**Wolbachia-Conferring Antiviral Protection Is Determined by Developmental Temperature**

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**ABSTRACT** Wolbachia is a maternally transmitted bacterium that is widespread in arthropods and filarial nematodes and confers strong antiviral protection in *Drosophila melanogaster* and other arthropods. Wolbachia-transinfected *Aedes aegypti* mosquitoes are currently being deployed to fight transmission of dengue and Zika viruses. However, the mechanism of antiviral protection and the factors influencing are still not fully understood. Here, we show that temperature modulates Wolbachia-conferring protection in *Drosophila melanogaster*. Temperature after infection directly impacts *Drosophila* C virus (DCV) replication and modulates Wolbachia protection. At higher temperatures, viruses proliferate more and are more lethal, while Wolbachia confers lower protection. Strikingly, host developmental temperature is a determinant of Wolbachia-conferved antiviral protection. While there is strong protection when flies develop from egg to adult at 25°C, the protection is highly reduced or abolished when flies develop at 18°C. However, Wolbachia-induced changes during development are not sufficient to limit virus-induced mortality, as Wolbachia is still required to be present in adults at the time of infection. This developmental effect is general, since it was present in different host genotypes, Wolbachia variants, and upon infection with different viruses. Overall, we show that Wolbachia-confered antiviral protection is temperature dependent, being present or absent depending on the environmental conditions. This interaction likely impacts Wolbachia-host interactions in nature and, as a result, frequencies of host and symbiont in different climates. Dependence of Wolbachia-mediated pathogen blocking on developmental temperature could be used to dissect the mechanistic bases of protection and influence the deployment of Wolbachia to prevent transmission of arboviruses.

**IMPORTANCE** Insects are often infected with beneficial intracellular bacteria. The bacterium Wolbachia is extremely common in insects and can protect them from pathogenic viruses. This effect is being used to prevent transmission of dengue and Zika viruses by Wolbachia-infected mosquitoes. To understand the biology of insects in the wild, we need to discover which factors affect Wolbachia-conferring antiviral protection. Here, we show that the temperature at which insects develop from eggs to adults can determine the presence or absence of antiviral protection. The environment, therefore, strongly influences this insect-bacterium interaction. Our work may help to provide insights into the mechanism of viral blocking by Wolbachia, deepen our understanding of the geographical distribution of host and symbiont, and incentivize further research on the temperature dependence of Wolbachia-conferring protection for control of mosquito-borne disease.

**KEYWORDS** Wolbachia, *Drosophila*, virus, temperature, symbiosis, development

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The environment affects not only individual organisms but also the relationships between them (1). Numerous physical and biological factors have been described as important for insect-microbe symbioses. These include insect population density (2–4), nutrient availability (5, 6), and interactions between factors with either synergistic or antagonistic effects (7). Temperature is a major factor that affects several different host-microbe symbioses and is relevant to understand host and symbiont distributions and to predict future outcomes of interactions in a scenario of global warming (1, 8–18).

Wolbachia is a maternally inherited intracellular bacterium that infects a wide range of arthropods and some nematodes. This endosymbiont induces strong phenotypes in many of its hosts, which may contribute to its success in invading and being maintained in host populations. wMel, the Wolbachia strain present in Drosophila melanogaster, confers strong protection against a wide range of RNA viruses, which can be a fitness benefit in nature (19, 20). This protection extends to nonnative Wolbachia-host associations, including in mosquito vectors of human disease (21). Recently, Wolbachia has become one of the most promising approaches to control dengue and Zika viruses in the wild. It has been shown that the release of Wolbachia-infected Aedes aegypti mosquitoes can reduce the number of dengue cases in both areas where dengue is endemic or nonendemic (22–26). Although the molecular mechanisms of Wolbachia-conferred antiviral protection are not yet known, identification of factors influencing protection can contribute to understanding the association of Wolbachia and hosts in natural populations, as well as inform Wolbachia-based field interventions.

Wolbachia-conferred antiviral protection is influenced by host and bacterial genetics. Different Wolbachia strains in the same host genetic background differ in protection (27–31). In general, differences in Wolbachia titers are correlated with differences in protection, with higher titers conferring higher antiviral protection (27–31). However, some Wolbachia strains do not provide protection despite high titers (32). Host genetic variation can also contribute to the strength of Wolbachia-conferred protection, as seen in Aedes aegypti (33).

Environmental factors also affect this symbiont-mediated protection, either through modulation of Wolbachia titer or by titer-independent mechanisms. A host diet rich in cholesterol reduces antiviral protection in Drosophila melanogaster (6). Similarly, antibiotic treatment reducing Wolbachia titer reduces the antiviral protection (34). Finally, temperature has been shown to modulate Wolbachia-conferred protection to parasites in an engineered Wolbachia-host association; in Anopheles stephensi, temperature and somatic Wolbachia infection determined Plasmodium titer in mosquitoes (14). In Aedes aegypti mosquitoes stably transinfected with wMel, the comparison of one constant and two fluctuating temperature regimes showed that the antiviral protection is robust across conditions and is unlikely to be compromised in antiviral field trials (35). However, a more recent study indicates that at a high temperature, wMel is less protective (36). This is associated with lower titers of wMel in A. aegypti mosquitoes reared at high temperatures, which may also affect the strength of cytoplasmic incompatibility and the vertical transmission of Wolbachia (36–39). On the other hand, different temperature regimes also affect dengue transmission independently of Wolbachia (40).

Despite two studies tackling the effect of temperature on Wolbachia-conferred protection in artificial associations, the impact of temperature on antiviral protection in natural Wolbachia-host symbiosis remains unknown. However, it is known that temperature can affect the interaction between Wolbachia and Drosophila. For instance, higher temperatures lead to greater proliferation, and, in the case of the pathogenic variants wMelPop and wMelOctoless, higher cost, in D. melanogaster (8, 41–43). Similarly, lower developmental temperatures lead to lower titers of wYak in Drosophila yakuba, which in turn increases imperfect vertical transmission of this Wolbachia strain (44). Lower temperatures also affect the fitness of flies with Wolbachia after reproductive dormancy (45). The effect of temperature on Wolbachia-related phenotypes may explain why different Drosophila species with different Wolbachia strains or variants
prefer different temperatures (46–48). The geographic distribution of Wolbachia in D. melanogaster populations could be explained by its relative fitness effects under varying thermal conditions (49, 50). Antiviral protection could be a temperature-dependent fitness benefit, which would be balanced against the potential cost of highly protective high-titer symbionts.

