Wolbachia Modifies Thermal Reference in Drosophila Melanogaster

Amy Michelle Truitt  
*Portland State University*

Martin Kapun  
*Université de Fribourg*

Rupinder Kaur  
*Medical University of Vienna*

Wolfgang J. Miller  
*Medical University of Vienna*

Follow this and additional works at: https://pdxscholar.library.pdx.edu/esm_fac

Part of the Environmental Sciences Commons

Let us know how access to this document benefits you.

Citation Details

Truitt, Amy Michelle; Kapun, Martin; Kaur, Rupinder; and Miller, Wolfgang J., "Wolbachia Modifies Thermal Reference in Drosophila Melanogaster" (2018). *Environmental Science and Management Faculty Publications and Presentations*. 278.  
https://pdxscholar.library.pdx.edu/esm_fac/278

This Pre-Print is brought to you for free and open access. It has been accepted for inclusion in Environmental Science and Management Faculty Publications and Presentations by an authorized administrator of PDXScholar. Please contact us if we can make this document more accessible: pdxscholar@pdx.edu.
Wolbachia modifies thermal preference in Drosophila melanogaster

Amy M. Truitt¹, Martin Kapun², Rupinder Kaur³, Wolfgang J. Miller³*

1. Department of Environmental Science & Management, Portland State University, Portland Oregon, USA, amtruitt@pdx.edu
2. Department of Biology, Université de Fribourg, Switzerland, martin.kapun@unifr.ch
3. Department of Cell & Developmental Biology, Medical University of Vienna, Vienna, Austria, rupinder.kaur@meduniwien.ac.at, wolfgang.miller@meduniwien.ac.at

*Corresponding author: Wolfgang J. Miller, email: wolfgang.miller@meduniwien.ac.at, Tel: +43 1 40160 37750, Fax: +43 1 40160 37790, address: Department of Cell & Developmental Biology, Center of Anatomy and Cell Biology, Medical University of Vienna, Schwarzspanierstrasse 17, A-1090 Vienna, Austria.

Running Title: Wolbachia and host thermal preferences
ABSTRACT

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 to uninfected Drosophila. Moreover, $T_p$ varied with respect to three investigated Wolbachia variants (wMel, wMelCS and wMelPop). While uninfected individuals preferred 24.4°C, we found significant shifts of -1.2°C in wMel- and -4°C in flies infected either with wMelCS or wMelPop. We, therefore, postulate that Wolbachia-associated $T_p$ variation within a host species might represent a behavioral accommodation to host-symbiont interactions and trigger behavioral self-medication and bacterial titer 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 & Sgrò 2011; Huey et al. 2012; Rajpuhrot and Schmidt 2016). In addition, variation in temperature can have fundamental effects on ecological interactions among organisms and their symbiotic microbes. Titers 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...
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. wMel, wMelCS, and wMelPop, 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 wMel and wMelCS infect natural populations of D. melanogaster. Historically, wMelCS existed globally at higher prevalence, but in the recent past wMel has almost completely replaced the more ancestral wMelCS strain in world-wide populations (Riegler et al. 2005; Nunes et al. 2008; Richardson et al. 2012; Ilinsky 2013; Early and Clark 2014). In contrast, wMelPop was isolated from a laboratory stock of D. melanogaster during a survey of genetic mutations and represents a pathogenic variant of wMelCS (Min and Benzer 1997; Richardson et al. 2012; Chrostek et al. 2013). Depending on rearing temperature, wMelPop 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 wMelPop but also its natural predecessor wMelCS have significantly higher cellular densities and growth rates than wMel 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 titer - and hence more costly - wMelCS variant was replaced by the low-titer wMel variant in natural D. melanogaster populations (Chrostek et al. 2013). Thereby, flies infected with the more recent wMel variant have higher fitness due to
lower Wolbachia titers compared to flies infected with wMelCS. Alternatively, the highly protective wMelCS variant may have been replaced by wMel 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 wMelCS by wMel in worldwide populations of D. melanogaster remains elusive.

