Wolbachia has no effect on thermal preference at different life-stages in highly inbred Drosophila melanogaster

Anton Strunov[1,*], Charlotte Schoenherr[1], Martin Kapun[1,2,*]

1 Center for Anatomy and Cell Biology, Medical University of Vienna, Austria
2 Central Research Laboratories, Natural History Museum of Vienna, Austria

* co-corresponding authors

Contact:
anton.strunov@meduniwien.ac.at
martin.kapun@meduniwien.ac.at

Running title: *Wolbachia and thermal preference of Drosophila*

Keywords: *Drosophila*, *Wolbachia*, thermal preference, development

Abstract (195 words)

Temperature fluctuations are challenging for ectotherms which are not able to regulate body temperature by physiological means and thus have to adjust their thermal environment via behavior. However, little is yet known about whether microbial symbionts influence thermal preference (T_p) in ectotherms by modulating their physiology. Several recent studies have demonstrated substantial effects of *Wolbachia* infections on host T_p in different *Drosophila* species. These data indicate that the direction and strength of thermal preference variation is strongly depend on host and symbiont genotypes and highly variable among studies. By employing highly controlled experiments, we investigated the impact of several environmental factors including humidity, food quality, light exposure, and experimental setup that may influence T_p measurements in adult flies. Additionally, we assessed the effects of *Wolbachia* infection on T_p of *Drosophila* at different developmental stages, which has not been done before. We find no effects of *Wolbachia* on host T_p at any life stage. Instead, we show that environmental variation has a substantial influence on T_p measurements which demonstrates the necessity of careful experimental design and cautious interpretations of T_p measurements together with a thorough description of the methods and equipment used to conduct behavioral studies.

Introduction

Temperature modulates many physiological processes and has direct effects on development, survival and reproduction of any organism (Huey and Berrigan, 2001; Hoffmann, 2010). Ectotherms lack the ability to regulate their body temperature by physiological means and are particularly affected by temperature variation (Angiletta et al., 2004). Their thermoregulation is thus often mediated through behavior (Stevenson, 1985) so ectotherms tend to occupy environmental niches close to their optimal thermal conditions to survive and propagate. Every organism exhibits a thermal preference (T_p), which is the preferred body temperature or temperature range that is chosen in the absence of other ecological constraints (Gilbert and Miles, 2017). However, estimates of T_p may be strongly influenced by interactions with ecological factors and even with symbionts. Endotherms, for example, combat bacterial and viral infections with fever by physiologically increasing their body temperature outside the thermal optimum of their infectious agents. Several studies show that also ectotherms employ similar behavioral strategies by changing their T_p (Hart, 1988; Watson et al., 1993; Inglis et al., 1996; Roode and Lefevre, 2012; Poulin, 2010; Fedorka et al., 2016; Curtis, 2014). For example, crickets actively seek higher temperatures when infected with the pathogenic bacterium *Serratia marcescens*, which may have physiological effects similar to fever in response to pathogenic infections. Three studies report the opposite behavior in *Drosophila melanogaster* infected with the bacterial endosymbiont *Wolbachia* (Truitt et al., 2019; Arnold et al., 2019; Hague et al., 2020). Depending on the *Wolbachia* variant, infected flies chose colder temperatures than uninfected individuals. This may suggest that the hosts alleviate detrimental effects of high titer infections by choosing ambient temperatures outside the optimal physiological range of the symbiont. It, however, remains uncertain if such behavioral patterns are restricted to adults or are also found at juvenile life stages. Finding the optimal temperature for development is pivotal since any major disturbance during juvenile stages might severely decrease survival and reproduction chances of the imago (Mirth et al., 2021). Manipulating thermal preference at this life stage of the host might thus be risky yet beneficial for the microbial symbiont, by “setting up” its thermal environment early in the host development. However, to the best of our knowledge, no study has yet investigated the effects of *Wolbachia* on *Drosophila* T_p at juvenile life stages.

Measuring thermal behaviour in *Drosophila* is complex, and many factors that potentially influence the T_p measurement have to be accounted for (Dillon et al., 2009). This becomes evident when comparing the results of three studies assessing the T_p of *D. melanogaster* in the presence of *Wolbachia* infections (Truitt et al., 2019; Arnold et al., 2019; Hague et al., 2020). The absolute T_p values for the same *Wolbachia* strain vary strongly

among the studies (up to 5-6°C), which is potentially influenced by different host genetic backgrounds, experimental conditions or temperature ranges used in these studies. Assessing the effects of different environmental factors on thermal preference in the *Wolbachia-Drosophila* system is therefore crucial for an informed analysis and interpretation of behavioral measurements.

Here, we demonstrate that several environmental factors, such as humidity, food, light exposure, and the structure of the thermal gradient device have a major impact on T_p measurements in adult *D. melanogaster* flies. Additionally, we investigate - for the first time - the effect of *Wolbachia* on juvenile T_p and find that it is not affected by infection in early and late fly larvae and at pupariation. Moreover, in contrast to previous studies, we also do not find any effect of *Wolbachia* on T_p in adult flies.

Materials and Methods

Fly lines

In our experiments, we used four highly inbred, long-term laboratory strains of *D. melanogaster* which were uninfected (*w*-) or infected with either two of the most common natural *Wolbachia* variants (*w*Mel and *w*MelCS) or with the *w*MelPop lab-variant that were previously investigated by Truitt et al. (2019) for thermal preference. All fly lines were initially established by Luis Teixeira (Teixeira et al., 2008) in a DrosDel w^{1118} isogenic background. Flies were maintained on a custom fly medium based on agar-agar, molasses, and yeast at 24°C with 12h:12h light:dark cycle and an average humidity of 50%. Prior to experiments, the infection status was confirmed by PCR using *Wolbachia*-specific primers amplifying parts of the *wsp* gene: forward - tggtccaataagtgatgaagaaactagcta and reverse - aaaaattaaacgctactccagcttctgcac (Zhou et al., 1998). *Wolbachia* variants infecting the fly strains were distinguished with diagnostic VNTR-141 PCR primers: forward - ggagtattattgatatgcg and reverse - gactaaaggttattgcat. PCR products vary in length among *Wolbachia* strains (Riegler et al., 2012).