Here, we tested how temperature affects Wolbachia protection against viruses in natural Wolbachia-Drosophila associations. First, we asked how different virus doses and postinfection temperatures affect Wolbachia densities and antiviral protection. Then, we dissected the effect of developmental temperature on Wolbachia-carrying Drosophila melanogaster. We show strong interaction between this environmental variable and Wolbachia-conferred protection against viruses.

RESULTS

To test how different infection temperatures affect Wolbachia-conferred protection to Drosophila C virus (DCV), we used the Drosophila melanogaster Drosdel w1118 isogenic line (iso) carrying a natural Wolbachia variant, wMelCS_b (Wolb+), and a matching Wolbachia-free control (Wolb−) (19, 27). Flies were raised at 25°C, and 3- to 6-day-old flies were challenged with serial dilutions of DCV and subsequently placed at either 18°C or at 25°C (Fig. 1A and B). Virus-induced mortality was higher at 25°C than at 18°C at all doses except the lowest one, where there is almost no mortality associated with the infection (Cox hazard ratio [CHR], temperature effect, \( P \leq 0.001 \) for all comparisons, except with an infection dose of \( 10^2 \) 50% tissue culture infective dose [TCID\(_{50}\)/ml]). Importantly, Wolbachia protection against DCV varies with temperature (Wolbachia−temperature interaction effect, \( P < 0.001 \)), and it is stronger when the temperature at infection is 18°C (CHR = −1.84; \( P < 0.001 \)) rather than 25°C (CHR = −0.75; \( P < 0.001 \)). Overall, the temperature of infection affects the survival of the flies, and Wolbachia is more protective at a lower infection temperature (Fig. 1A and B).

To test if the interaction of Wolbachia protection with temperature is also reflected in viral loads, we measured viral titers in flies at 3 days postinfection (dpi) by quantitative reverse transcription-PCR (RT-qPCR) (Fig. 1C). We detected a strong interaction between Wolbachia presence, temperature of infection, and dose of the virus (linear model [LM], \( P < 0.001 \) for the interaction). At the lower temperature, Wolbachia conferred more resistance at higher viral doses, as viral titers stayed very low at lower doses in both Wolb+ and Wolb− flies. At the higher temperature, it conferred more resistance at the lower doses, as virus titers were very high in Wolb+ and Wolb− flies exposed to high virus doses. The mean viral load was higher at 25°C than at 18°C (LM, \( P < 0.001 \)) and was lower in the presence of Wolbachia (LM, \( P < 0.001 \)). On average, Wolbachia induced higher resistance at 18°C than at 25°C. There was a 550-fold reduction in viral load at 18°C (LM, \( P < 0.001 \)) and a 50-fold reduction at 25°C (LM, \( P < 0.001 \)), producing an approximately 11-fold difference between these two conditions (LM, \( P = 0.003 \)). These results show that the strength of Wolbachia-conferred protection against DCV, in terms of both survival and viral loads, depends on the postinfection temperature. Protection is higher at a lower temperature. Since postinfection temperature affects viral infection independently of Wolbachia, the lesser capacity of Wolbachia to protect at higher postinfection temperatures may be related to higher replication rates of the virus.

However, antiviral protection is also usually positively correlated with Wolbachia levels (27, 28, 30, 34, 51, 52), so we tested Wolbachia levels at the day of infection and after 3 days in DCV-infected, buffer-pricked (control), or unmanipulated flies, at both temperatures (Fig. 1D). Wolbachia levels were not affected by virus, buffer, or temperature of infection (LM, \( P > 0.425 \) for effect of temperature or treatment). Thus, the difference in protection is likely independent of Wolbachia levels, as these are not significantly affected within 3 days of the viral challenge.

We extended our analysis by testing how preinfection temperature, which includes that during egg laying, larval and pupal development, and the first days of adulthood,
affects *Wolbachia* protection (Fig. 2). We followed the survival of infected flies under all four possible combinations of preinfection and postinfection temperatures of 18°C and 25°C (Fig. 2A). There is a strong interaction between preinfection temperature and *Wolbachia* (CHR, *P* = 0.009). Remarkably, in flies raised at 18°C, *Wolbachia* does not protect against DCV infection (CHR, *P* = 0.207), while in flies raised at 25°C it does (CHR = −1.38.
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\[ P < 0.001 \], irrespective of the postinfection temperature. Also, in the absence of Wolbachia, the preinfection temperature has no effect on the survival after DCV infection (CHR, \( P = 0.276 \)). This strong interaction between Wolbachia-conferred protection against DCV and preinfection temperature is also reflected in DCV loads (see Fig. 2B and Fig. S1 in the supplemental material; linear mixed-effects model [LMM], \( P < 0.001 \)). On average, Wolbachia reduced viral titers 14-fold at a preinfection temperature of 18°C and 1,900-fold at 25°C (LMM, difference between reductions, \( P < 0.001 \)). In these assays, preinfection temperature also did not affect viral loads in the absence of Wolbachia (LMM, \( P = 0.534 \)). Therefore, in contrast to postinfection temperature, preinfection temperature does not directly impact virus performance and modulates only Wolbachia protection. In summary, in flies raised at a lower temperature, Wolbachia confers reduced resistance to DCV in terms of viral titers and no protection in terms of survival.

Next, we measured Wolbachia levels in flies raised at 18°C and 25°C (Fig. 2C). Flies raised at 18°C had approximately 33% less Wolbachia than the flies raised at 25°C (LM, \( P < 0.001 \)). Although this difference may contribute to the difference in protection, a larger, 50% difference in Wolbachia titers between wMel- and wMelCS-harborining flies did not abolish the protection (27). Thus, differences in Wolbachia titers do not seem to fully explain the difference in protection between flies raised at different temperatures.

