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Wolbachia modifies thermal preference in *Drosophila melanogaster*

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Summary

Environmental variation can have profound and direct effects on fitness, fecundity, and host–symbiont interactions. Replication rates of microbes within arthropod hosts, for example, are correlated with incubation temperature but less is known about the influence of host–symbiont dynamics on environmental preference. Hence, we conducted thermal preference (T_p) assays and tested if infection status and genetic variation in endosymbiont bacterium *Wolbachia* affected temperature choice of *Drosophila melanogaster*. We demonstrate that isogenic flies infected with *Wolbachia* preferred lower temperatures compared with uninfected *Drosophila.* Moreover, T_p varied with respect to three investigated *Wolbachia* variants (*w*Mel, *w*MelCS, and *w*MelPop). While uninfected individuals preferred 24.4°C, we found significant shifts of −1.2°C in *w*Mel- and −4°C in flies infected either with *w*MelCS or *w*MelPop. We, therefore, postulate that *Wolbachia*-associated T_p variation within a host species might represent a behavioural accommodation to host–symbiont interactions and trigger behavioural self-medication and bacterial titre regulation by the host.

Introduction

Environmental variations through intrinsic (e.g., physiology, reproduction, metabolism) and extrinsic (e.g., food sources, predation risk, immunity) factors impose a strong impact on the fitness of all organisms (e.g., Levins, 1968; Endler, 1977, 1986; Fox *et al*., 2001). Temperature is one of the most important environmental abiotic factors that affect the physiology and life history traits in many organisms (Huey and Berrigan, 2001; Hoffmann, 2010; Bozinovic *et al*., 2011; Amarasekare and Savage, 2012). Ectotherms, such as terrestrial insects, depend on ambient conditions to maintain their body temperature within a thermoregulatory range (Angilletta *et al*., 2004). For example, thermal preference (T_p) in *Drosophila melanogaster*, a dipteran model species of world-wide distribution, varies with geography and elevation, and is thus potentially shaped by selection (Martin and Huey, 2008; Dillon *et al*., 2009; Garrity *et al*., 2010; Hoffmann and Sgrò, 2011; Huey *et al*., 2012; Rajpurohit and Schmidt, 2016). In addition, variation in temperature can have fundamental effects on ecological interactions among organisms and their symbiotic microbes. Titres of endosymbiotic *Wolbachia* bacteria are highly temperature-dependent in various arthropod hosts. For example, some *Wolbachia* strains have increased replication rates at warmer temperatures (Clancy and Hoffmann, 1998; Hurst *et al*., 2000; Mouton *et al*., 2006; Correa and Ballard, 2012; Strunov *et al*., 2013a), while others are highly sensitive to heat stress (Van Opijnen and Breeuwer, 1999; Wiwatanaratanabutr and Kittayapong, 2009).

Endosymbionts of the genus *Wolbachia* are widespread and found in more than 50% of all investigated terrestrial and some aquatic insects (Zug and Hammerstein, 2012; Weinert *et al*., 2015; Sazama *et al*., 2017). *Wolbachia* have garnered extensive interest due to reproductive manipulations they can inflict on their hosts, i.e., inducing parthenogenesis, male killing, feminization, and cytoplasmic incompatibility (CI). By acting as reproductive parasites these bacteria boost their own transmission (reviewed by Werren *et al*., 2008). However, *Wolbachia* can also behave as facultative or obligate mutualists (reviewed by Zug and Hammerstein, 2015) by enhancing host fecundity and fitness (Dedeine *et al*., 2001; Hosokawa *et al*., 2010; Miller *et al*., 2010) and by providing protection against RNA viruses (Hedges *et al*., 2008; Teixeira *et al*., 2008; Moreira *et al*., 2009; Osborne *et al*., 2009). Several closely related genetic variants of *Wolbachia* have been isolated from natural and laboratory populations of *D. melanogaster. w*Mel,