Thermal gradient machine

To carry out T_p measurements in *D. melanogaster* larvae, during pupariation and in adult flies, we designed and built a new thermal gradient apparatus with two narrow arenas for precise temperature measurements and with limited space for movement along the thermal gradient. The “plate” device consisted of an elongated aluminum plate as the bottom piece resting on two peltier elements, which are either heating or cooling to generate a temperature gradient. On top, we used an equisized plexiglass plate as the cover, which contained holes to apply test subjects. Two narrow arenas with 6 mm heights and widths

were established by placing three thin plexiglass dividers between the two plates, which contained holes for inserting temperature sensors. We restricted the length of the arena to 30 cm by adding foam stoppers at the ends of each lane to prevent flies from escaping and to maintain airflow. In addition, Whatman paper was used as a bottom layer to cover the aluminum plate in the arenas. We built two identical devices with the aforementioned design, which allowed us to investigate T_p in four arenas in parallel during one experimental assay (see https://github.com/capoony/DrosophilaThermalGradient for a detailed description).

For T_p measurements in adult flies, we additionally designed and built a second "tube" thermal gradient device with a tubular shape following Rajpurohit and Schmidt (2016) and complementing the design as described in Truitt et al. (2019). This apparatus consisted of a central cylindrical aluminum rod connected to quadratic aluminum bases at both ends that conduct heat from two peltier elements, which are either heating or cooling. The aluminum rod was placed inside a transparent plexiglass tube, which left an open space of approximately 4 cm in diameter. The plexiglass tube was attached to the aluminum rod with Teflon covers which enclosed the fly arena on both sides. The plexiglass tube contained several holes to insert flies and to attach sensors for temperature measurement. A Whatman paper was inserted right above the aluminum rod to divide the arena into two halves. Flies were kept in the upper half, which facilitated imaging the location of the flies within the cylindrical arena around the central rod from atop during T_P measurements (see https://github.com/capoony/DrosophilaThermalGradient for a detailed description).

Peltier elements (P&N Technology, Xiamen Fujian China) generating heat were attached to small heat sinks (Fischer Electronic, Germany) connected to a ventilator (Oezpolat, Germany) to stabilize the temperature. Cooling peltier elements produce excess waste heat and were therefore placed on top of a bigger heat sink (Fischer Electronic, Germany), which was partially submerged in a bowl with cold water that was constantly cooled down by pipes supplying cold water from a distant water bath (fbc630, Fisher scientific, USA).

20 min prior to each experiment, we attached the peltier elements to power supplies set to 2 V and 3 V for cooling and heating, respectively, to stabilize the temperature gradient within the thermal gradient apparatus. We found that the temperature gradient remained stable for at least 1 h, which generally exceeded the duration of an experimental run (app. 20-30 min). After each experiment, the plexiglass parts of the apparatus were cleaned with soap and hot water to remove any potential pheromones and other odorants, which might interfere with thermal preference behavior of flies.

Each experiment was carried out in a dark isolated room with no, or very low noise distraction. The room was equipped with air-conditioning to keep the ambient temperature stable at 21-23°C, which was constantly monitored with a data logger. The temperature

146 within the devices was measured at three points (in the center and at the hot and cold
147 edges) with digital thermal sensors (Analog devices, USA). The data from thermal sensors
148 were collected with a custom python script on a Raspberry pi 3B+ computer (Raspberry Pi
149 Foundation, UK) in 10 sec intervals and saved as text files. The position of the flies within
150 the devices were assessed by an infrared camera attached atop of the thermal gradient
151 apparatus. Images were taken every 30 sec and saved to the Raspberry pi computer for
152 each run. We developed a custom python script to estimate T_p for every fly using information
153 of an individual's position relative to the linear temperature gradient between the
154 temperature measurement points. The coordinates of each individual fly and of the thermal
155 sensors were manually assessed in ImageJ (Schneider et al., 2012) based on infrared
156 images captured 20 min after the onset of each experiment. The python scripts for
157 quantifying thermal preference from the coordinates including a test dataset, a detailed
158 description and all raw images from the thermal preference assays are available at GitHub
159 (https://github.com/capoony/DrosophilaThermalGradient).
160
161 *Temperature preference measurements in larvae*
162 To assess larval T_p, we used early and late 3rd instar larvae, 72 h, and 120 h after
163 egg laying (AEL), respectively, and focused on two fly strains that were either uninfected (w-
164) or infected with *w*MelCS for thermal preference experiments (see *Fly lines* section in
165 Materials and methods). To obtain tightly synchronized cohorts of larvae, we maintained 40-
166 50 adult female flies at 24°C in glass vials with the medium for 3-6 h for egg laying and then
167 transferred these to a fresh medium for collecting another replicate. After 72 or 120 h, larvae
168 were moved to an 18% sucrose solution (in dH_2O) using a spatula. Floating larvae were
169 gently collected with a cut 1 mL pipette tip and transferred to dH_2O. After washing three
170 times with dH_2O, larvae were transferred into empty plastic Petri dishes (d=2.5 cm) and kept
171 for 10-20 min to recover from the stress.
172 For T_p measurements, we covered the bottom of each lane in the thermal gradient
173 machine with a 1mm layer of 2% agarose gel to create a habitable environment for larvae
174 that allowed them to crawl freely. We started the experiment when the thermal gradient was
175 constant and linear from 18°C to 28°C (1°C per 3cm). Experiments were carried out in a
176 dark room at 24-25°C, with 30% humidity at different times of the day (from 11 am to 4 pm).
177 Each run included two replicates of the *w*- and the *w*MelCS lines. 50-100 larvae were
178 introduced in the middle of each lane (23°C) with a wet brush and a Plexiglass cover was
179 applied on top of the machine to prevent larvae from escaping. The position of larvae was
180 analyzed on site after 30 min by quantifying the numbers of larvae located in a certain
181 temperature zone marked with a pen beforehand.

182 *Measurement of pupariation Tp*

183 For assessing T_p at pupariation, we used late 3rd-instar larvae, 14 0h after egg laying
184 (AEL) close to pupariation. Again, we focused on the *w*- and *w*MelCS strains for the
185 experiments and obtained synchronized cohorts of 140 h-old larvae as explained above.