FIG 2 Preinfection temperature determines Wolbachia-conferred antiviral protection. (A) Wolb\(^+\) and Wolb\(^-\) flies, 50 per Wolbachia status per condition, were infected with DCV (10\(^8\) TCID\(_{50}\)/ml) and checked for survival every day. Flies were raised and kept at 18°C or 25°C before DCV infection (preinfection temperature) and placed at either 18°C or 25°C after infection (postinfection temperature). (B) DCV titers in flies at 3 dpi measured by RT-qPCR. Flies were raised and kept at either 18°C or 25°C before infection and at 18°C after infection. A replicate of this experiment is shown in Fig. S1 in the supplemental material. (C) Wolbachia levels measured by qPCR in 3- to 6-day-old flies raised at either 18°C or 25°C. (B, C) Each dot represents a sample of 10 flies. Horizontal lines show medians of the samples.
To test the robustness of preinfection temperature effect on Wolbachia-conferred protection, we tested flies with distinct host genetic backgrounds, Aljezur1 and Oregon-R W-20 (19), each harboring its original wMel-like Wolbachia strain (Fig. 3A and B and Fig. S2A and B). In agreement with the previous results, there was a significant effect of preinfection temperature on Wolbachia-conferred antiviral protection against DCV in both lines (CHR, P < 0.001 for both). Wolbachia in Aljezur1 line conferred protection only when the preinfection temperature was 25°C (P = 0.763 for 18°C; CHR = −1.19, P < 0.001 for 25°C). In the Oregon-R W-20 line, Wolbachia still conferred some protection when the preinfection temperature was 18°C, although it was significantly less than at 25°C (CHR = −1.21, P < 0.001 for 18°C; CHR = −3.02, P < 0.001 for 25°C; CHR difference = 1.8, P < 0.001). Next, we investigated if the preinfection temperature effect was specific to DCV or if it was also present upon challenge with another RNA virus, Flock House virus (FHV) (Fig. 3C and Fig. S2C). There was an interaction between Wolbachia, preinfection temperature, and dose in this assay (CHR, P = 0.013). Wolbachia protection was significantly higher in flies raised at 25°C for all doses of virus except for the lowest one (contrast of CHR < −2.84, P < 0.001, for 10^7 to 10^8 TCID50/ml). At the lowest dose, 10^6 TCID50/ml, there was an overall low mortality of flies, and the difference in survival was not statistically significant (contrast of CHR = −1.00, but P = 0.421). The preinfection temperature had no effect on survival against FHV in the absence of Wolbachia (CHR, P = 1, in all comparisons), as observed for DCV. In conclusion, the strong influence of preinfection temperature on Wolbachia-conferred protection is a general effect observed
in different host genetic backgrounds, *Wolbachia* variants, and with the different viruses used.

Since there is a strong interaction between protection and constant preinfection temperature, we asked if protection exists when temperature is cycling daily between 18°C and 25°C (Fig. S3). *Wolbachia* protects against DCV infection at the cycling temperature (CHR = 20.67, \( P = 0.014 \)). This protection seems to be intermediate between the protection seen in flies developed at 18°C or 25°C. This result shows that under conditions of daily cycling temperatures, *Wolbachia* can protect against viruses.

In the above-described protocols, flies developed from egg to adult at a given temperature, and after collection they were aged for three more days at the same temperature before infection with viruses. To dissect at which of these stages, development and/or aging, temperature influences *Wolbachia* protection, we tested the four possible combinations of the two different temperatures for these two stages (Fig. S4). Developmental temperature strongly influenced *Wolbachia* protection, which was higher at 25°C (CHR = −1.16, \( P < 0.001 \)). Aging of the flies at 25°C also interacted with *Wolbachia* protection, but increased it only slightly compared to that with aging at 18°C (CHR = −0.431, \( P = 0.011 \)). Therefore, *Wolbachia* protection against viruses is mainly dependent on the temperature of development from egg to adult.

Since preinfection events are crucial for *Wolbachia*-conferring resistance, we asked how quickly the difference in virus titers arises between flies with and without *Wolbachia* (Fig. 4A). We observed a *Wolbachia*-induced resistance of approximately 120-fold as soon as we could detect viral RNA, at 12 h after infection, (LM, \( P < 0.001 \) at 12 h; \( P < 0.002 \) for all posterior time points in Fig. 4A and Fig. S5A). Thus, *Wolbachia* reduces viral titers very early in the course of DCV infection. We observed a similar pattern of early FHV blocking by *Wolbachia* from 1 day postinfection onwards, but with smaller viral titer differences (*Wolbachia*-infected flies had 2.3 to 5.5 times less FVH from the first day onwards; LM, \( P < 0.028 \) for all these comparisons; Fig. S5B).

**FIG 4** *Wolbachia* presence in adults is required for antiviral protection. (A) Time course analysis of DCV titers in *Wolb*+ and *Wolb*− flies raised at 25°C and kept at 18°C after DCV infection. Relative DCV levels were determined by RT-qPCR. Time zero corresponds to the time of infection. Each dot represents a sample consisting of 10 flies; lines indicate medians. (B) *Wolbachia* levels in single flies raised at 25°C, at eclosion (day 0), after 10 days of different antibiotic and control treatments at 25°C (day 10), and after additional 20 days of treatment at 18°C (day 30), measured by qPCR. A replicate of this experiment is shown in Fig. S5C. (C) Survival of *Wolb*+ and *Wolb*− flies, 50 per *Wolbachia* status per treatment, developed at 25°C, collected at eclosion, fed antibiotic-containing food for 10 days at 25°C, infected with DCV (10⁸ TCID₅₀/ml), and placed at 18°C on antibiotic-containing food. Mortality was recorded daily. A replicate of this experiment is shown in Fig. S5E.
Developmental temperature determines protection, and this protection can be detected as early as we detect viral replication in flies. Thus, we asked if changes in the fly caused by Wolbachia during development are sufficient to block viral infection in adults. To answer this, adult flies developed at 25°C were subsequently treated with antibiotics for 10 days to remove Wolbachia before viral infection (Fig. 4B and Fig. S5C). Wolbachia levels were reduced approximately 10-fold during 10 days of treatment with tetracycline and rifampicin (LMM, $P < 0.001$ for both) and stayed low until the end of the experiment at 30 days (LMM, $P < 0.001$ for both). These treatments eliminated antiviral protection from the flies ($CHR, P = 1.00$ for both; Fig. 4C and Fig. S5E). The control treatments with the antibiotics ampicillin and streptomycin did not affect Wolbachia levels (LMM, $P > 0.254$ for both; Fig. 4B and Fig. S5C) and did not affect endosymbiont-mediated protection (under both treatments, Wolbachia protection is significant [$P < 0.001$] and not different from Wolbachia protection in controls [$P > 0.301$]; CHR; Fig. 4C and Fig. S5E). As bacteria in the fly gut were efficiently cleared by all antibiotic treatments (Fig. S5D), we conclude that it is Wolbachia loss that leads to the loss of protection and not differential effect of the antibiotics on gut-associated bacteria. These data show that Wolbachia presence in adults is required for the Wolbachia-conferred antiviral protection, even though the presence or absence of protection is determined by the temperature during development, before the challenge occurs.