Host-symbiont conflicts may arise from disparities between physiological requirements of Wolbachia and those of their hosts. For example, some insects induce behavioral fever (Louis et al. 1986) or behavioral 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 behavioral 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 wMel, wMelCS_b, and wMelPop (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
Our results provide compelling evidence that Wolbachia infections can affect host thermal preference behavior, 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 Fig. S1-4). 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+B and Table 2A+B and 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
flies. In contrast, flies infected with wMelCS or wMelPop 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, Table 2C and Table 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 Tp 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 wMel-infected flies preferred 23.2°C, and both wMelCS- and wMelPop-infected flies preferred 20.6°C and 20.5°C respectively.

Tp 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 wMelCS variant is being globally replaced by the wMel -variant (Riegler et al. 2005; Nunes et al. 2008; Richardson et al. 2012; Early and Clark 2013). Rare cases of the wMelCS infection type were recently detected in the wild (Nunes et al. 2008; Ilinsky 2013), thus replacement by wMel is still incomplete. Although the reason for the worldwide turn-over remains elusive, it has been hypothesized that wMel, which persists in hosts at significantly lower densities than wMelCS at 25°C (Chrostek et al. 2013), has better adapted to D. melanogaster. Accordingly, wMel infections are less costly to the host compared to the more ancestral wMelCS variant (Chrostek et al. 2013; reviewed by Miller 2013).
Insects can actively reduce or avoid costs of potentially fitness-reducing symbionts or parasites by behavioral adjustments such as changing egg deposition (Kacsoh et al. 2013) or mating behavior (reviewed by Wedell 2013). We find compelling evidence for Wolbachia-induced behavioral 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 harboring $w$Mel exhibit thermal preferences that are closer to uninfected flies under natural conditions compared to flies infected with $w$MelCS. Since Drosophila development is strictly temperature dependent (approximately 14 days of egg-to-adult development at 20°C and 9 days at 24°C; Ashburner 1989), flies infected with $w$Mel should have shorter generation times and thereby produce more generations per year resulting in higher net fecundity compared to $w$MelCS infected flies.

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 $\geq$ 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 behavior or behavioral chill (Fedorka et al. 2016) to attenuate the fitness costs associated with deleterious effects of Wolbachia over-proliferation and high cell densities (Chrostek et al. 2013; Strunov et al. 2013a; Strunov et al. 2013b).
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 titers are a temperature sensitive trait (Hoffmann et al. 1990; Reynolds et al. 2003; Mouton et al. 2006; Mouton et al. 2007; Bordenstein & Bordenstein 2011; Correa and Ballard 2012; Chrostek et al. 2013; Strunov et al. 2013a; Strunov et al. 2013b; 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 behavioral 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; $w$Mel, $w$MelCS_b, and
wMelPop all set in the DrosDel *w^{118}* isogenic background, which were kindly provided by Luis Teixeira and previously described by Teixeira *et al.* (2008) and Chrostek *et al.* (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 *Tp* differences (see Supporting Information Table S1 and Supporting Information File 1). In addition to testing for sex-class *Tp* differences, we performed assays to test for age-specific *Tp* 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 *wMelPop* 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 *wMelPop*-infected fly line did not produce enough flies to conduct all three age-class assays. Therefore, we excluded *wMelPop* 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 141bp tandem repeats, VNTR-141 loci (Riegler *et al.* 2005). The PCR reactions for *wsp* amplification were carried out in a total volume of 10µl containing 2µl Promega 5x Green GoTaq buffer, 4mM Promega MgCl₂, 0.8µM of forward and reverse
primers, 35µM of each dNTP, 0.04 U Promega GoTaq DNA Polymerase, and 1µl of genomic DNA template. Diagnostic VNTR-141 PCR reactions were each a total of 10µl comprised of the following: 2µl Promega 5x Green GoTaq buffer, 1.5mM Promega MgCl₂, 0.3µM of forward and reverse primers, 35µM of each dNTP, 0.04 U Promega GoTaq DNA Polymerase, and 1µl 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 wMelPop 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 aluminum rod (length 74.93cm, diameter 3.02cm; Part #R31-316 Metals Depot, Winchester, KY) was encased within a 58.76cm long and 6.35cm 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 aluminum rod to create a temperature gradient inside the thermal preference chamber. Temperatures along the gradient were measured at seven points that were 8.39cm apart using K-type thermocouples and two four-channel thermocouple recorders. We recorded temperatures on the aluminum 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 aluminum rod, 58.76 cm distance (Supporting Information Fig. S4). Along the aluminum
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 minutes and remained stable for at least 3 hours. 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 CO₂ treatment on Drosophila behavior (Barron 2000). Each assay was conducted for thirty minutes. Between assays, the temperature gradient chamber was taken apart and thoroughly cleaned to avoid contamination from any pheromone particles. All aluminum 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 four-minute 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
seconds for the duration of each treatment (30 minutes). 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 mins before they settled on either the aluminum rod or polycarbonate tube surfaces. Therefore, we selected images for analysis of fly distribution at the 20-minute time point as representative of the 30-minute 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 $\chi^2$ tests using the R function anova (see Supporting Information File 1 for full R code).