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*w*MelCS, and *w*MelPop, which represent three of the most well-studied *Wolbachia* variants in *D. melanogaster* (Riegler *et al*., 2005), cause very weak, if any, CI in their native host (Hoffmann, 1988; Reynolds *et al*., 2003; Veneti *et al*., 2003; Fry *et al*., 2004; Yamada *et al*., 2007), but provide virus protection to varying degrees (Chrostek *et al*., 2013; Martinez *et al*., 2014). Both *w*Mel and *w*MelCS infect natural populations of *D. melanogaster.* Historically, *w*MelCS existed globally at higher prevalence, but in the recent past *w*Mel has almost completely replaced the more ancestral *w*MelCS strain in worldwide populations (Riegler *et al*., 2005; Nunes *et al*., 2008; Richardson *et al*., 2012; Ilinsky, 2013; Early and Clark, 2013). In contrast, *w*MelPop was isolated from a laboratory stock of *D. melanogaster* during a survey of genetic mutations and represents a pathogenic variant of *w*MelCS (Min and Benzer, 1997; Richardson *et al*., 2012; Chrostek *et al*., 2013). Depending on rearing temperature, *w*MelPop infections can lead to a strong reduction of host lifespan with respect to uninfected controls (Min and Benzer, 1997; McGraw *et al*., 2002; Reynolds *et al*., 2003; Chrostek *et al*., 2013). This detrimental effect is caused by over-proliferation in host tissues, such as the brain, retina, and muscles (Min and Benzer, 1997; Strunov *et al*., 2013b). Importantly, not only *w*MelPop but also its natural predecessor *w*MelCS have significantly higher cellular densities and growth rates than *w*Mel when assayed in the same fly genetic background at 25°C (Table 1**;** Chrostek *et al*., 2013). While high *Wolbachia* densities result in augmented antiviral protection, they also have negative effects by reducing their host's lifespan. Accordingly, it has been proposed that the higher titre – and hence more costly – *w*MelCS variant was replaced by the low-titre *w*Mel variant in natural *D. melanogaster* populations (Chrostek *et al*., 2013). Thereby, flies infected with the more recent *w*Mel variant have higher fitness due to lower *Wolbachia* titres compared with flies infected with *w*MelCS. Alternatively, the highly protective *w*MelCS variant may have been replaced by *w*Mel independent of the symbiont's capacity for virus resistance but because of better adaptation to viruses at the host level (Martins *et al*., 2014). In line with this hypothesis, a recent study failed to find correlations between RNA virus prevalence and *Wolbachia* frequency in natural populations of *D. melanogaster* (Webster *et al*., 2015). However, the main causalities explaining the well-documented global almost complete replacement of *w*MelCS by *w*Mel in worldwide populations of *D. melanogaster* remains elusive.

Table 1. Comparison of strain type titre levels, growth rates and effects on host's lifespan at 25°C.

Strain type	Relative amount of *Wolbachia*	Effects on host's lifespan
*w*Mel	Lowest titre level and growth rate	No reduction
*w*MelCS	Approximately double the titre level compared with *w*Mel and higher growth rate	Some reduction
*w*MelPop	Titre level 20 times higher compared with *w*MelCS	Reduction by approximately half

Note: Information on titre levels, growth rate, host's lifespan effects for *w*Mel and *w*MelCS from Chrostek and colleagues (2013), information on *w*MelPop's effects on host's lifespan from Reynolds and colleagues (2003).

Host–symbiont conflicts may arise from disparities between physiological requirements of *Wolbachia* and those of their hosts. For example, some insects induce behavioural fever (Louis *et al*., 1986) or behavioural chill (Fedorka *et al*., 2016) as an immune strategy to fight bacterial pathogen infections. Conversely, some bacterial symbionts are known to alter their host's thermal tolerance range in an adaptive manner (Russell and Moran, 2006; Dunbar *et al*., 2007; reviewed by Wernegreen, 2012). We, therefore, speculate that additional ecological and behavioural factors, such as host temperature preference, may play a pivotal role in determining *Wolbachia* prevalence and the dynamics of their strain replacement in natural *D. melanogaster* populations.