**DISCUSSION**

Here, we show that temperature is a strong modulator of Wolbachia-conferred antiviral protection in the natural host D. melanogaster. The temperature at which the infection progresses influences this interaction, with Wolbachia giving more resistance and increasing survival at lower temperatures. However, the most striking phenotype we report is the effect of host developmental temperature on this interaction. While development at 25°C leads to a strong antiviral protection in terms of survival and resistance to DCV, development at 18°C abrogates or strongly reduces protection. This is observed with different genotypes of D. melanogaster, different variants of Wolbachia (wMel and wMelCS), and different viruses, and is therefore likely to be a general phenomenon.

This complex interaction between temperature and Wolbachia protection against viruses may play a role in the natural environment. The outcome of Wolbachia-host interactions, in terms of antiviral protection, may differ in regions with different climates or across seasons in the same region. Wolbachia may provide more or less of a fitness benefit, depending on the conditions, and therefore the balance between the cost of harboring Wolbachia and the benefit may change with place and time. Our results lead to a prediction that Wolbachia would be more protective under warmer conditions, given the strong reduction in antiviral protection with a low developmental temperature. We also observed lesser protection at a higher temperature of infection, but this effect was weaker than the effect of temperature of development. We have suggested before that the antiviral protection is a fitness advantage that may explain the prevalence of Wolbachia in D. melanogaster populations (19). Selection for hosts carrying Wolbachia would, therefore, be stronger at higher temperatures. Consequently, geography and seasonality could impact the frequency of Wolbachia in a population. Interestingly, there is large variation in the frequency of Wolbachia in D. melanogaster natural populations and a clinal distribution of this frequency. At lower latitudes, and therefore in warmer climates, the frequency of Wolbachia is higher (45). However, the relationship between Wolbachia frequency, temperature and other environmental parameters in D. melanogaster is more complex at a smaller geographic scale, Wolbachia frequency is the highest in regions with a mean annual temperature of 22 to 26°C (53). In insects, in general, there is also a positive correlation between temperature and Wolbachia frequency, but only in temperate climates (50).

Despite ample laboratory data on Wolbachia protection against viruses in D. melanogaster, there is a lack of evidence for this in natural populations. A survey of
presence of viruses in different *D. melanogaster* populations did not detect a correlation between frequencies of *Wolbachia* and different viruses (54). However, several reasons may have led to this lack of correlation, including lack of power to detect it. Moreover, the effect of *Wolbachia* on viruses may be more quantitative rather than be defined by presence/absence (see reference 54 for more). Moreover, it is difficult to predict what the correlation would be. Should *Wolbachia* presence be driven to high frequency in populations with a high frequency of viral infection, or should the frequency of viruses decrease in population with a high frequency of *Wolbachia*? The interaction between *Wolbachia* and viruses may lead to cyclic fluctuations and requires longitudinal sampling of natural populations to understand the relationships between *Wolbachia* and viruses in nature. A direct comparison of viral titers between *Wolbachia*-carrying and *Wolbachia*-free *D. melanogaster* flies from the same natural population could be an approach to test the impact of *Wolbachia* in nature. The comparison of these groups of flies from several locations in Australia did not detect an effect of *Wolbachia* on viral infection (55). However, viral presence and infection frequency assessments were performed in F1 flies raised in the lab at 19°C. This low temperature of development may have prejudiced the expression of the antiviral protection induced by *Wolbachia*. There is a need for further studies to understand the impact of *Wolbachia* on viruses in natural populations of *D. melanogaster*. Our results show that this impact may be very variable and strongly dependent on environmental conditions and may help to define how to test this effect in natural populations.

Other reasons may explain or contribute to clinal variation in *Wolbachia* frequency in natural populations. For instance, wYak, which is closely related to wMel, is imperfectly transmitted in *Drosophila yakuba* when flies are raised at a lower temperature (20°C versus 25°C), probably due to lower *Wolbachia* titers in these flies (44). This may explain why maternal transmission of wYak in *D. yakuba* is lower in flies collected at higher altitudes than in those collected at lower altitudes. Also, *Wolbachia*-carrying *D. melanogaster* flies have lower fecundity and viability following dormancy induced by cold temperatures in laboratory experiments (45). On the other hand, cytoplasmic incompatibility decreases with higher temperature in *Drosophila simulans* males carrying wRi (56), showing that temperature has different effects on *Wolbachia*-induced phenotypes in different host-*Wolbachia* strain combinations.

These results are also relevant for the deployment of *Wolbachia*-carrying mosquitoes to block dengue, Zika, chikungunya, and other arboviruses (21, 26, 57–59). It is already known that heat stress impacts wMel in transinfected *Aedes aegypti* mosquitoes, reducing its titers (37, 39), that heatwaves can impact titers and frequency of *Wolbachia* in these mosquito populations (60), and that at low cycling temperatures *Wolbachia* titers decrease (61). On the other hand, temperatures varying between 25°C and 28°C do not seem to affect protection against viruses in this system (35). It would be important, however, to assess how lower temperatures influence *Wolbachia*-induced pathogen blocking in mosquitoes. Determining the temperature range of effectiveness of *Wolbachia*-deploying antiviral field interventions will be crucial to plan where to use them and when to combine them with other complementary approaches (e.g., insecticides).