At first, we excluded flies infected with wMelPop, 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: $T_i = \text{wol} + \text{age} + \text{wol} \times \text{age} + \text{Rep} + \varepsilon_i$. Here, $T$ is the continuous response variable “Temperature”, $\text{age}$ is a nominal fixed factor with three levels each ($\text{age}$: 3-4 days, 5-7 days and 10-14 days post eclosion), $\text{wol}$ is a nominal fixed factor “Wolbachia” with three levels (un-infected, wMel and wMelCS), $\text{wol} \times \text{age}$ is the interaction term, $\text{Rep}$ is a nominal random factor “Replicate” for replicate trials and $\varepsilon_i$ 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
wMelPop (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 Tp 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: $T_i = w + sex + w \times
sex + Rep + \epsilon_i$ Here, $T$ is the continuous response variable “Temperature”, $sex$ is a nominal
fixed factor with two levels (male and female), $w$ is a nominal fixed factor “Wolbachia”
with three levels (wMel, wMelCS, and wMelPop), $w \times age$ is the interaction term, $Rep$ is a
nominal random factor “Replicate” for replicate trials and $\epsilon_i$ 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: $T_i = w + Rep + \epsilon_i$, where $T$ is the continuous response variable “Temperature”, $w$ is
a nominal fixed factor “Wolbachia” with four levels (un-infected, wMel, wMelCS, and
wMelPop), $Rep$ is the nominal random factor “Replicate” and $\epsilon_i$ 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 $\alpha$
threshold ($\alpha' = 0.05/3 = 0.017$) to account for multiple testing.

Acknowledgement

The authors want to thank Luis Teixeira for providing the Drosophila melanogaster white-
isogenic DrosDel w1118 lines infected with wMel, wMelCS-b and wMelPop. The authors
declare no conflict of interest.
Author contributions:

A.T. and W.J.M. conceived and planned the study, A.T. performed the experiment, A.T., R.K., and M.K. analyzed the data and A.T., W.J.M., and M.K. wrote the paper.

Funding Source

This work was supported by the National Science Foundation award (0948041) “Cascades to Coast GK-12: Enhancing STEM Education through Environmental Sustainability” awarded to AMT and the FWF grant (P28255-B22) from the Austrian Science Fund to WJM.
REFERENCES

Amarasekare P. and Savage V. (2012) A framework for elucidating the temperature dependence of fitness. *Am Nat* **179**: 178–191. https://doi.org/10.1086/663677

Angilletta, M.J., Steury, T. D., and Sears, M.W. (2004) Temperature, growth rate, and body size in ectotherms: Fitting pieces of a life-history puzzle. *Integr Comp Biol* **44**: 498–509. http://doi.org/10.1093/icb/44.6.498

Ashburner, M. (1989) *Drosophila: A Laboratory Manual*. Cold Spring Harbor Laboratory Press.