186 For T_p measurements, we covered the bottom of the four lanes of the gradient
187 machine with a 1 mm thick sheet of odorless black plastic to create a contrasting
188 background and to supply a solid surface for pupariation. We inserted larvae when the
189 gradient was constant and linear from 18°C to 28°C (1°C per 3cm). Experiments were
190 carried out in a dark room at 24-25°C with 30% humidity for approximately 12h until every
191 larva had pupariated. Each run included two replicate lines at the same time (2 times *w*- and
192 2 times *w*MelCS). 50-100 larvae were introduced in the middle of the lane (23°C) by wet
193 brush and a plexiglass cover was used to seal the top of the machine to prevent larvae from
194 crawling out of the arenas. Thermal preference at pupariation was analyzed after 6-12 h by
195 quantifying the numbers of pupae in certain temperature zones as described above.

196

197 *Temperature preference measurements in adults*

198 Prior to the experiments, we selected cohorts of 20 females and 20 males, which
199 were one week old (± two days), for each of the four strains (*w*-, *w*Mel, *w*MelCS and
200 *w*MelPop). We transferred these to fresh glass vials with medium by gently applying CO_2
201 and then allowed the flies to recover for two days. To reduce the possibility of an
202 experimenter bias, we anonymized the ID's of the cohorts prior to experiments by an
203 independent researcher. For each assay on the "plate" device, we included one replicate of
204 all four fly lines and placed each of the groups in one of the four lanes. For experiments
205 using the "tube" device, we carried out two replicate assays in parallel, using randomly
206 drawn cohorts. Flies were gently collected from the vial with a handmade fly aspirator and
207 transferred to the thermal gradient apparatus through a hole in the center. Then, the hole
208 was tightly sealed with cotton, the light was turned off and measurements were taken during
209 20 min. An image of the whole apparatus was taken every 30 seconds with an infrared
210 camera attached atop the setup. After the experiment was finished, flies were anesthetized
211 with CO_2 within the apparatus and then collected into 1.5ml tubes and frozen at -20°C for
212 further analyses.

213 Because time of the day can have a strong influence on T_p in *Drosophila* (Chen et al.,
214 2022), we performed a maximum of 4 runs per day to avoid potential bias from circadian
215 rhythms. The time of the runs during the day was usually as follows: first two runs from 10
216 am to 12 am, last two runs from 1 pm to 3.30 pm. To quantify T_p of individual flies in the
217 arena, we used the images of fly locations taken 20 min after the onset of the experiment
218 similar to Truitt et al. (2019).

In addition to the experiments outlined above, we sought to test how environmental variation influences T_p. We accounted for four different factors: humidity, light, food, and type of the gradient machine. All four factors were tested in at least three replicates with the "tube" device, except for the last one where both types were used for comparison. When testing for the effects of humidity, we used a standard humidifier to increase the humidity from 30%, which we usually measured without any adjustment, to stable 60% humidity in the test room. To test the influence of light on thermal preference, we positioned the gradient machine at the center above a ceiling lamp and performed thermal preference runs with the light on and off using different biological replicates. When testing for the effect of food quality on T_p, we kept the flies for at least three generations on different diets before conducting the experiment. We compared the effects of our in-house food recipe, described above, to Carolina 4-24 instant food, obtained directly from the manufacturer (Carolina, USA). To test how the type of device affects T_p, we performed the runs in the two different devices in the same room with similar ambient temperatures to keep environmental conditions consistent.

Statistical analyses

The effects of *Wolbachia* infection and other environmental factors on T_p were analyzed using generalized linear mixed models (GLMMs) with a Poisson error structure in *R* (R Core Team, 2021) based on the "glmer" function in the lme4 package (Bates et al., 2015). In our models, we considered T_p of every individual as the dependent variable, and infection status as the fixed factor. For the larval T_p experiment, we additionally included the larval age (72 h and 120 h) as a fixed factor and the interaction of larval age with infection status in our model. Moreover, for most of the models, we included the time of the run of each replicate during the day as a random factor. We used the *Anova* function in *R* to test for a significant effect of infection status on thermal preference and calculated a Type-III ANOVA using the *car* package (Fox and Weisberg, 2019) to test for significant effects of fixed factors and interaction in the T_p experiments. Whenever the factor *infection status* had a significant effect, we performed post-hoc tests using Tukey's HSD method as implemented in the *emmeans* package (Lenth, 2021). We also inferred the skewness of T_p distributions using the *moments* package (Kommsta & Novometsky 2022) and compared the average skewness among experimental setups with Wilcoxon Rank tests in *R*. The raw data table (Strunov_etal_WolbTP_2023_RawData.xslx) and all *R* code (Strunov_etal_WolbTP_2023_Rcode.zip) are available as Supplementary Materials.

Results and Discussion

Wolbachia infection has no effect on T_p in 3rd-instar Drosophila larvae and on pupariation T_p in D. melanogaster

Using the newly designed thermal gradient apparatus (see Materials and Methods), we first tested if our experimental setup allowed us to reliably assess thermal preference variation in *Drosophila*. We therefore repeated an experiment previously reported in Sokabe et al. (2016), who found that thermal preference in 3rd-instar *Drosophila* larvae sharply declined from 25°C to 18°C prior to pupariation. We found that T_p in early (72h AEL; n=281) and late (120h AEL; n=658) 3rd-instar larvae significantly shifted from a median T_p of 22-23°C to a median T_p of 19°C with progression of development (GLMM: p=7.8e-12, x^2=51.2, df=2; **Table 1**, **Figure 1A**). Our results were consistent with previous findings in Sokabe et al. (2016) and indicated that our setup is suitable to reliably measure T_p. However, we neither observed an effect of *Wolbachia* on T_p in 3rd-instar larvae (GLMM; p=0.34, x^2=2.1, df=2) nor an interaction of *Wolbachia* with larval age (GLMM; p=0.17, x^2=1.9, df=1). Since many larvae clustered at the coldest spot at the wall in the gradient machine (18-19°C), we repeated the experiment for the 120h age class, which exhibited lower T_p, and extended the lower temperature range to 15°C. Analysis of 529 infected and 332 uninfected 3rd-instar larvae at 120h AEL similarly showed no effect of *Wolbachia* infection on T_p of the host (GLMM: p=0.38, x^2=0.76, df=1; **Figure 1B**, **Table 1**), although the median T_p decreased by approximately 1°C with the majority of larvae of both infection types (*w*- and *w*MelCS) clustering at temperatures between 16°C and 18°C (median T_p of 18°C).