Preinfection temperature has a drastic and enigmatic effect on *Wolbachia*-conferred antiviral protection. Lower temperatures during host development determine the level of protection in adult life. Understanding the molecular mechanism of this effect will elucidate how the environment interacts with host-microbe symbioses and may be key to understanding *Wolbachia*-conferred antiviral protection. The effect of development temperature acts solely on the interaction between virus and *Wolbachia* and does not affect viral infection by itself. The onset of the protection conferred by *Wolbachia* seems to be immediately or very soon after viral infection. We observed a difference in viral titers as soon as we detected viral replication in *Wolbachia*-free flies, 12 h after infection. Thus, *Wolbachia* likely inhibits the viral entry or first replication cycle in vivo. *Wolbachia* interference in early stages of viral infection is in agreement with cell culture
sect the mechanism of protection against viruses, indicates that temperature could be a crucial determinant of the cost-benefit ratio of carrying Wolbachia. Therefore, temperature could deeply impact the D. melanogaster-Wolbachia interaction in natural populations. Moreover, these results may have important implications for the deployment of Wolbachia-carrying mosquitoes to fight arbovirus transmission and may lead to new approaches to disrupt the mechanism of Wolbachia-conferred antiviral protection.

Temperature affects many insect-symbiont interactions and their phenotypes (1), including protective symbiosis (65–67). Therefore, this environmental factor may play a general critical role in determining the outcome of complex host-endosymbiont-pathogen interactions and shape the geographic distribution of insects and their symbionts. The phenotypic variation we report here, ranging from no protection to strong protection itself, may underlie the phenotypic differences. It would, therefore, be interesting to characterize in detail the spatial distribution of Wolbachia and virus in flies developed at the different temperatures. Comparative transcriptomic and metabolomic analysis of D. melanogaster and Wolbachia under these two conditions could elucidate not only how temperature affects protection, but also the mechanism of Wolbachia antiviral protection itself.

MATERIALS AND METHODS

Fly strains and husbandry. DrosDel w1118 isogenic D. melanogaster (iso) with wMelCS_b Wolbachia (Wolb-) and the matching control without Wolbachia (Wolb+) were described elsewhere (19, 27, 68). Aljezur 1 and W-20 D. melanogaster lines (Wolb- and Wolb+) were also described previously (19). We determined that the Wolbachia variants in both of these lines lack an ISS1 transposon insertion in gene WD1310, based on the primers described in Riegler et al. (69). This insertion is present in all wMel-like variants, but not in wMel variants (27, 69), and therefore Aljezur-1 and W-20 are both wMel-like Wolbachia variants. Stocks were maintained at a constant temperature of 25°C on a diet consisting of: 45 g molasses, 75 g sugar, 70 g commeal, 20 g yeast extract, 10 g agar, 1,100 ml water, and 25 ml of 10% Nipagin, with the addition of live yeast (Sigma).

Virus infection experiments. DCV and FHV were produced and titrated in cell culture as described previously (19, 27). Flies for experiments were produced by placing 12 females and 6 males per bottle of food without live yeast. Flies were aged for 3 more days at the developmental or otherwise indicated temperatures. These 3- to 6-day-old flies were pricked intrathoracically with virus diluted in 50 mM Tris-HCl (pH 7.5). After infection, flies were placed at the indicated temperatures. Survival was monitored daily, and vials were changed every 5 days.

Nucleic acids extractions and real-time qPCR. DNA for the quantification of Wolbachia was extracted from pools of 10 flies using the DrosDel protocol (https://drosdel.org.uk/molecular_methods.php) (68) or from single flies with a protocol described previously (70). RNA for assessment of viral titers was extracted using TRIzol (Invitrogen), and cDNA was prepared using Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega), as described previously (27). Real-time qPCRs were carried out in a 7900HT Fast real-time PCR system (Applied Biosystems) with the iQ SYBR green supermix (Bio Rad) or in a QuantStudio 7 Flex real-time PCR system (Applied Biosystems) with iTaq universal SYBR green supermix (Bio-Rad). Wolbachia was quantified using wsp as the target gene and Drosophila Rpl32 as the reference gene. DCV was quantified with primers for DCV as the target gene and Drosophila rpl32 as the reference gene. The primers used were as follows: Wolbachia wsp, 5'-CATGGTGGTGGTGTTGGTG-3' and 5'-ACCGAATACGACGCCGAGC-3; DCV, 5'-TCATGGTGGTGTTGGTG-3' and 5'-GCAGAACCAGCAGGATGTG-3'; FHV, 5'-AACCG-3'. The thermal cycling protocol for Wolbachia amplification was as follows: initial 50°C for 2 min, denaturation for 10 min at 95°C followed by 40 cycles of 30 s at 95°C, 1 min at 59°C, and 30 s at 72°C. For DCV and FHV, the same conditions were used, except for an annealing temperature of 56°C. Relative levels of Wolbachia or DCV were calculated by the Pfaffl method (71).

Antibiotic treatment of flies. Wolb+ and Wolb- flies were raised at 25°C, collected as 0- to 1-day-old adults, and placed on fly food with 100 mg/ml of tetracycline hydrochloride, rifampicin, ampicillin
sodium, or streptomycin sulfate (all from Sigma) or control food with antibiotic solvent (water or etha-
nol). At day 10 of treatment, flies were challenged with DCV and placed at 18°C, and survival was fol-
lowed for an additional 20 days. Food vials were changed every 3 days. At day 0, day 10, and day 30, flies
were collected to assay Wolbachia levels by qPCR. At day 31, guts from three Wolb+ flies per condition
were dissected to assess the effect of antibiotics on the gut-associated bacteria. Guts were homogenized
in 250 μl of sterile LB, and 30 μl was plated on mannitol agar plates. This medium sustains growth of
the main gut-associated bacteria in lab D. melanogaster, Acetobacter, and Lactobacillus species (72). CFU
were counted after 5 days of incubation at 26°C.