Barron, A.D. (2000) Anaesthetising *Drosophila* for behavioural studies. *J Insect Physiol* **44**: 439-442. https://doi.org/10.1016/S0022-1910(99)00129-8

Bates, D., Maechler, M., Bolker, B.M., and Walker, S.C. (2015) Fitting Linear Mixed-Effects Models Using *lme4*. *J Stat Softw* **67**: 1–48. http://doi: 10.18637/jss.v067.i01

Blanford, S. and Thomas, M.B. (1999) Host thermal biology: the key to understanding host-pathogen interactions and microbial pest control? *Agric For Entomol* **1**: 195–202. http://doi.org/10.1046/j.1461-9563.1999.00027.x

Bordenstein, S.R. and Bordenstein, S.R. (2011) Temperature affects the tripartite interactions between bacteriophage WO, *Wolbachia*, and cytoplasmic incompatibility. *PLoS ONE* **6**: e29106 http://doi.org/10.1371/journal.pone.0029106

Bozinovic F., Bastías, D., Boher, F., Clavijo-Baquet, S., Estay, S.A., and Angilletta Jr., M.J. (2011) The mean and variance of environmental temperature interact to determine physiological tolerance and fitness. *Physiol Biochem Zool* **84**: 543–552. http://doi.org/10.1086/662551

Chrostek, E., Marialva, M.S.P., Esteves, S.S., Weinert, L.A., Martinez, J., Jiggins, F.M., and Teixeira, L. (2013) *Wolbachia* variants induce differential protection to viruses in *Drosophila melanogaster*: A phenotypic and phylogenomic analysis. *PLoS Genet* **9**: e1003896. http://doi.org/10.1371/journal.pgen.1003896
Chrostek, E.A. (2014) Genomic and environmental factors influence Wolbachia-Drosophila symbiosis. Chapter 5: Temperature dependence of Wolbachia-conferred antiviral protection. Dissertation. Instituto de Tecnologia Química e Biológica. https://run.unl.pt/handle/10362/14860

Chrostek, E. and Teixeira, L. (2015) Mutualism Breakdown by Amplification of Wolbachia Genes. *PLoS Biol* 13: e1002065. http://doi.org/10.1371/journal.pbio.1002065

Clancy, D.J. and Hoffmann, A.A. (1998) Environmental effects on cytoplasmic incompatibility and bacterial load in Wolbachia infected *Drosophila simulans*. *Entomol Exp Appl* 86: 13–24. http://doi.org/10.1046/j.1570-7458.1998.00261.x

Correa, C.C. and Ballard, J.W.O. (2012) *Wolbachia* gonadal density in female and male *Drosophila* vary with laboratory adaptation and respond differently to physiological and environmental challenges. *J Invertebr Pathol* 111: 197–204. http://doi.org/10.1016/j.jip.2012.08.003

Dedeine, F., Vavre, F., Fleury, F., Loppin, B., Hochberg, M.E. and Boulétreau, M. (2001) Removing symbiotic *Wolbachia* bacteria specifically inhibits oogenesis in a parasitic wasp. *Proc Natl Acad Sci U S A* 98: 6247–6252. http://doi.org/10.1073/pnas.101304298

Dillon, M.E., Wang, G., Garrity, P.A., and Huey, R.B. (2009) Thermal preference in *Drosophila*. *J Therm Biol* 34: 109 – 119. http://doi.org/10.1016/j.jtherbio.2008.11.007

Dunbar, H.E., Wilson, A.C.C., Ferguson, N.R., and Moran, N.A. (2007) Aphid thermal tolerance is governed by a point mutation in bacterial symbionts. *PLoS Biol* 5: e96. http://doi.org/10.1371/journal.pbio.0050096

Dutra, H.L., Rocha, M.N., Dias, F.B., Mansur, S.B., Caragata, E.P., and Moreira, L.A. (2016) *Wolbachia* blocks currently circulating Zika virus isolates in Brazilian *Aedes aegypti* mosquitoes. *Cell Host Microbe* 19: 771-774. doi: 10.1016/j.chom.2016.04.021
Early, A.M. and Clark, A.G. (2013) Monophyly of Wolbachia pipientis genomes within Drosophila melanogaster: geographic structuring, titre variation, and host effects across five populations. Molecular Ecology, 22: 5765–5778. https://doi.org/10.1111/mec.12530

Endler, J.A. (1977) Geographic Variation, Speciation, and Clines. Princeton University Press.

Endler, J.A. (1986) Natural Selection in the Wild. Princeton University Press.

