λ = 0.778 [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 Tp, and the one B-group strain increased host Tp 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 Tp 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 (wRi, wHa, wSh, and wTei) to a cool temperature should reduce Wolbachia titer in host bodies (i.e., behavioral chill), whereas shifting D. mauritiana infected with wMau to a warm temperature should reduce Wolbachia titer (i.e., behavioral fever). We tested whether shifting infected hosts toward their Tp 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 cool (18°C) or warm (25°C) incubator for 24 h, after which we measured Wolbachia titer.

For wRi-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 wHa-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 wMau-infected D. mauritiana females (W = 14, P = 0.589) or males (W = 14, P = 0.589). For wSh-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).
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 wMelCS or wMel 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* ~ 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

![Boxplots of relative Wolbachia density from temperature shift experiments for the five Wolbachia strains showing main effects on host Tp (Table 1).](image-url)
other B-group strains that infect hosts in the *D. melanogaster* subgroup (wNo and wSn) almost always occur as coinfections with other *Wolbachia* (41). wNo co-occurs with wHa in *D. simulans* (83–86), and wSn co-occurs with wSh 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, wTei and wYak diverged only about 1,500 years ago and share very high sequence similarity (0.0039% third-position pairwise differences) (32), yet wTei altered the $T_p$ of *D. teissieri* and wYak had no effect on *D. yakuba* (Fig. 2). Similarly, wHa and wSh 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 wHa in *D. simulans* (157.1) and wSh 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 wRi (mean = 23.13°C) and wHa (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 wMelCS (27.9°C) and wMel (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 wMelCS-infected *D. melanogaster* (25.06°C versus 25.78°C for uninfected flies), and Truitt and colleagues (47) found that a wMelCS 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 wMelCS 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 R* line that was originally established in the 1920s (8, 98, 99). Our wMelCS-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). wSh-infected *D. sechellia* and wTei-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 wSh- and wTei-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 wSh or wTei (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 wYak 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 wMelCS is costly to the host because the strain has a higher titer and growth rate than wMel (97) and that wMelCS-infected D. melanogaster prefers colder temperatures to reduce Wolbachia titer and limit costly infections. The authors did not measure wMelCS titer or estimate host fitness components to test this hypothesis (47), although very recent work has demonstrated that wMelCS-infected D. melanogaster has reduced Wolbachia titer when raised at 18°C compared to 25°C (108).

We found no effects of wMelCS or wMel 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 wAlbB 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 wAlbB 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 wMel-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, wMel 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 wRi 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: wRi- and wHa-infected D. simulans and wMelCS- and wMel-infected D. melanogaster. With the exception of the wMelCS D. melanogaster line (Canton S Berkeley), all of 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. wMelCS 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 wMel in roughly the last 5,000 years (32, 99, 128, 129). wMelCS was originally identified in the common laboratory strain Canton Special (99–101), and a strain (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 × 13 × 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 airtight seal between the aluminum plate and the Plexiglas lid using double-sided tape and C-clamps. To keep flies on the plate that allows flies to move around on the thermal gradient. We created an airtight seal between the aluminum plate and the Plexiglas lid using double-sided tape and C-clamps. To keep flies on the thermal gradient comprised a 44

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 cycle (Pericival model I-360L) 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 "glmmer" 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 section 7 were modeled as 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 C. 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 recomputed 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 phylogenetic analysis.** We conducted a phylogenetic analysis to characterize the evolutionary relationships among Wolbachia strains included in this study. Hosts infected with A-group Wolbachia (wRi, wHa, wSh, and wTei) preferred cooler temperatures, whereas D. mauritiana infected with B-group wMau 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 wRi (138), wHa (139), wMau (41), and wYak and wTei (32). We also obtained raw Illumina reads for the wSh-infected D. sechellia individual from a previously published data set (NCBI:SRX accession no. SRX3029362 (140). Importantly, two divergent Wolbachia strains may infect D. sechellia: A-group wSh and B-group wSn. In nature, wSh singly infects some individuals, but it also occurs as a coinfection with wSn (85). We confirmed that our D. sechellia genotype (Pmuseumbananath) is singly infected with wSh using qPCR primers described below, which can distinguish between A-group and B-group Wolbachia. Finally, we sequenced our wMelCS- and wMel-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 N50 and the fewest scaffolds (Table S6). The wMelCS, wMel, and wSh genomes, along with the five previously published genomes were annotated using Prokka version 1.1.11, 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 genomes sequenced. Genes were identified as single copy if they uniquely matched a bacterial reference genome 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 181,488bp met these criteria.

We also repeated this analysis to include the wMelCS and wMel genomes used in Truitt et al. (47). Here, we restricted our analysis to only wMelCS and wMel 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 wMel-like Wolbachia was relatively high, with a total of 720 genes totaling 733,923bp that met our criteria.

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 (\( \lambda \)) (67). We used a likelihood ratio test to compare our fitted value of \( \lambda \) 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 \( \lambda \) estimate using 1,000 bootstrap replicates in the R package pmc (146). To evaluate whether larger phylogenies increase the accuracy of \( \lambda \) estimation, we simulated trees with an increasing number of Wolbachia strains (\( N = 25, 50, \) and 100) and our \( \lambda \) estimate of 0.778 using the “sim.bdtree” and “sim.char” functions in the geiger R package (147). We then reestimated confidence intervals surrounding \( \lambda \) 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 \( \Delta Ct = Ct_{\text{Wolbachia}} - Ct_{\text{CtWolbachia}} \) (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.
ACKNOWLEDGMENTS

We thank Tim Wheeler for assistance in the lab and Will Conner for help with bioinformatic analyses. Isaac Humble helped construct the thermal gradient apparatus. Dave Begun, Michael Turelli, and Daniel Matute kindly provided the flies used in this study. The Cooper lab group, Michael May, and Gregg Thomas provided valuable feedback that improved the quality of the manuscript. We thank the Genomics Core and the Environmental Control for Organismal Research (ECOR) Laboratories at the University of Montana for their support. An invited editor and two anonymous reviewers provided comments that improved the manuscript.