Table 1. Analysis of *Drosophila* T_p at different stages of development. Significant results are highlighted in bold.

(A) T_p of 72h vs 120h larvae			
Effect	**χ^2**	***df***	***p*-Value**
age	51.2	2	**7.8E-12**
infection	2.1	2	0.34
age*infection	1.9	1	0.17
(B) T_p of 120h larvae			
Effect	**χ^2**	***df***	***p*-Value**
infection	0.8	1	0.38
(C) T_p of 140h larvae			
Effect	**χ^2**	***df***	***p*-Value**
infection	0.2	1	0.64
(D) T_p of adult (flat)			
Effect	**χ^2**	***df***	***p*-Value**
infection	5.8	3	0.12

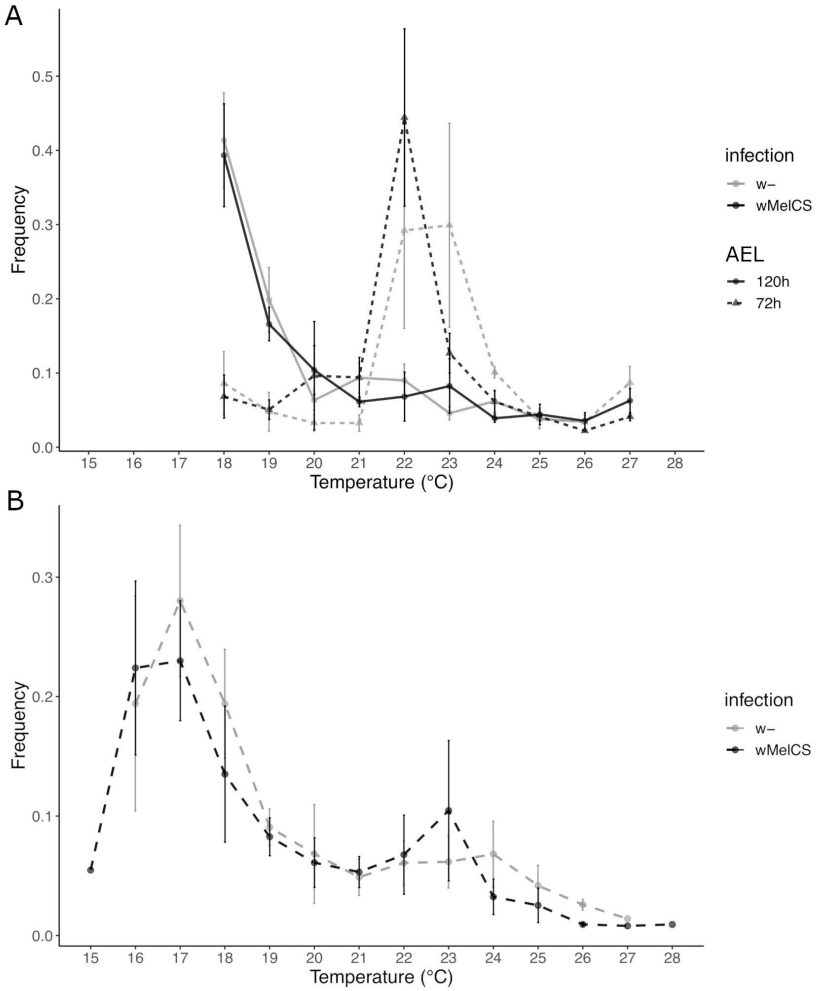

Figure 1. T_p of early and late *D. melanogaster* 3rd-instar larvae. (A) Temperature shift in early and late 3rd-instar larvae. Note the absence of *Wolbachia* effects on thermal preference at both larval stages. (B) T_p of late 3rd-instar larvae measured at a broader temperature range. Note the absence of *Wolbachia* effect on thermal preference in both cases. AEL = after egg laying.

Pupariation in *Drosophila* is a crucial developmental stage during which the fly is immobilized for several days and undergoes complete metamorphosis. Thus, for larvae to properly develop choosing the optimal pupariation temperature is of great importance (Al-Saffar et al., 1996; Dillon et al., 2009). We analyzed the T_p of 192 infected and 142 uninfected larvae about to pupariate (140h AEL). In comparison to T_p in late 3rd-instar larvae (Median T_p of 18°C for both infection types), the pupariation T_p shifted to warmer temperatures (**Figure 2**, median T_p of 20°C for uninfected, and 21°C for infected individuals). However, similarly to 3rd-instar larvae, we found no significant effect of *Wolbachia* on thermal preference at pupariation stage (GLMM: p=0.64, x^2=0.21, df=1) (**Table 1**).