Fedorka, K.M., Kutch, I.C., Collins, L., and Musto, E. (2016) Cold temperature preference in bacterially infected Drosophila melanogaster improves survival but is remarkably suboptimal. J Insect Physiol 93–94: 36–41. http://doi.org/10.1016/j.jinsphys.2016.08.005

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

Garrity, P., Goodman, M., Samuel, A., and Sengupta, P. (2010) Running hot and cold: behavioral strategies, neural circuits, and the molecular machinery for thermotaxis in C. elegans and Drosophila. Genes Dev 24: 2365–2382. http://doi.org/10.1101/gad.1953710

Hedges, L.M., Brownlie, J.C., O’Neill, S.L., and Johnson, K.N. (2008) Wolbachia and virus protection in insects. Science 322: 702. http://doi.org/10.1126/science.1162418

Hoffmann, A.A. (1988) Partial cytoplasmic incompatibility between two Australian populations of Drosophila melanogaster. Entomol Exp Appl 48(1):61–67.

Hoffmann, A.A. (2010) Physiological climatic limits in Drosophila: patterns and implications. J Exp Biol 213: 870–80. http://doi.org/10.1242/jeb.037630

Hoffmann, A.A., Turelli, M., and Harshman, L.G. (1990) Factors affecting the distribution of cytoplasmic incompatibility in Drosophila simulans. Genetics 126: 933–948.
Hoffmann, A.A. and Sgró, C.M. (2011) Climate change and evolutionary adaptation. Nature 470: 479–85. http://doi.org/10.1038/nature09670

Hosokawa, T., Koga, R., Kikuchi, Y., Meng, X., and Fukatsu, T. (2010) Wolbachia as a bacteriocyte-associated nutritional mutualist. Proc Natl Acad Sci U S A 107: 769-774. http://doi.org/10.1073/pnas.0911476107

Huey, R.B., and Berrigan, D. (2001) Temperature, demography, and ectotherm fitness. Am Nat 158: 204–210. http://doi.org/10.1086/321314

Huey, R.B., Kearney, M.R., Krockenberger, A., Holtum, J.A.M., Jess, M., and Williams, S.E. (2012) Predicting organismal vulnerability to climate warming: roles of behaviour, physiology and adaptation. Philos Trans R Soc Lond B Biol Sci 367: 1665–1679. http://doi.org/10.1098/rstb.2012.0005

Hurst, G. D, Johnson, A.P., Schulenburg, J.H., and Fuyama, Y. (2000) Male-killing Wolbachia in Drosophila: a temperature-sensitive trait with a threshold bacterial density. Genetics 156: 699–709.

Ilinsky, Y. (2013) Coevolution of Drosophila melanogaster mtDNA and Wolbachia genotypes. PLoS ONE 8: e54373. https://doi.org/10.1371/journal.pone.0054373

Iturbe-Ormaetxe, I., Walker, T., and O’ Neill, S.L. (2011) Wolbachia and the biological control of mosquito-borne disease. EMBO Rep 12: 508–518. http://doi.org/10.1038/embor.2011.84

Jeyaprakash, A., and Hoy, M.A. (2000) Long PCR improves Wolbachia DNA amplification: wsp sequences found in 76% of sixty-three arthropod species. Insect Mol Biol 9: 393–405. http://doi.org/10.1046/j.1365-2583.2000.00203.x

Kacsoh, B.Z., Lynch, Z.R., Mortimer, N.T., and Schlenke, T.A. (2013) Fruit flies medicate offspring after seeing parasites. Science 339: 947-950. http://doi.org/10.1126/science.1229625.

Levins, R. (1968) Evolution in Changing Environments. Princeton University Press.
Louis, C., Jourdan, M., and Cabanac, M. (1986) Behavioral fever and therapy in a *rickettsia*-infected *Orthoptera*. *Am J Physiol* **250**: R991–R995.

https://doi.org/10.1152/ajpregu.1986.250.6.R991

Martin, T.L., and Huey, R.B. (2008) Why "suboptimal" is optimal: Jensen’s inequality and ectotherm thermal preferences. *Am Nat* **171**: 102–118. http://doi.org/10.1086/527502