Research reported in this publication was supported by the National Institute of General Medical Sciences of the National Institutes of Health (NIH) under award number R35GM124701 to B.S.C.

REFERENCES

1. Hoffmann AA, Turelli M. 1997. Cytoplasmic incompatibility in insects, p 42–80. In O'Neill SL, Hoffmann AA, Werren JH (ed), Influen
tial passenges: 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.59.030804.120141.

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 TB, Gao J-J, Eisen MB, Chiu JC, Conner WR, Hoffmann AA. 2018. Rapid global spread of \textit{Wolbachia} across multiple \textit{Drosophila}. Curr Biol 28:963–971.e8. https://doi.org/10.1016/j.cub.2018.02.015.
Wolbachia Modifies Host Temperature Preference

Wolbachia modifies host temperature preference: a case study in Drosophila simulans.

Cooper BS, Vanderpool D, Conner WR, Matute DR, Turelli M. 2019. 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/10.1086/693369.2014.119415.

Hoffmann A. 2010. Physiological climatic limits in Drosophila: patterns and implications. J Exp Biol 213:870–880. https://doi.org/10.1242/jeb.03763.

Löfqvist M, Durate EH. 2019. Titer regulation in arthropod-Wolbachia symbioses. FEMS Microbiol Lett 366:fnz232. https://doi.org/10.1093/femsec/fnz232.

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

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

Ross PA, Wiwatanaaratanaubt 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.

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.

Foo U-J, 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.

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/jtaa074.

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.

Refinetti R, Menaker M. 1992. The circadian rhythm of body temperature. Physiol Behav 51:613–637. https://doi.org/10.1016/0031-9384(92)01088-8.

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.

Pagel M. 1999. Inferring the historical patterns of biological evolution. Nature 401:877–884. https://doi.org/10.1038/44676.

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

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.

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.

Kellermann V, Loeschcke V, Hoffmann AA, Kristensen TN, Floegaard 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.1558-5646.2012.01665.x.

Adlon 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.

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

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

Quinton I, Wiens JJ. 2013. Rates of projected climate change dramat-
Hague et al.

The maternal effect gene Wds controls... 

bacterial titer in... 

the expression of cytoplasmatic incompatibility in... and D. mauritiana. Genetics 140:1307–1317.

Balla 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/s002390010064.

Fukunouseru Y, Kanayama O, Shinaya K, Bordenstein SR. 2018. The maternal gene effect Wds controls Wolbachia titer in Nasonia. Curr Biol 28:1692–1702. https://doi.org/10.1016/j.cub.2018.04.010.

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.

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.6800054.

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.

Gruntenko NE, Karpova EV, Andreenkova OV, Burdina EV, Ilinsky YY, Bykov RA, Menshanov PN, Ilinskaya BY, Bykov RA, Menshanov PN, Rauschenbach IY. 2019. Wolbachia-induced cytoplasmic incompatibility in Drosophila simulans: dynamics and parameter estimates from natural populations. Genetics 140:1319–1338.

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:e224. https://doi.org/10.1371/journal.pbio.0050224.

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

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.

Mouton L, Henri H, Charif D, Bouletreau 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.

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.

Sumi T, Miyura 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.

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.

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. Kiesner 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. Martínez J, Longdon B, Bauer S, Chan Y-S, Miller WJ, Bourtizs 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, Rieger M, Witzenburg JJJ, 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 of 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 evidence for metabolic provisioning by Wolbachia wMel in Drosophila melanogaster. Mol Biol Evol 25:2493–2498. https://doi.org/10.1093/molbev/msn199.

127. Richardson MF, Weinert LA, Welch JI, Linheiro RS, Magwire MM, Jiggins FM, Bergman CM. 2012. Population genomics of the Wolbachia endosymbiont in Drosophila melanogaster. PLoS Genet 8:e1003381. https://doi.org/10.1371/journal.pgen.1003381.

128. Dankev H, Wang L, Hooper 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.

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

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. Ellegaard KM, Klasson L, Näslund K, Bourtzis K, Andersson SG. 2013. Comparative genomics of Wolbachia and the bacterial species concept. Proc Natl Acad Sci USA 110:5725–5730. https://doi.org/10.1073/pnas.1307531010.

135. Fascioli J, Trumper C, Johnson KL, Petrov DA. 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.

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

137. 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.

138. Klasson L, Westberg J, Sämslund K, Lutnaes Y, Darby AC, Veneti Z, Chen L, Braig HR, Garrett R, Bourtizs 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. Proc Natl Acad Sci USA 110:5725–5730. https://doi.org/10.1073/pnas.0810753106.

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, Durocher D, Lander ES. 2008. GEIGER: a general evolutionary inference framework for comparative genomic analysis. Genome Res 18:1327–1330. https://doi.org/10.1101/gr.075211.108.

143. Seemann T. 2014. Prokka: rapid prokaryotic genome annotation. Bioinformatics 30:2666–2668. https://doi.org/10.1093/bioinformatics/btu153.

144. Hönna S, Landis MJ, Heath TA, Boussau B, Martiottl N, Moore BR, Hulsenbeck 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: 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.