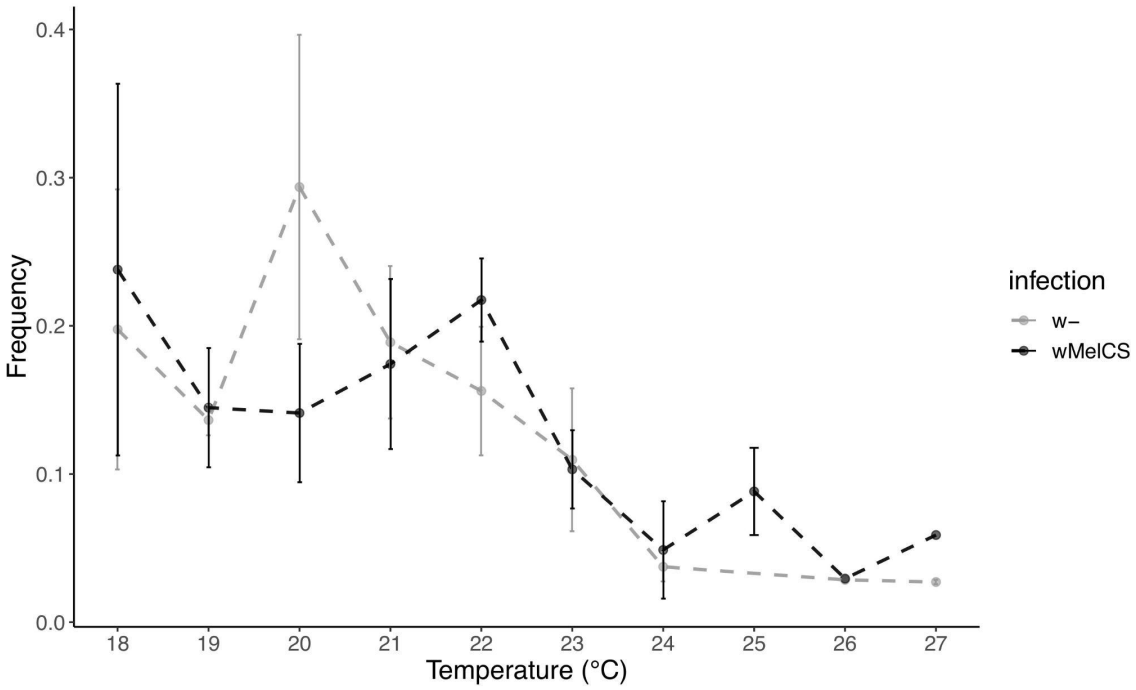

Figure 2. Thermal preference of *D. melanogaster* larvae at the onset of pupariation (140h AEL). Note the absence of *Wolbachia* effect on the thermal preference of pupae.

The absence of *Wolbachia* effects on T_p in early and late 3rd-instar larvae and on pupariation T_p might indicate that infection does not impose significant costs at least in the developmental stages investigated here. It might be correlated with bacterial titer, which is known to influence the fitness of symbiotic association when it reaches high levels (Min and Benzer, 1997; Osborne et al., 2009; Strunov et al., 2013a; Chrostek et al., 2013; Martinez et al., 2017). Although little is known about *Wolbachia* titer dynamics during fly development, our findings could imply dormancy of infection at juvenile stages of the host. Strunov et al. (2013a), for example, showed that the titer of the pathogenic *w*MelPop strain in *D. melanogaster* is significantly lower in larvae than in the imago and stable across development without any effects of rearing temperature. However, an influence of temperature on *Wolbachia* titer has been previously observed in adults after metamorphosis (Strunov et al., 2013b, Chrostek et al., 2021), indicating that bacterial multiplication and pronounced interaction with the host may take place during late pupariation stage. In line with this hypothesis, it was shown that *Wolbachia* interferes with pheromone production in females during pupariation and affects female-to-male communication (Pontier et al., 2015). Additionally, two recent papers demonstrated the absence of *Wolbachia* effects on locomotion and fitness at juvenile stage of wild-caught *D. nigrosparsa* (Detcharoen et al., 2020) and *D. melanogaster* flies (Strunov et al., 2022). The absence of *Wolbachia* effects on host juvenile traits is challenged by a study, which reported stable abundance of *Wolbachia* transcripts at all stages of host development (Gutzwiller et al., 2015), although there was no

data on translation of the transcripts. Thus, *Wolbachia* have presumably no or very low and undetected effects on developing flies. Additionally, other cues like foraging for food (Sokolowski et al., 1984) might represent stronger behavioral stimuli, which could mask subtle effects of *Wolbachia* on thermal behavior.

Wolbachia infection has no effect on thermal preference in adult flies.

Using our new experimental setup, we sought to reproduce previous experiments which reported that *Wolbachia* infection can lower thermal preference in adult *D. melanogaster* flies depending on the *Wolbachia* variant studied (Truitt et al., 2019; Arnold et al., 2019; but see Hague et al., 2020). We used the same fly lines, initially established by Teixeira et al. (2008), as examined by Truitt et al. (2019). We investigated five replicate cohorts of each fly line (*w*-, *w*Mel, *w*MelCS, and *w*MelPop) with our new thermal gradient device under highly controlled environmental conditions. Unexpectedly, we were unable to reproduce the previous results in Truitt et al. (2019), since none of the lines differed in thermal preference (GLMM: $p>0.05$, $x^2=5.8$, $df=3$; **Figure 3**). Median T_p was 18.3°C, 18.8°C, 18.1°C, and 18.2°C for *w*-, *w*Mel, *w*MelCS, and *w*MelPop, respectively. The variation among the replicates was quite high with 2.4 to 3°C standard deviation among the mean T_p, depending on the infection type studied.

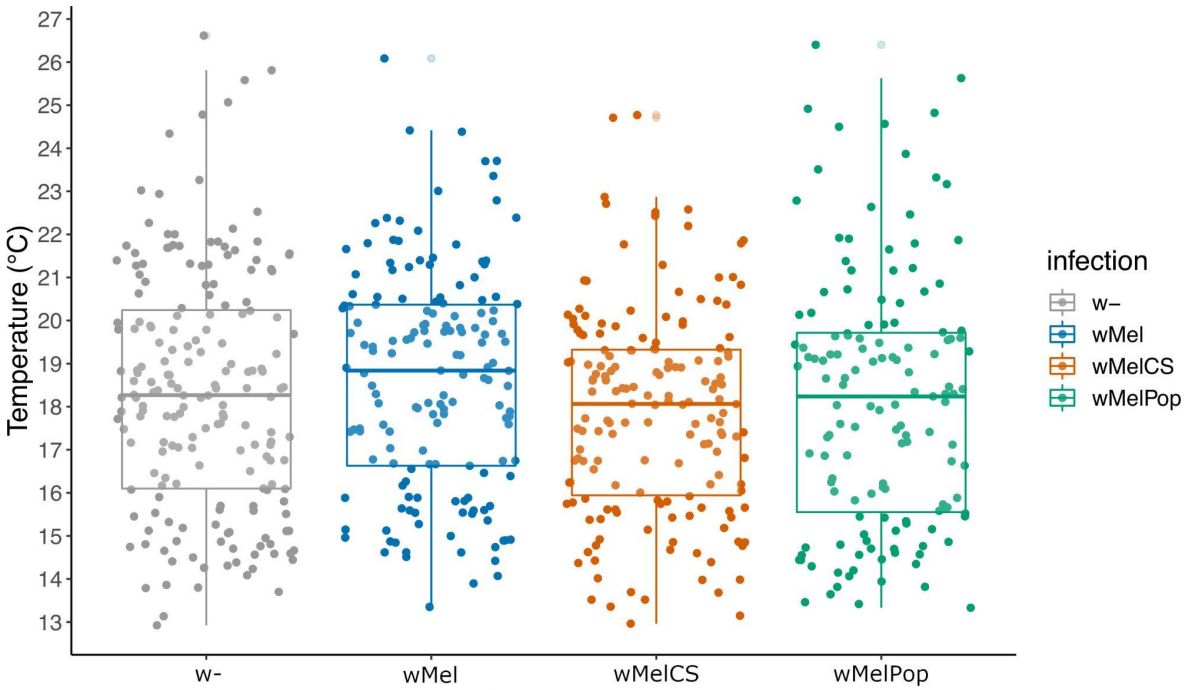

Figure 3. Thermal preference of adult *D. melanogaster* with different infection types.