Martinez, J., Longdon, B., Bauer, S., Chan, Y.S., Miller, W.J., Bourtzis, K., *et al.* (2014) Symbionts commonly provide broad spectrum resistance to viruses in insects: A comparative analysis of *Wolbachia* strains. *PLoS Pathog* **10**: e1004369.

http://doi.org/10.1371/journal.ppat.1004369

Martins, N.E., Faria, V.G., Nolte, V., Schlötterer, C., Teixeira, L., Succena, É., and Magalhães, S. (2014) Host adaptation to viruses relies on few genes with different cross-resistance properties. *Proc Natl Acad Sci U S A* **111**: 5938–5943.

http://doi.org/10.1073/pnas.1400378111

Matute, D.R., Novak, C.J., and Coyne, J.A. (2009) Temperature-based extrinsic reproductive isolation in two species of *Drosophila*. *Evolution* **63**: 595–612.

http://doi.org/10.1111/J.1558-5646.2008.00588.X

McGraw, E.A., Merritt, D.J., Droller, J.N., and O'Neill, S.L. (2002) *Wolbachia* density and virulence attenuation after transfer into a novel host. *Proc Natl Acad Sci U S A* **99**: 2918-2923. http://doi.org/10.1073/pnas.052466499

Miller, W.J. (2013) Bugs in transition: The dynamic world of *Wolbachia* in insects. *PLoS Genet* **9**: e1004069. http://doi.org/10.1371/journal.pgen.1004069

Miller, W.J., Ehrman, L., and Schneider, D. (2010) Infectious speciation revisited: Impact of symbiont-depletion on female fitness and mating behavior of *Drosophila paulistorum*. *PLoS Pathog* **6**: e1001214 . http://doi.org/10.1371/journal.ppat.1001214

Min, K.T., and 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–
Moreira, L.A., Iturbe-Ormaetxe, I., Jeffery, J.A., Lu, G., Pyke, A.T., Hedges, L.M., et al. (2009) A Wolbachia symbiont in Aedes aegypti limits infection with dengue, Chikungunya, and Plasmodium. Cell 139: 1268-1278. https://doi.org/10.1016/j.cell.2009.11.042

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

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

Murdock, C.C., Blanford, S., Hughes, G.L., Rasgon, J.L., & Thomas, M.B. (2014) Temperature alters Plasmodium blocking by Wolbachia. Sci Rep 4: 3932. http://doi.org/10.1038/srep03932

Nunes, M.D.S., Nolte, V., and Schlötterer, C. (2008) Nonrandom Wolbachia infection status of Drosophila melanogaster strains with different mtDNA haplotypes. Mol Biol Evol 25: 2493–2498. http://doi.org/10.1093/molbev/msn199.

O’Neill, S.L., Giordano, R., Colbert, A.M., Karr, T.L., and Robertson, H.M. (1992) 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. Proc Natl Acad Sci USA 89: 2699–2702.

Osborne, S.E., Leong, Y.S., O’Neill, S.L., and Johnson, K.N. (2009) Variation in antiviral protection mediated by different Wolbachia strains in Drosophila simulans. PLoS Pathog 5: e1000656. http://doi.org/10.1371/journal.ppat.1000656

R Development Core Team (2009) R: A Language and Environment for Statistical Computing. R-project.org.

Rajpurohit, S., and Schmidt, P.S. (2016) Measuring thermal behavior in smaller insects: a
case study in *Drosophila melanogaster* demonstrates effects of sex, geographic origin, and rearing temperature on adult behavior. *Fly* **10**: 149-161

http://doi.org/10.1080/19336934.2016.1194145

Reynolds, K.T., Thomson, L.J., and Hoffmann, A.A. (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.