To test our hypothesis, we conducted laboratory-based temperature preference assays using isogenic *D. melanogaster* w^{1118} strains that are either uninfected (*w*-) or infected with one of the three common *Wolbachia* strains *w*Mel, *w*MelCS_b, and *w*MelPop (Teixeira *et al*., 2008; Chrostek *et al*., 2013) and determined if *Wolbachia* affects the temperature preference of its native host *D. melanogaster.* To this end, we built a custom thermal gradient apparatus and determined the temperature preference of replicated fly populations with varying *Wolbachia* infection statuses along the thermal gradient ranging from 17°C to 32°C. Our experiments demonstrate that the temperature preference of *D. melanogaster* is neither sex- nor age-dependent but is highly dependent on the *Wolbachia* infection status and on the symbiont genotype. Our results provide compelling evidence that *Wolbachia* infections can affect host thermal preference behaviour, at least under strict laboratory conditions in *D. melanogaster* strains.

Results

To determine whether T_p of adult *D. melanogaster* varies with *Wolbachia* infection status and *Wolbachia* genotype, we conducted lab-based experiments using a custom-built temperature gradient apparatus for assaying flies of the isogenic lab-strain w^{1118} that were either uninfected (*w*-) or infected with one of the *Wolbachia* strains *w*Mel, *w*MelCS, or *w*MelPop (Supporting Information Figs. S1–S4). We first investigated whether age (3–4, 5–7, or 10–14 days post-eclosion) and *Wolbachia* infections or sex (males or females) and *Wolbachia* infections had an

influence on T_p by means of two-way mixed-effect Poisson regressions. We neither found significant effects of age or sex nor significant interactions of either factor with *Wolbachia* infections (see Fig. 1A and B and Table 2A, B; Supporting Information Fig. S5; Poisson regression: $p > 0.05$ for factors age and sex and both interaction terms respectively). In contrast, both two-way regressions revealed highly significant effects of *Wolbachia* infections on T_p (Poisson regression $p < 0.001$ for factor *Wolbachia* in both analyses). Since both aforementioned analyses were carried out on different subsets of the data which did not include all four infection types (*w*-, *w*Mel, *w*MelCS, and *w*MelPop), we further investigated all data jointly irrespective of sex and host age and evaluated the effect of symbiont genetic variation on T_p by means of post-hoc pairwise comparisons based on Tukey's honestly significant differences (HSD). We found that temperature preference of *D. melanogaster* strongly depended on (1) the infection status of the flies and (2) on the *Wolbachia* strain used for infections: Uninfected flies (*w*-) exhibited the highest mean T_p at 24.4°C (Median: 25°C; Mode: 26°C), while *w*Mel-infected flies preferred average temperatures at 23.2°C (Median: 24°C; Mode: 24°C), which is 1.2°C lower than uninfected (*w*-) flies. In contrast, flies infected with *w*MelCS or *w*MelPop showed highly similar thermal preferences at 20.6°C and 20.5°C (Median: 19°C and Mode: 18°C for both) respectively, which were both approximately 4°C lower than to *w*- (see Fig. 1C**,** Tables 2C, and 3).

Discussion

In this study, we, for the first time, investigated the relationship between temperature preference of *D. melanogaster* and *Wolbachia* infection under laboratory conditions. Using a custom-built thermal gradient apparatus, we conducted temperature preference assays and showed that the T_p of *D. melanogaster* is shifted to lower temperatures when flies are infected with *Wolbachia.* Uninfected *D. melanogaster* flies preferred an average temperature of 24.4°C, whereas *w*Mel-infected flies preferred 23.2°C and both *w*MelCS- and *w*MelPop-infected flies preferred 20.6°C and 20.5°C respectively.