340 To rule out that the absence of T_p variation among strains infected with different
341 *Wolbachia* variants is an artifact of our experimental setup, we newly built a device similar to
342 the design in Truitt et al. (2019) and repeated the T_p assays. Overall, median T_p was
343 approximately 3°C higher than the measurements from the "plate" device (21.7°C, 22°C,
344 21.5°C and 21.5°C average T_p for *w*-, *w*Mel, *w*MelCS, and *w*MelPop, respectively), which
345 indicates a strong influence of the design on absolute T_p (GLMM: p<2.2e-16, x^2=754.5, df=4;
346 **Supp. Table S1**). Additionally, we found significant differences among infection types
347 (GLMM: p<0.0001, x^2=24.7, df=3), albeit in the opposite direction according to our
348 expectations: the T_p of the *w*Mel variant increased by approximately 1°C compared to *w*-
349 (Tukey's post hoc test: p<0.003). In contrast, *w*MelCS and *w*MelPop variants did not show
350 any effect on T_p compared to the uninfected control lines (Tukey's post hoc test: p>0.05),
351 which is in stark contrast to substantial T_p differences (2-4°C) among variants previously
352 described by Truitt *et al*. (2019) and Arnold *et al.* (2019). Thus, despite using a similar
353 design for the thermal gradient device as in Truitt *et al.* (2019), we were not able to
354 reproduce the previously obtained results. Keeping the lines in the lab for 4-5 years with high
355 inbreeding prior to the repeated thermal gradient assays might have potentially influenced
356 the results through mutation accumulation, titer reduction or other unknown genetic factors
357 (Charlesworth, Willis, 2009). Moreover, differences in the environmental conditions during
358 our experiment and the experiments in Truitt *et al.* (2019) may strongly confound thermal
359 behavior. For example, the experiments in Truitt *et al.* (2019) were neither controlled for light
360 cues nor for the effects of humidity nor ambient temperature variation. While the
361 experiments in Arnold *et al.* (2019) were carried out in complete darkness, these assays
362 were also neither controlled for humidity variation nor ambient temperature variation. Such
363 environmental factors may have a strong influence on behavior during experimental assays,
364 which could potentially overwhelm subtle variation in thermal preference.
365
366 *Humidity, diet and light substantially influence thermal preference of flies independent of*
367 *Wolbachia infection*
368 The analyses presented above show that the design of the thermal gradient device
369 plays an important role in T_p measurements. The tubular device consisted of more spare
370 metal parts than the plate device, which negatively affected the temperature conductivity.
371 Accordingly, we found that the effective temperature range along the gradient was much
372 narrower in the tubular compared to the plate device. The minimum temperature in the tube
373 never decreased below 17°C (maximum temperature: 27°C), whereas the plate reached
374 temperatures as low as 13°C (maximum temperature: 27°C). We therefore tested if the flies
375 were limited by the temperature range and were unable to choose preferred temperatures
376 below the minimum temperature in the tube. In this case, flies may cluster at the lower end

377 of the gradient in the tube device, which would result in T_p distributions skewed towards the
378 lower end, compared to the plate device. However, when comparing the skewness of T_p
379 distributions in replicate experiments among the two devices we did not find significant
380 differences (Wilcoxon Rank test, W=258, p=0.128) which suggests that the temperature
381 range within the tube did not limit the behavior of the test flies.
382 We thus speculate that other environmental factors associated with the design of the
383 devices may strongly affect thermal behavior. According to Dillon *et al.* (2009), sex, age,
384 humidity, light, circadian rhythms, number of individuals tested at once and feeding status all
385 influence thermal behavior. To further investigate how experimental design influences T_p
386 measurement, we compared 15 studies on thermal behavior in *D. melanogaster* flies. This
387 qualitative meta-analysis revealed high variation in measured T_p, which apparently depends
388 on the design of the gradient device, the range or the temperature gradient, rearing and
389 experimental conditions, food, age of flies and number of individuals tested (all summarized
390 in **Supp. Table S2**).
391 To better understand the influence of specific environmental stimuli on thermal
392 preference assays, we experimentally investigated how humidity, food and light affect T_p
393 measurement in the context of varying *Wolbachia* infection status. We observed a highly
394 significant effect of humidity (GLMM: p<2.2e-16, x^2=268.4, df=4; **Figure 4**) on T_p of adult
395 flies. Higher humidity (60%) resulted in increased thermal preferences, elevated by 1-2°C.
396 The median T_p at 30% humidity ranged from 21.5°C (*w*MelCs and *w*MelPop) to 22°C (*w*-),
397 whereas the median T_p at 60% humidity ranged from 22.7°C (*w*-) to 23.5°C (*w*Mel, *w*MelCS).
398 A positive correlation between humidity and T_p was similarly observed already in other
399 insects (Gunn and Cosway, 1938; Deal, 1941).
400 Diet also had a strong influence on thermal behavior. We found that flies on in-house
401 food preferred 1°C lower temperatures than counterparts maintained on Carolina instant
402 food (GLMM: p=8.83e-6, x^2=23.3, df=2; **Figure 4**). Differences in diet quality or feeding
403 status (fed versus fasting) are known to influence thermal preference in ectotherms, where
404 rich food diet increases T_p compared to poor food or fasting conditions (Thomson, 1938;
405 Chapman, 1965; Angilletta Jr. et al., 2002; Pulgar et al., 2003). Our results match these
406 previous findings. While we did not assess the exact nutrient composition in our in-house fly
407 food, the recipe is considered less rich in carbohydrates and proteins than Carolina 4-24
408 instant food. Additionally, diet is also known to affect the titer of *Wolbachia* in different
409 tissues: Protein-rich food has been found to boost titer levels in somatic tissues, but not in
410 ovaries. Conversely, carbohydrate-rich food can increase titer levels in ovaries only (Serbus
411 et al., 2015; Ponton et al., 2015). If variation in titer levels may have a strong influence on
412 thermal preference, as speculated previously (Truitt et al. 2019; Hague et al. 2020), the food
413 quality may indirectly influence T_p by modulating titer levels.