Richardson, M.F., Weinert, L.A., Welch, J.J., Linheiro, R.S., Magwire, M.M., Jiggins, F.M., and Bergman, C.M. (2012) Population genomics of the *Wolbachia* endosymbiont in *Drosophila melanogaster*. *PLoS Genet* **8**: e1003129. http://doi.org/10.1371/journal.pgen.1003129

Riegler, M., Iturbe-Ormaetxe, I., Woolfit, M., Miller, W.J., and O’Neill, S.L. (2012) Tandem repeat markers as novel diagnostic tools for high resolution fingerprinting of *Wolbachia*. *BMC Microbiol* **12 Suppl 1**: S12. http://doi.org/10.1186/1471-2180-12-S1-S12

Riegler, M., Sidhu, M., Miller, W.J., and O’Neill, S.L. (2005) Evidence for a global *Wolbachia* replacement in *Drosophila melanogaster*. *Curr Biol* **15**: 1428–1433. http://doi.org/10.1016/j.cub.2005.06.069

Russell, J.A., and Moran, N.A. (2006) Costs and benefits of symbiont infection in aphids: variation among symbionts and across temperatures. *Proc Biol Sci* **273**: 603–610. http://doi.org/10.1098/rspb.2005.3348

Sazama, E.J., Bosch, M.J., Shouldis, C.S., Ouellette, S.P., and Wesner, J. S. (2017) Incidence of *Wolbachia* in aquatic insects. *Ecol Evol* **7**: 1165–1169. http://doi.org/10.1002/ece3.2742

Strunov, A. A., Ilinskii, Y. Y., Zakharov, I. K., & Kiseleva, E. V. (2013a). Effect of high temperature on survival of *Drosophila melanogaster* infected with pathogenic strain of *Wolbachia* bacteria. *Russ J Genet Appl Res* **3**: 435–443. http://doi.org/10.1134/S2079059713060099
Strunov, A., Kiseleva, E., and Gottlieb, Y. (2013b) Spatial and temporal distribution of pathogenic Wolbachia strain wMelPop in Drosophila melanogaster central nervous system under different temperature conditions. J Invertebr Pathol 114: 22–30. http://doi.org/10.1016/j.jip.2013.05.001

Teixeira, L., Ferreira, Â., and Ashburner, M. (2008) The bacterial symbiont Wolbachia induces resistance to RNA viral infections in Drosophila melanogaster. PLoS Biol 6: e2. http://doi.org/10.1371/journal.pbio.1000002

van Opijnen, T.V. and Breeuwer, J.A.J. (1999) High temperatures eliminate Wolbachia, a cytoplasmic incompatibility inducing endosymbiont, from the two-spotted spider mite. Exp Appl Acarol 23: 871–881.

Veneti, Z., Clark, M.E., Zabalou, S., Karr, T.L., Savakis, C., and Bourtzis, K. (2003) Cytoplasmic incompatibility and sperm cyst infection in different Drosophila-Wolbachia associations. Genetics 164: 545–552.

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

Webster, C.L., Waldron, F.M., Robertson, S., Crowson, D., Ferrari, G., Quintana, J.F., et al. (2015) The discovery, distribution, and evolution of viruses associated with Drosophila melanogaster. PLoS Biol 13: e1002210. http://doi.org/10.1371/journal.pbio.1002210

Wedell, N. (2013) The dynamic relationship between polyandry and selfish genetic elements. Philos Trans R Soc Lond B Biol Sci 368: 20120049. http://doi.org/10.1098/rstb.2012.0049

Weinert, L.A., Araujo-jnr, E.V, Ahmed, M.Z., Welch, J.J., and Welch, J.J. (2015) The incidence of bacterial endosymbionts in terrestrial arthropods. Proc Biol Sci 282: 20150249. http://dx.doi.org/10.1098/rspb.2015.0249

Wernegreen, J.J. (2012) Mutualism meltdown in insects: Bacteria constrain thermal
adaptation. Curr Opin Microbiol 15(3), 255–262.

http://doi.org/10.1016/j.mib.2012.02.001

Werren, J. H., Baldo, L., and Clark, M.E. (2008) *Wolbachia*: master manipulators of invertebrate biology. Nat Rev Microbiol 6: 741–751. http://doi.org/10.1038/nrmicro1969

Werren, J.H., Zhang, W., and Guo, L.R. (1995) Evolution and phylogeny of *Wolbachia*: reproductive parasites of arthropods. Proc Biol Sci 281: 55-63.

http://doi.org/10.1098/rspb.1995.0117

Wiwatanaratanabutr, I. and Kittayapong, P. (2009) Effects of crowding and temperature on *Wolbachia* infection density among life cycle stages of *Aedes albopictus*. J Invertebr Pathol 102: 220–224. http://doi.org/10.1016/j.jip.2009.08.009