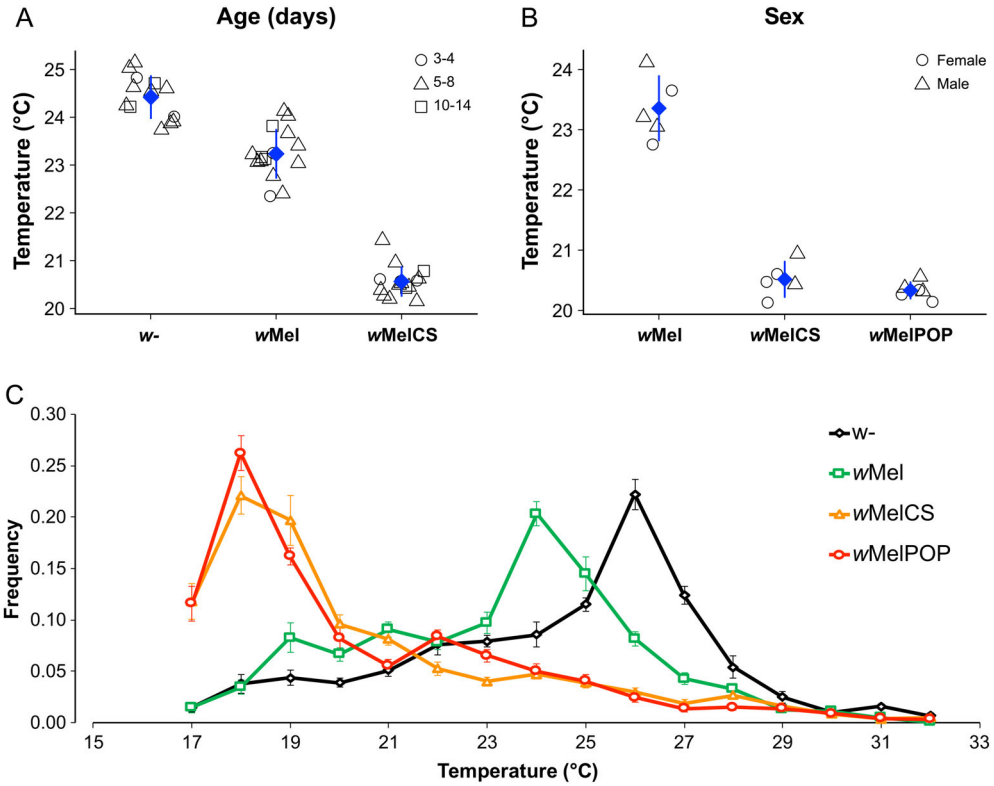

Fig. 1. Thermal preference of *Drosophila* with and without *Wolbachia* infections. Panels A and B show average T_p (blue diamonds) with respect to age (3–4, 5–7, or 10–14 days post eclosion, $n = 4370$ excluding flies infected with *w*MelPop) and sex (male or female; $n = 1718$, excluding uninfected flies) respectively. Each symbol represents the average T_p for a replicate at a given factor level of either age (circle: 3–4 days, triangle: 5–8 days and square: 10–14 days) or sex (circle: females, triangle: males). Panel C shows line plots with relative proportions of flies observed at a given temperature. Each line represents the average proportion of flies which were either uninfected (*w*-; black diamonds) or infected with *w*Mel (green circles), *w*MelCS (orange triangles), or *w*MelPop (red squares). The error bars represent standard errors for average frequencies at a given temperature across all replicated experiments carried out for each infection type. We found that infected flies exhibit significantly lower thermal preference compared with uninfected flies. [Color figure can be viewed at wileyonlinelibrary.com]

Table 2. Table showing the results of three analyses based on generalized linear mixed models with a Poisson error structure to account for the statistical properties of count data.

Analysis	Model	Factor	*N*	df	χ^2	*p*-value
A	***wol + age + wol × age***	***wol***	**4370**	**6**	**119.71**	**1.87E-23**
A	*wol + age + wol × age*	*age*	4370	6	2.10	0.91
A	*wol + age + wol × age*	*wol × age*	4370	4	1.31	0.86
B	***wol + sex + wol × sex***	***wol***	**1718**	**6**	**61.19**	**2.58E-11**
B	*wol + sex + wol × sex*	*sex*	1718	4	2.12	0.71
B	*wol + sex + wol × sex*	*wol × sex*	1718	3	1.90	0.59
C	***wol***	***wol***	**5717**	**3**	**168.69**	**2.44E-36**

The columns show ID's for the different analyses (A-C), the models, the individual factors and interactions tested, the samples size, the degrees of freedom for the χ^2 test of the analysis of deviance, the χ^2 value and the corresponding *p*-value. Note that analyses with significant effects after Bonferroni correction (adjusted $\alpha = 0.017$) are highlighted in bold.