We further observed that light significantly influenced the T_p, albeit to a lower extent (GLMM: p=0.02, x^2=7.9, df=2; **Figure 4**) and only in uninfected flies. Higher light intensities were previously found to shift preferences to cooler temperatures in ectotherms (Regal et al., 1974; Dillon et al., 2009). In contrast, we found that uninfected individuals preferred 1°C higher temperatures under light exposure compared to dark conditions (Tukey's post hoc test: p<0.03). Additionally, we found weak interactions between light and infection variants (GLMM: p<0.03, x^2=4.8, df=1), which may suggest that the presence or absence of light may influence how *Wolbachia* infections influence T_p.

Interestingly, in contrast to the experiments performed in our newly designed "plate" gradient device, the follow-up experiments in the "tube" testing for the effects of light and humidity variation revealed significant effects of *Wolbachia* infections on T_p (see **Supp. Table S1**). However, the observed patterns were opposite to expectations. Flies infected with *w*MelCS preferred warmer temperatures than uninfected flies in both experiments (Tukey's post hoc test: p<0.003; **Table 2**; **Figure 4**). These findings contradict previously observed results where flies infected with *w*MelCS variant preferred lower temperatures than uninfected counterparts (Truitt et al., 2019; Arnold et al., 2019). Our results are, however, in line with the data observed by Hague et al. (2020), showing no shift of preference to higher temperatures in flies artificially infected with *w*MelCS. In summary, these results further suggest that T_p measurement is strongly influenced by the experimental setup and that T_p variation with respect to *Wolbachia* infections is probably very subtle.

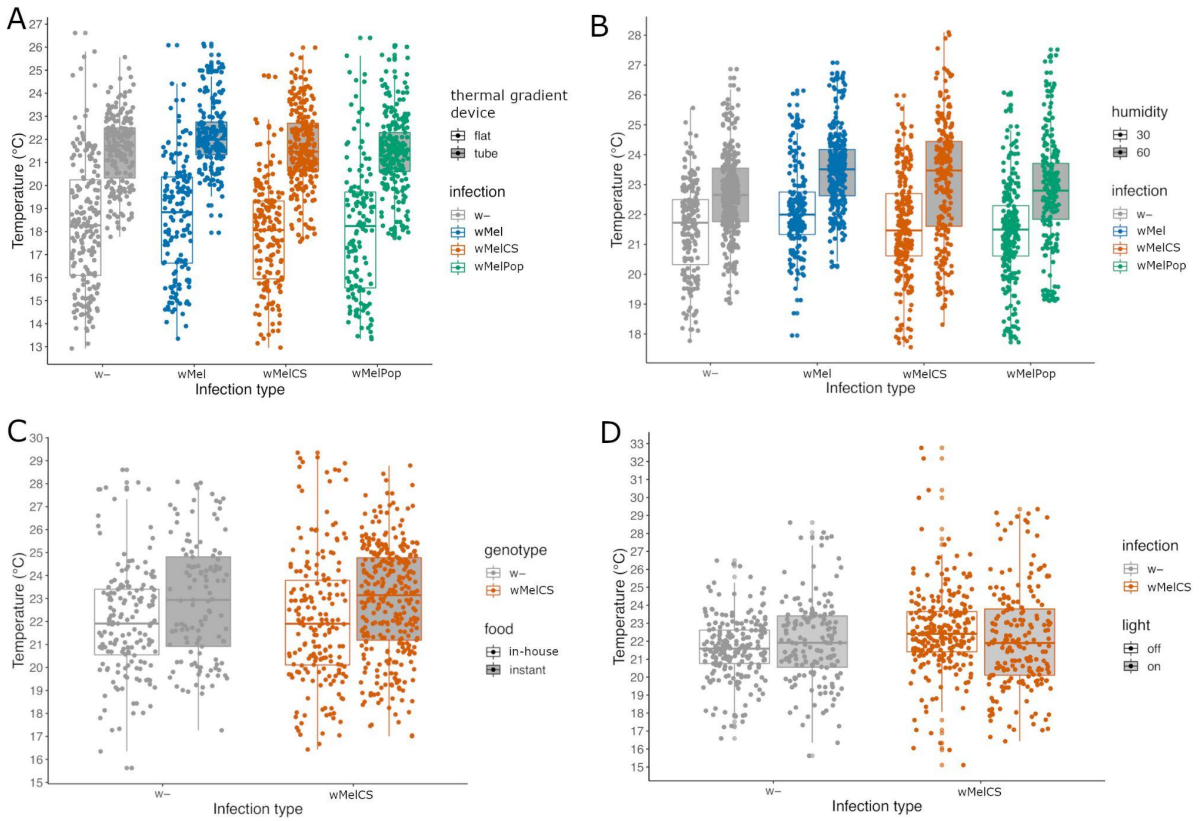

Figure 4. Influence of humidity, the structure of a gradient machine, food, and light on measurement of *Tp* in *D. melanogaster*.