Yamada, R., Floate, K.D., Riegler, M., and Neill, S.L.O. (2007) Male development time influences the strength of *Wolbachia*-induced cytoplasmic incompatibility expression in *Drosophila melanogaster*. Genetics 177: 801–808.

http://doi.org/10.1534/genetics.106.068486

Zhou, W., Rousset, F., and O’Neil, S. (1998) Phylogeny and PCR-based classification of *Wolbachia* strains using *wsp* gene sequences. Proc Biol Sci 265: 509–515.

http://doi.org/10.1098/rspb.1998.0324

Zug, R. and Hammerstein, P. (2012) Still a host of hosts for *Wolbachia*: analysis of recent data suggests that 40% of terrestrial arthropod species are infected. PloS One, 7: e38544.

http://doi.org/10.1371/journal.pone.0038544

Zug R. and Hammerstein P. (2015) Bad guys turned nice? A critical assessment of *Wolbachia* mutualisms in arthropod hosts. Biol Rev Camb Philos Soc 90: 89-111. doi: 10.1111/brv.12098
Table 1. Comparison of strain type titer levels, growth rates, and effects on host’s lifespan at 25°C.

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. 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 \( \chi^2 \) test of the analysis of deviance, the \( \chi^2 \) value and the corresponding \( P \)-value. Note that analyses with significant effects after Bonferroni correction (adjusted \( \alpha = 0.017 \)) are highlighted in bold.

Table 3: Table showing z-values from post-hoc pairwise comparisons with Tukey’s HSD for the factor Wolbachia (Analysis C; see Experimental Procedures) with four levels (non-infected, wMel, wMelCS, and wMelPop). Bold type indicates significance after Bonferroni correction (adjusted \( \alpha' = 0.017 \)). * \( p < 0.05 \); ** \( p < 0.01 \); *** \( p < 0.001 \).

Figure 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 wMelPop) 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), or infected with wMel (red), wMelCS (blue) or wMelPop (green). 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 to uninfected flies.
Table 1.

| Strain type | Relative amount of Wolbachia                                      | Effects on host’s lifespan       |
|-------------|------------------------------------------------------------------|----------------------------------|
| wMel        | Lowest titer level and growth rate                               | No reduction                     |
| wMelCS      | Approximately double the titer level compared to wMel and higher growth rate | Some reduction                   |
| wMelPop     | Titer level 20 times higher compared to wMelCS                   | Reduction by approximately half  |

Note: Information on titer levels, growth rate, host’s lifespan effects for wMel and wMelCS from Chrostek et al. 2013, information on wMelPop’s effects on host’s lifespan from Reynolds et al. 2003.
Table 2

| Analysis | Model                      | Factor     | n   | Df | $\chi^2$ | P-value  |
|----------|----------------------------|------------|-----|----|----------|----------|
| A        | $\text{wol + age + wol x age}$ | $\text{wol}$ | 4370 | 6  | 119.71   | 1.87E-23 |
| A        | $\text{wol + age + wol x age}$ | $\text{age}$ | 4370 | 6  | 2.10     | 0.91     |
| A        | $\text{wol + age + wol x age}$ | $\text{wol x age}$ | 4370 | 4  | 1.31     | 0.86     |
| B        | $\text{wol + sex + wol x sex}$ | $\text{wol}$ | 1718 | 6  | 61.19    | 2.58E-11 |
| B        | $\text{wol + sex + wol x sex}$ | $\text{sex}$ | 1718 | 4  | 2.12     | 0.71     |
| B        | $\text{wol + sex + wol x sex}$ | $\text{wol x sex}$ | 1718 | 3  | 1.90     | 0.59     |
| C        | $\text{wol}$                      | $\text{wol}$ | 5717 | 3  | 168.69   | 2.44E-36 |
Table 3

|       | w-     | wMel   | wMelCS  | wMelPop |
|-------|--------|--------|---------|---------|
| w-    | -      | -      | -       | -       |
| wMel  | -6.76*** | -     | -       | -       |
| wMelCS| -21.93*** | -15.49*** | -       | -       |
| wMelPop| -21.5*** | -15.35*** | -0.6    | -       |