T_p can vary significantly between populations of the same species (Matute *et al*., 2009; Rajpurohit and Schmidt, 2016) and can have profound effects on immune function, fitness, and fecundity (Huey and Berrigan, 2001; Martin and Huey, 2008; Hoffmann, 2010). Recent population analyses of *Wolbachia* and mitochondria from *D. melanogaster* have provided evidence that over the past few thousand years, the *w*MelCS variant is being globally replaced by the *w*Mel-variant (Riegler *et al*., 2005; Nunes *et al*., 2008; Richardson *et al*., 2012; Early and Clark, 2013). Rare cases of the *w*MelCS infection type were recently detected in the wild (Nunes *et al*., 2008; Ilinsky, 2013), thus replacement by *w*Mel is still incomplete. Although the reason for the worldwide turnover remains elusive, it has been hypothesized that *w*Mel, which persists in hosts at significantly lower densities than *w*MelCS at 25°C (Chrostek *et al*., 2013), has better adapted to *D. melanogaster.* Accordingly, *w*Mel infections are less costly to the host compared with the more ancestral *w*MelCS variant (Chrostek *et al*., 2013; reviewed by Miller, 2013).

Insects can actively reduce or avoid costs of potentially fitness-reducing symbionts or parasites by behavioural adjustments such as changing egg deposition (Kacsoh *et al*., 2013) or mating behaviour (reviewed by Wedell, 2013). We find compelling evidence for *Wolbachia*-induced behavioural changes in host T_p, which may provide an alternative explanation for the recent global replacement of *w*MelCS by *w*Mel independent of density costs or anti-viral effects: we propose that *w*Mel is less costly for the host than *w*MelCS-infections because flies harbouring *w*Mel exhibit thermal preferences that are closer to uninfected flies under natural conditions compared with flies infected with *w*MelCS. *Drosophila* development is strictly temperature dependent (~ 14 days of egg-to-adult development at 20°C and 9 days at 24°C; Ashburner, 1989). Due to cooler thermal preference, infections with *w*MelCS may, thus, result in slower development and lead to longer generation times compared with *w*Mel-infected flies. Variance in generation times as a function of *Wolbachia* infections may, thus, have a substantial impact on fitness if *w*Mel-infected flies produce more generations per year resulting in higher net fecundity compared with flies infected with *w*MelCS.

Table 3. Table showing z-values from post-hoc pairwise comparisons with Tukey's HSD for the factor Wolbachia (Analysis C; see section on 'Experimental Procedures') with four levels (non-infected, wMel, wMelCS and wMelPop).

	w-	*w*Mel	*w*MelCS	*w*MelPop
***w*-**	–			
***w*Mel**	**−6.76*****	–		
***w*MelCS**	**−21.93*****	**−15.49*****	–	
***w*MelPop**	**−21.5*****	**−15.35*****	−0.6	–

Bold type indicates significance after Bonferroni correction (adjusted $\alpha' = 0.017$). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Small fluctuations in temperature can cause considerable modifications to host–symbiont interactions (Blanford and Thomas, 1999). Pathogenicity of *w*MelPop is attributed to its active proliferation in host tissues at temperatures ≥ 19°C. The increase of *w*MelPop density confers strong anti-viral protection but leads to a significant reduction in host lifespan at 25°C (Chrostek *et al*., 2013). However, at temperatures < 19°C, pathogenicity of *w*MelPop is eliminated (Reynolds *et al*., 2003). Similarly, but less dramatically *w*MelCS, the progenitor of *w*MelPop, is also costly by reducing host lifespan due to high symbiont densities at 25°C (Chrostek *et al*., 2013). We, therefore, speculate that the adjustment of lower temperature preference in *D. melanogaster* as a response to the *w*MelCS and *w*MelPop infections represents a physiological self-medicating behaviour or behavioural chill (Fedorka *et al*., 2016) to attenuate the fitness costs associated with deleterious effects of *Wolbachia* overproliferation and high cell densities (Chrostek *et al*., 2013; Strunov *et al*., 2013a, b).