Statistical analysis. All of the statistical analysis was performed in R (73). The data sets and script of
the statistical analysis and the output of this analysis are available from figshare (https://doi.org/10

6804/m9.figshare.13123271.v1) (64).

Analysis of survival data was performed with Cox proportional hazard mixed-effects models. Fixed
effects, depending on the experiment, included temperature, dose of DCV, presence/absence of
Wolbachia, and antibiotic treatment, while replicate vials within the same experiment or full experimen-
tal replicates were considered random effects. Model fitting was performed using the coxme package in
R (74).

Wolbachia and DCV titers were analyzed with log-transformed qPCR data and linear models or gen-
eral linear models. Model fitting was performed using lm or the lme4 package in R (75).

The effect of interaction between factors in the models was determined by analysis of variance
(ANOVA). Post hoc analysis of marginal (least-squares) means was used to compare between the condi-
tions of interest using the lsmeans package in R (76).

Figures were produced using the ggplot2 package (77).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, TIF file, 1.3 MB.
FIG S2, TIF file, 1.5 MB.
FIG S3, TIF file, 2 MB.
FIG S4, TIF file, 1 MB.
FIG S5, TIF file, 1.7 MB.

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REFERENCES

1. Corbin C, Heyworth ER, Ferrari J, Hurst GDD. 2017. Heritable symbionts in
a world of varying temperature. Heredity (Edinb) 118:10–20. https://doi
.org/10.1038/hdy.2016.71.
2. Dutton TJ, Sinkins SP. 2004. Strain-specific quantification of Wolbachia
density in Aedes albopictus and effects of larval rearing conditions. Insect
Mol Biol 13:317–322. https://doi.org/10.1111/j.0962-1075.2004.00490.x.
3. Wiwatanaratanabutr I, Kittayapong P. 2009. Effects of crowding and tem-
perature on Wolbachia infection density among life cycle stages of Aedes
albopictus. J Invertebr Pathol 102:220–224. https://doi.org/10.1016/j.jip
2009.08.009.
4. Ross PA, Asfod JK, Richardson KM, Endersby-Harshman NM, Hoffmann
AA. 2017. Maintaining Aedes aegypti mosquitoes infected with Wolbachia.
JoVe https://doi.org/10.3791/56124.
5. Caragata EP, Rancès E, O’Neill SL, McGraw EA. 2014. Competition for
amino acids between Wolbachia and the mosquito host, Aedes aegypti.
Microb Ecol 67:205–218. https://doi.org/10.1007/s00248-013-0339-4.
6. Caragata EP, Rancès E, Hedges LM, Gofton AW, Johnson KN, O’Neill SL,
McGraw EA. 2013. Dietary cholesterol modulates pathogen blocking by
Wolbachia. PLoS Pathog 9:e1003459. https://doi.org/10.1371/journal.ppat
.1003459.
7. Triggs A, Knell RJ. 2012. Interactions between environmental variables
determine immunity in the Indian meal moth Plodia interpunctella. J
Anim Ecol 81:386–394. https://doi.org/10.1111/j.1365-2656.2011.01920.x.
8. Reynolds KT, Thomson LJ, Hoffmann AA. 2003. The effects of host age,
host nuclear background and temperature on phenotypic effects of the
virulent Wolbachia strain popcorn in Drosophila melanogaster. Genetics
164:1027–1034. https://doi.org/10.1093/genetics/164.3.1027.
9. Mouton L, Henri H, Bouletreau M, Vavre F. 2006. Effect of temperature on
Wolbachia density and impact on cytoplasmic incompatibility. Parasitol-
ology 132:49–56. https://doi.org/10.1017/S0031182005008723.
10. 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.
11. Guruprasad N, Mouton L, Puttaraju H. 2011. Effect of Wolbachia infection and temperature variations on the fecundity of the Uziﬂy Exorista sorbillans (Diptera: Tachinidae). Symbiosis 54:151–158. https://doi.org/10.1007/s10319-011-0138-y.
12. 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.00029106.
13. Kusminarsih ES. 2012. Effects of tetracycline and temperature on Drosophila melanogaster infected with Wolbachia inducing the popcorn effect. Microbiol Indones 6:130–134. https://doi.org/10.4534/mi.6.3.6.
14. Murdoc CC, Blanford S, Hughes GL, Rasgon JL, Thomas MB. 2014. Temperature alters Plasmidum blocking by Wolbachia. Sci Rep 4:3932. https://doi.org/10.1038/srep03932.
15. Dunbar HE, Wilson ACC, 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.
16. Anbutu H, Goto S, Fukatsu T. 2008. High and low temperatures differentially affect infection density and vertical transmission of male-killing Spiroplasma symbioses in Drosophila host. Appl Environ Microbiol 74:6053–6059. https://doi.org/10.1128/AEM.01503-08.
17. Brown BE. 1997. Coral bleaching: cause and consequences. Coral Reefs 16:5129–5138. https://doi.org/10.1007/s003380050529.
18. Renoz F, Pons I, Hance T. 2019. Evolutionary responses of mutualistic insect–bacterial symbioses in a world of fluctuating temperatures. Curr Opin Insect Sci 35:20–26. https://doi.org/10.1016/j.cois.2019.06.006.