Wolbachia's ability to provide anti-viral protection to their hosts has emerged as the most promising approach to combatting insect-vector borne pathogens that pose serious health risks to humans, such as dengue fever

and Zika (Moreira *et al*., 2009; Iturbe-Ormaetxe *et al*., 2011; Dutra *et al*., 2016). However, because the strength of anti-viral protection is associated with higher *Wolbachia* densities (Chrostek *et al*., 2013; Martinez *et al*., 2014) and bacterial titres are a temperature sensitive trait (Hoffmann *et al*., 1990; Reynolds *et al*., 2003; Mouton *et al*., 2006; 2007; Bordenstein and Bordenstein, 2011; Correa and Ballard, 2012; Chrostek *et al*., 2013; Strunov *et al*., 2013a; Murdock *et al*., 2014; Versace *et al*., 2014), it is feasible that under certain thermal conditions such as lower environmental temperatures, *Wolbachia*-induced virus protection could be attenuated or absent (Chrostek, 2014). Furthermore, our findings, as demonstrated in a highly inbred lab strain of *D. melanogaster*, need to be tested first in different host backgrounds, which are naturally or artificially infected with the endosymbiont.

In conclusion, we present experimental support for a potential ecological conflict between host and symbiont that may have profound effects on host physiology. Our results provide a novel conceptual platform from which to further investigate host temperature preference or behavioural chill, in other *Wolbachia*-infected insect hosts. Future studies should examine if host temperature preference has a direct impact on *Wolbachia* density regulation. Additionally, it is important to determine any effects that host T_p has on the strength of anti-viral protection that *Wolbachia* provide to some hosts.

Experimental procedures

Fly lines

For all assays, we used *D. melanogaster* without *Wolbachia* (*w*-) as well as flies infected with one of three genetic variants of the *Wolbachia* *w*Mel-strain; *w*-, *w*Mel, *w*MelCS_b, and *w*MelPop all set in the DrosDel w^{1118} isogenic background, which were kindly provided by Luis Teixeira and previously described by Teixeira and colleagues (2008) and Chrostek and colleagues (2013).

We used biological replicates of approximately 30 flies per vial, independently rearing each vial of flies at 25°C, in a 12:12 light–dark cycle with constant 45% humidity. Flies were raised on *Drosophila* Formula 4-24® Instant Medium (Carolina®, NC) that was supplemented with fresh yeast. Approximately equal numbers of male and female flies were used in each assay except for assays that explicitly tested sex-class T_p differences (see Supporting Information Table S1 and Supporting Information File 1). In addition to testing for sex-class T_p differences, we performed assays to test for age-specific T_p differences, thus all fly lines were segregated into three age-classes – 3–4 days, 5–7 days, and 10–14 days post-eclosion. Due to fitness costs to the host associated with infection by *w*MelPop at 25°C, possibly due to the onset of the life reducing phenotype (Min and Benzer, 1997) or increase in copy numbers of the Octomom repeat (Chrostek and Teixeira, 2015), our *w*MelPop-infected fly line did not produce enough flies to conduct all three age-class assays. Therefore, we excluded *w*MelPop from the statistical analyses of age-specific effects (see Supporting Information Table S1 and the description of statistical analyses).

Genotyping of Wolbachia *strains*

Genome sections that contain hypervariable loci or hypervariable regions covering tandem repeats were used as genetic markers to differentiate *Wolbachia* strains and strain variants (O'Neill *et al*., 1992; Werren *et al*., 1995; Zhou *et al*., 1998; Riegler *et al*., 2012). To confirm *Wolbachia*-infection status, we performed diagnostic PCR amplification using primers for a gene that encodes the *Wolbachia* surface protein, *wsp* (Jeyaprakash and Hoy, 2000) and for an intergenic region with 141 bp tandem repeats, VNTR-141 loci (Riegler *et al*., 2005). The PCR reactions for *wsp* amplification were carried out in a total volume of 10 μl containing 2 μl Promega 5× Green GoTaq buffer, 4 mM Promega $MgCl_2$, 0.8 μM of forward and reverse primers, 35 μM of each dNTP, 0.04 U Promega GoTaq DNA Polymerase and 1 μl of genomic DNA template. Diagnostic VNTR-141 PCR reactions were each a total of 10 μl comprised of the following: 2 μl Promega 5x Green GoTaq buffer, 1.5 mM Promega $MgCl_2$, 0.3 μM of forward and reverse primers, 35 μM of each dNTP, 0.04 U Promega GoTaq DNA Polymerase and 1 μl of genomic DNA template. PCR products were visualized on a 1% agarose gel. Presence/absence of the *wsp* signal and the size of the diagnostic VNTR-141 locus confirmed their respective infection type (Riegler *et al*., 2012). The proper infection status of the *w*MelPop isoline was verified by assaying flies for early mortality at 29°C.

Thermal gradient apparatus

Temperature preference assays were performed using a custom made thermal gradient apparatus that allowed the flies to move in a three-dimensional space (adapted from Rajpurohit and Schmidt, 2016; Supporting Information Fig. S2). An aluminium rod (length 74.93 cm, diameter 3.02 cm; Part #R31-316 Metals Depot, Winchester, KY) was encased within a 58.76 cm long and 6.35 cm inside diameter polycarbonate tube, creating an enclosed chamber allowing for three-dimensional movement. Constant voltage was applied to Peltier devices on each end of the aluminium rod to create a temperature gradient inside the thermal preference chamber. Temperatures along the gradient were measured at seven points that

were 8.39 cm apart using K-type thermocouples and two four-channel thermocouple recorders. We recorded temperatures on the aluminium rod and inside polycarbonate tube surfaces (bottom, top, and mid-point between the top and bottom surfaces; Supporting Information Fig. S3). The average temperatures from each thermocouple point on all surfaces from 57 different assays are depicted in Supporting Information Fig. S1. Mean temperatures increased linearly and ranged from 12°C at the coldest point to 40°C at the hottest point of the aluminium rod, 58.76 cm distance (Supporting Information Fig. S4). Along the aluminium rod, for every 4.2 cm from cold to hot, the temperature increased by 2°C. Temperatures along each of the measured polycarbonate tube surfaces (bottom, mid-point, and top) increased 1°C every 4.2 cm from cold to hot. The gradient reached thermal stability after approximately 20 min and remained stable for at least 3 h. Assays were conducted once the device had attained thermal stability.

Thermal preference assays

All assays were conducted in a room with a constant temperature of 24°C and constant 40% humidity. During several trial runs, we established that 75–100 flies for each assay resulted in distributions along the thermal gradient that avoided over-crowding in preferred temperature ranges, eliminating potential counting errors during analysis. Flies were introduced by aspiration into the thermal gradient chamber through a small hole located halfway along the top of the polycarbonate tube, where the temperature consistently averaged 25°C. Flies used for thermal preference assays were never anesthetized because of the strong effects from CO_2 treatment on *Drosophila* behaviour (Barron, 2000). Each assay was conducted for 30 min. Between assays, the temperature gradient chamber was taken apart and thoroughly cleaned to avoid contamination from any pheromone particles. All aluminium parts were cleaned using 95% ethanol. Because ethanol and polycarbonate are chemically incompatible, the polycarbonate tube and end caps were cleaned using hot water and soap, followed by a 4-min rinse with hot water to ensure that surfaces were free of soap residue.

Data collection

Using three GoPro HERO3+ cameras, we collected data for each assay in the form of digital images. To capture images of the entire thermal gradient and the flies within it, we mounted the cameras above, lateral to and below the apparatus, capturing images every 30 s for the duration of each treatment. Following Goda and colleagues (2014), treatment duration was 30 min to avoid any behavioural aberration from the desiccation and/or starvation of the flies. Images were analyzed using Adobe Photoshop CS6. All 60 images from each assay were reviewed, from which we determined that (a) the flies were highly active, retaining the ability to relocate as necessary, for the entire assay, and (b) after being introduced to the thermal gradient, actively flew around for up to 15 min before they settled on either the aluminium rod or polycarbonate tube surfaces. Therefore, we selected images for analysis of fly distribution at the 20-min time point as representative of the 30-min experiment. For each assay, we manually counted flies and marked the location of flies on a custom grid that delineated gradient surfaces and surface temperatures.

Statistical analyses

We calculated generalized linear mixed models (GLMM) with a Poisson error structure using the *R* (R Development Core Team, 2009) package *lme4* (Bates *et al*., 2015) to account for the statistical properties of count data from flies observed at different temperatures. To test for significance of a given predictor variable, we compared the full model including all factors to a reduced model excluding the given factor by analysis of deviance with χ^2 tests using the *R* function *anova* (see Supporting Information File 1 for full *R* code).