19. Teixeira L, Ferreira A, 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.
20. 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.
21. Moreira LA, Iturbe-Ormaetxe I, Jeffery JA, Lu G, Pyke AT, Holden KE, Yuen KY, Johnson KN, Kay BH, McLaugh EA, van den Hurk AF, Ryan PA, O'Neill SL, Hoffmann AA. 2020. High temperature cycles result in maternal transmission and dengue infection differences between Wolbachia strains in Aedes aegypti. bioRxiv doi:https://doi.org/10.1101/2020.06.11.117596.
22. Nazni WA, Hoffmann AA, Noor Afzah A, Cheong YL, Mancini MV, Golding N. 2019. Establishment of Wolbachia strain wAlbB in Malaysian populations of Aedes aegypti for dengue control. bioRxiv doi:https://doi.org/10.1101/2019.11.04.3268.
23. O'Neill SL. 2018. The use for Wolbachia by the World Mosquito Programme to interrupt transmission of Aedes aegypti transmitted viruses, p 355–360. In: Hulimenfeld R, Vasudevan SG (eds), Dengue and Zika: control and antiviral treatments. Springer Singapore, Singapore, pp 355–360. https://doi.org/10.1007/978-981-10-8840-7_28.
24. O'Neill SL, Ryan PA, Turley AP, Wilson G, Retzki K, Iturbe-Ormaetxe I, Dong Y, Kenny N, Paton CJ, Ritchie SA, Brown-Kenyon J, Stanford D, Wittmeier N, Jewell NP, Tanamas SK, Anders KL, Simmons CP. 2018. Wolbachia in Aedes aegypti limits infection with dengue, chikungunya, and Plasmodium. Cell 174:1268–1278. https://doi.org/10.1016/j.cell.2018.11.042.
25. Vaidyanath P, Santhanam R, O'Neill SL, Simmons CP. 2018. Wolbachia strain wAlbB in Malaysia populations of Aedes aegypti for dengue control. bioRxiv doi:https://doi.org/10.1101/2019.11.04.3268.
26. https://doi.org/10.1371/journal.pbio.0050060.
27. Ye YH, Carrasco AM, Dong Y, Sygo CM, McGraw EA. 2016. The effect of temperature on Wolbachia-mediated dengue virus blocking in Aedes aegypti. Am J Trop Med Hyg 94:812–819. https://doi.org/10.4269/ajtmh.15-0801.
28. Manfeito MV, Ant TH, Hance T, Tantowijoyo W, Murdoc EA, Mamaric SM, Maro E. 2020. Wolbachia induces resistance to RNA viral infections in Aedes aegypti infected mosquitoes. PLoS Negl Trop Dis 13:e0007357. https://doi.org/10.1371/journal.pntd.0007357.
29. Ulrich JN, Beier JC, Devine GJ, Hugo LE. 2016. Heat sensitivity of wMel Wolbachia during Aedes aegypti development. PLOS Negl Trop Dis 10: e1004873. https://doi.org/10.1371/journal.pntd.0004873.
30. Ross PA, Wiwanataranatubat L, Jxford JK, White VL, Endersby Harshman NM, Hoffmann AA. 2017. Wolbachia infections in Aedes aegypti differ markedly in their response to cyclonic heat stress. PLoS Pathog 13: e1006066. https://doi.org/10.1371/journal.ppat.1006066.
31. Ross PA, Ritchie SA, Jxford 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.
32. Lambrechts L, Paaijmans KP, Fansiri T, Carrington LB, Kramer LD, Thomas MB, Scott TW. 2011. Impact of daily temperature fluctuations on dengue virus transmission by Aedes aegypti. Proc Natl Acad Sci U S A 108:7460–7465. https://doi.org/10.1073/pnas.1101377108.
33. Min KT, Benzer S. 1997. Wolbachia, normally a symbiont of Drosophila, can be virulent, causing degeneration and early death. Proc Natl Acad Sci U S A 94:10792–10796. https://doi.org/10.1073/pnas.94.20.10792.
34. Sreekumar AA, Ilinski Y, Griesemer AA, Kiseleva EV. 2013. Effect of high temperature on survival of Drosophila melanogaster infected with pathogenic strain of Wolbachia bacteria. Russ J Genet Appl Res 3:435–443. https://doi.org/10.12134/rjgen.2020.997.
35. Duarte EH, Carvalho A, Lopez-Madrigal S, Costa J, Teixeira L. 2021. Forward genetics in Wolbachia: regulation of Wolbachia proliferation by the amplification and deletion of an addictive genomic island. PLoS Genet 17:e1009612. https://doi.org/10.1371/journal.pgen.1009612.
36. Hague MJ, Mavengere H, Maturé DR, Cooper BS. 2020. Environmental and genetic contributions to imperfect wMel-like Wolbachia transmission and frequency variation. Genetics 215:1117–1132. https://doi.org/10.1534/genetics.120.303330.
37. Krieger P, Conner WR, Weeks AR, Turelli M, Hoffmann AA. 2016. Persistence of a Wolbachia infection frequencycline in Drosophila melanogaster and the possible role of reproductive dormancy. Evolution 70:979–997. https://doi.org/10.1111/evo.12933.
38. Truitt AM, Kapun M, Kaur R, Miller WJ. 2019. Wolbachia modifies thermal preference in Drosophila melanogaster. Environ Microb Biol 21:3259–3268. https://doi.org/10.1111/1462-2920.14347.
39. Hague MJ, Caldwell CN, Cooper BS. 2020. Divergent effects of Wolbachia on host temperature preference. bioRxiv doi:https://doi.org/10.1101/2020.06.11.146997.
40. Arnold PA, Levin SC, Stevanovic AL, Johnson KN. 2019. Drosophila melanogaster infected with Wolbachia strain wMelCS prefer cooler temperatures. Ecol Entomol 44:287–290. https://doi.org/10.1111/een.12696.
41. Versace E, Volte 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.