At first, we excluded flies infected with *w*MelPop, since we failed to obtain sufficient flies to test for age-specific T_p at all three age-classes (3–4 days, 5–7 days, and 10–14 days post-eclosion; Supporting Information Table S1) and tested for age- and *Wolbachia*-specific differences in thermal preference with a two-way GLMM of the form: $T_i = wol + age + wol \times age + Rep + \varepsilon_i$. Here, *T* is the continuous response variable 'Temperature', *age* is a nominal fixed factor with three levels each (*age*: 3–4 days, 5–7 days, and 10–14 days post-eclosion), *wol* is a nominal fixed factor '*Wolbachia'* with three levels (un-infected, *w*Mel, and *w*MelCS), $wol \times age$ is the interaction term, *Rep* is a nominal random factor 'Replicate' for replicate trials and ε_i is the error (Table 2A**,** Fig. 1A). In a complementary analysis, we removed all flies of the age class 3–4 days and repeated the abovementioned analysis including all *Wolbachia* strains on two age classes (5–7 days and 10–14 days post eclosion) only. This latter analysis yielded qualitatively similar results to the former analysis including all age classes without *w*MelPop (Supporting Information Table S2).

Next, we censored flies with undetermined sex status and excluded uninfected flies (*w*-), since we failed to obtain sufficient replication to test for male-specific T_p for uninfected flies (Supporting Information Table S1). We then tested for sex- and *Wolbachia*-specific differences in thermal preference with a two-way GLMM of the form:

$T_i = wol + sex + wol \times sex + Rep + \varepsilon_l$ Here, *T* is the continuous response variable 'Temperature', *sex* is a nominal fixed factor with two levels (male and female), *wol* is a nominal fixed factor '*Wolbachia*' with three levels (*w*Mel, *w*MelCS, and *w*MelPop), *wol* × *age* is the interaction term, *Rep* is a nominal random factor 'Replicate' for replicate trials and ε_l is the error (Table 2B**;** Fig. 1B).

Finally, we included all flies, irrespective of age and sex status and tested for the effect of infection status and *Wolbachia* strain variation on thermal preference with a GLMM of the form: $T_i = wol + Rep + \varepsilon_i$, where *T* is the continuous response variable 'Temperature', *wol* is a nominal fixed factor '*Wolbachia'* with four levels (un-infected, *w*Mel, *w*MelCS, and *w*MelPop), *Rep* is the nominal random factor 'Replicate' and ε_i is the error (Table 2C**;** Fig. 1C). Here, we further tested for significant pair-wise comparisons among the level of the factor '*Wolbachia'* with Tukey's honestly significant difference (HSD) post-hoc tests using the *R* package *multcomp* (Table 3). We conservatively applied Bonferroni corrections to the α threshold ($\alpha' = 0.05/3 = 0.017$) to account for multiple testing.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1 Supporting Information

Fig. S1. *Thermal* gradient apparatus gradient depicting different temperature zones and fly dispersion (*w*MelPop).

Fig. S2. Schematic of the thermal gradient apparatus used for thermal gradient assays as adapted from Rajpurohit and Schmidt (). The polycarbonate tube and length of aluminium gradient within the tube were 58.76 cm and temperature was recorded with K-type thermocouples.

Fig. S3. Average + 0.5°C (SD) temperatures from 18 runs that were recorded at each surface measured using k-type thermocouples. There was a linear increase in temperature from cold to hot as measured at each of seven evenly spaced (8.39 cm).

Fig. S4. Plots showing linearity of temperature change for the different surfaces (a. aluminium rod, b. top, c. bottom, and mid-point of the polycarbonate tube) as measured with K-type thermocouples at regular intervals along the length of apparatus from the hottest end (H3) to the coldest (C3).

Fig. S5. Line plots showing the portion of flies observed at a given temperature for males and females in the left panel and age classes (*young*: 3–4 days or *old*: 10–14 days post eclosion) in the right panel. Each of the four subfigures shows the average proportion of flies with respect to different infection status (uninfected, *w*Mel, *w*MelCS and *w*MelPop). For *w*MelPop infected flies only two age groups were tested. Error bars represent standard errors for average frequencies at a given temperature across all replicated experiments carried out for a given infection type and levels of the factors sex or age.

Table S1. Counts of flies and number of replicates (in parentheses) per sex and age class.

Table S2. Results of two-way GLMM with independent factors age, Wolbachia and the interaction between them (see Table for more detail).