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

51. Frentiu FD, Robinson J, Young PR, McGraw EA, O'Neill SL. 2010. *Wolbachia*-mediated resistance to dengue virus infection and death at the cellular level. PLoS One 5:e13398. https://doi.org/10.1371/journal.pone.0013398.

52. Lu P, Bian G, Pan X, Xi Z. 2012. *Wolbachia* induces density-dependent inhibition to dengue virus in mosquito cells. PLoS Negl Trop Dis 6:e1754. https://doi.org/10.1371/journal.pntd.0001754.

53. Gora NV, Serga SV, Maistrenko OM, Shnir T. 2012. Impact of *Wolbachia* infection frequencies in natural populations of *Drosophila melanogaster*. Cytol Genet 54:189–198. https://doi.org/10.3103/S00954527201200044.

54. Webster CL, Waldron FM, Robertson S, Crowson D, Ferrari G, Quintana JF, Bouguil J-M, Bayne EH, Longdon B, Buck AH, Lazzaro BP, Akorli J, Haddrell PR, O'Neill SL. 2015. The discovery, distribution, and evolution of viruses associated with *Drosophila melanogaster*. PLoS Biol 13:e1002210. https://doi.org/10.1371/journal.pbio.1002210.

55. Shi M, White VL, Schlub T, Eden J-S, Hoffmann AA, Holmes EC. 2018. No detectable effect of *Wolbachia* on the prevalence and abundance of the RNA virome of *Drosophila melanogaster*. Proc R Soc B 285:20181165. https://doi.org/10.1098/rspb.2018.1165.

56. Clancy DJ, Hoffmann AA. 1998. Environmental effects on cytoplasmic incompatibility and bacterial load. Entomologia Experimentalis Et Applicata 86:13–24. https://doi.org/10.1111/j.1570-7458.1998.tb00261.x.

57. van den Hurk AF, Hall-Mendelin S, Pyke AT, Frentiu FD, McElroy K, Day A, Higgins S, O'Neill SL. 2012. Impact of *Wolbachia* on infection with chikungunya and yellow fever viruses in the mosquito vector *Aedes aegypti*. PLoS Negl Trop Dis 6:e1892. https://doi.org/10.1371/journal.pntd.0001892.

58. Allota MT, Peinado SA, Velez ID, Osorio JE, Campos GS, Bandea AC. 2016. The *wMel* strain of *Wolbachia* reduces transmission of Zika virus by *Aedes aegypti*. Sci Rep 6:28792. https://doi.org/10.1038/srep28792.

59. Dutra HLC, Rocha MN, Dias FBS, Mansur SB, Caragata EP, Moreira LA. 2016. *Wolbachia* blocks currently circulating Zika virus isolates in Brazilian *Aedes aegypti* mosquitoes. Cell Host Microbe 19:771–774. https://doi.org/10.1016/j.chom.2016.04.021.

60. Ross PA, Axford JK, Yang Q, STAUNTON KM, Ritchie SA, Richardson KM, Hoffmann AA. 2020. Heatwaves cause fluctuations in *wMel Wolbachia* densities and frequencies in *Aedes aegypti*. PLoS Negl Trop Dis 14:e0007958. https://doi.org/10.1371/journal.pntd.0007958.

61. Lau M-J, Ross PA, Endersby-Harshman NM, Hoffmann AA. 2020. Impacts of low temperatures on *Wolbachia* (Rickettsiales: Rickettsiaceae)-infected *Diptera: Culicidae*). J Med Entomol 57:1567–1574. https://doi.org/10.1093/jme/jtaa074.

62. Rainey SM, Martinez J, McFarlane M, Juneja P, Sarkies P, Lulla A, Schnettler E, Varjak M, Merits A, Miska EA, Jiggins FM, Kohl A. 2016. *Wolbachia* blocks viral genome replication early in infection without a transcriptional response by the endosymbiont or host small RNA pathways. PLoS Pathog 12:e1005336. https://doi.org/10.1371/journal.ppat.1005336.

63. Schultz MJ, Tan AL, Gray CN, Isen S, Michael SF, Frydman HM, Connor JH. 2018. *Wolbachia* w*Stri* blocks Zika virus growth at two independent stages of viral replication. mBio 9:e00738-18. https://doi.org/10.1128/mBio.00738-18.

64. Ekwudu O, Devine GJ, Aaskov JG, Frentiu FD. 2020. *Wolbachia* strain wAlbB blocks replication of flaviviruses and alphaviruses in mosquito cell culture. Parasit Vectors 13:54. https://doi.org/10.1186/s13071-020-3936-3.

65. Doremus MR, Smith AH, Kim KL, Holder AJ, Russell JA, Oliver KM. 2018. Breakdown of a defensive symbiosis, but not endogenous defences, at elevated temperatures. Mol Ecol 27:2138–2151. https://doi.org/10.1111/mec.14399.

66. Heyworth ER, Ferrari J. 2016. Heat stress affects facultative symbiont-mediated protection from a parasitoid wasp. PLoS One 11:e0167180. https://doi.org/10.1371/journal.pone.0167180.

67. Corbin C, Jones JE, Chrostek E, Fenton A, Hurd G. 2021. Thermal sensitivity of the *Spiroplasma-Drosophila lydioides* protective symbiosis: the best of climes, the worst of climes. Mol Ecol 30:1336–1344. https://doi.org/10.1111/mec.15799.

68. Ryder E, Blows F, Ashburner M, Bautista-Llacer R, Coulson D, Drummond J, Webster J, Gubb D, Gunton N, Johnson G, O’Kane CJ, Huen D, Sharma P, Asztalos Z, Baisch H, Schulze J, Kube M, Kittlaus K, Reuter G, Moray P, Szidonya J, Rasmussen-Lestander A, Ekstrom K, Dickson B, Hugentobler C, Stocker H, Hafen E, Lepesant JA, Pfugfelder G, Heisenberg M, Mechler B, Serras F, Cominomas M, Schneuwly S, Pretat R, Troote J, Russell S. 2004. The DrosDel collection: a set of P-element insertions for generating custom chromosomal aberrations in *Drosophila melanogaster*. Genetics 167:797–813. https://doi.org/10.1534/genetics.104.026658.

69. 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.

70. Chrostek E, Teixeira L. 2015. Mutualism breakdown by amplification of *Wolbachia* genes. PLoS Biol 13:e1002065. https://doi.org/10.1371/journal.pbiol.1002065.

71. 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.

72. Pais IS, Valetana RS, Sporniai M, Teixeira L. 2018. *Drosophila melanogaster* establishes a species-specific mutualistic interaction with stable gut-colonizing bacteria. PLoS Biol 16:e2005710. https://doi.org/10.1371/journal.pbiol.2005710.

73. R Core Team. 2019. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

74. Therneau T. 2012. coxme: mixed effects Cox models. R package, version 2.2-3. www.cran.R-project.org/package=coxme.

75. Bates D, Mächler M, Bolker BM, Walker SC. 2015. Fitting linear mixed-effects models using lme4. J Stat Soft 67. https://doi.org/10.18637/jss.v067.i01.

76. Lenth RV. 2016. Least-squares means: the R package lsmeans. J Stat Soft 69. https://doi.org/10.18637/jss.v069.i01.

77. Wickham H. 2016. ggplot2: elegant graphics for data analysis. Springer, New York, NY.