440 **Table 2**. Tukey's post hoc pairwise comparison of *Wolbachia* effects on host T_p.

factor tested	comparison pair	mean *Tp* (°C)	*p* value	influence of high *Wolbachia* titer on *Tp*
"plate" gradient	wMel wMelCS	18.6 17.9	0.02	*Tp*↓
light	w- wMelCS	21.6 22.6	0.003	*Tp*↑
humidity 30%	w- wMel	21.5 22.2	0.002	*Tp*↑
	wMel wMelCS	22.2 21.6	0.003	*Tp*↓
	wMel wMelPop	22.2 21.4	0.001	*Tp*↓
humidity 60%	w- wMel	22.7 23.5	<0.0001	*Tp*↑
	w- wMelCS	22.7 23.2	0.046	*Tp*↑
	wMel wMelPop	23.5 22.8	<0.0001	*Tp*↓

441

442 **Conclusions**

443

444 In our thermal behavior assays we found that *Wolbachia* does not influence thermal
445 preference in early and late 3rd-instar *Drosophila* larvae, consistent with previous studies
446 suggesting that *Wolbachia* has no, or only very weak effects on juvenile traits of *D.*
447 *melanogaster*. We also found no effect of *Wolbachia* on thermal preference in adult *D.*
448 *melanogaster*, which is inconsistent with previous data and indicates that T_p measurement
449 assays are potentially influenced and confounded by uncontrolled environmental cues. Such
450 unwanted behavioral stimuli can overwhelm subtle thermal preference differences during
451 behavioral experiments. As we show in our study, factors such as humidity, light, diet and
452 design of the gradient device substantially influence measurements of thermal preference in
453 *D. melanogaster* infected with different *Wolbachia* variants. Our findings demonstrate the
454 necessity of a careful experimental design and cautious interpretations of T_p measurements
455 together with a thorough description of the methods and equipment used to conduct
456 behavioral studies. Our data and findings thus provide important considerations when
457 planning future behavioral assays to assess thermal preference not only in *Drosophila* but
458 also in other insects infected with *Wolbachia*, such as vectors of human diseases like
459 mosquitoes.

460

Acknowledgments

We are especially grateful to Wolfgang Miller who helped with the design of the experiment, who provided lab space and who conceptually supported this project. We are also indebted to Marcel Freund at the University of Zürich who not only hand-crafted the thermal gradient devices used in this study but also helped with their design. Moreover, we thank Thomas Flatt, Elisabeth Haring and Roman Arguello for helpful comments on previous versions of this manuscript. We are very grateful to Fabian Gstöttenmayr, Janis Jeschgo, Julia Mras, Elina Koivisto and Jasmin Jester who helped with fly maintenance and fly food cooking. This project was funded by a standalone grant from the Austrian Science Fund (FWF P32275) to Martin Kapun.

Authors Contributions

Anton Strunov involved in conceptualization, investigation, data curation, visualization, formal analysis, validation, writing—original draft; Charlotte Schoenherr involved in investigation, data curation and writing—review & editing; Martin Kapun involved in conceptualization, formal analysis, visualization, supervision, funding acquisition, writing—review & editing and project administration.

482 **References**

483

484 Al-Saffar, Z.Y., Grainger, J.N.R., and Aldrich, J. (1996) Temperature and humidity affecting
485 development, survival and weight loss of the pupal stage of Drosophila
486 melanogaster, and the influence of alternating temperature on the larvae. *Journal of*
487 *Thermal Biology* 21: 389–396.
488 Angilletta, M.J., Niewiarowski, P.H., and Navas, C.A. (2002) The evolution of thermal
489 physiology in ectotherms. *Journal of Thermal Biology* 27: 249–268.
490 Angilletta, M.J., Steury, T.D., and Sears, M.W. (2004) Temperature, growth rate, and body
491 size in ectotherms: fitting pieces of a life-history puzzle. *Integr Comp Biol* 44: 498–
492 509.
493 Arnold, P.A., Levin, S.C., Stevanovic, A.L., and Johnson, K.N. (2019) Drosophila
494 melanogaster infected with Wolbachia strain wMelCS prefer cooler temperatures.
495 *Ecological Entomology* 44: 287–290.
496 Bates, D., Mächler, M., Bolker, B., and Walker, S. (2015) Fitting Linear Mixed-Effects Models
497 Using lme4. *Journal of Statistical Software* 67: 1–48.
498 Chapman, R.F. (1965) The Behaviour of Nymphs of Schistocerca Gregaria (Forskål)
499 (Orthoptera, Acrididae) in a Temperature Gradient, With Special Reference To
500 Temperature Preference. *Behaviour* 24: 283–317.
501 Charlesworth, D. and Willis, J.H. (2009) The genetics of inbreeding depression. *Nat Rev*
502 *Genet* 10: 783–796.
503 Chrostek, E., Martins, N., Marialva, M.S., and Teixeira, L. (2021) Wolbachia-Conferred
504 Antiviral Protection Is Determined by Developmental Temperature. *mBio* 12:
505 e02923-20.
506 Complex effects of environment and Wolbachia infections on the life history of Drosophila
507 melanogaster hosts - Strunov - 2022 - Journal of Evolutionary Biology - Wiley
508 Online Library.
509 Curtis, V.A. (2014) Infection-avoidance behaviour in humans and other animals. *Trends*
510 *Immunol* 35: 457–464.
511 Detcharoen, M., Arthofer, W., Jiggins, F.M., Steiner, F.M., and Schlick-Steiner, B.C. (2020)
512 Wolbachia affect behavior and possibly reproductive compatibility but not
513 thermoresistance, fecundity, and morphology in a novel transinfected host,
514 Drosophila nigrosparsa. *Ecol Evol* 10: 4457–4470.
515 Dillon, M.E., Wang, G., Garrity, P.A., and Huey, R.B. (2009) Thermal preference in
516 Drosophila. *Journal of Thermal Biology* 34: 109–119.

517 Fedorka, K.M., Kutch, I.C., Collins, L., and Musto, E. (2016) Cold temperature preference in
518 bacterially infected Drosophila melanogaster improves survival but is remarkably
519 suboptimal. *Journal of Insect Physiology* 93–94: 36–41.
520 Fox J, Weisberg S (2019). *An R Companion to Applied Regression*, Third edition. Sage,
521 Thousand Oaks CA
522 Gilbert, A.L. and Miles, D.B. (2017) Natural selection on thermal preference, critical thermal
523 maxima and locomotor performance. *Proceedings of the Royal Society B: Biological*
524 *Sciences* 284: 20170536.
525 Giraldo, D., Adden, A., Kuhlemann, I., Gras, H., and Geurten, B.R.H. (2019) Correcting
526 locomotion dependent observation biases in thermal preference of Drosophila. *Sci*
527 *Rep* 9: 3974.
528 Gutzwiller, F., Carmo, C.R., Miller, D.E., Rice, D.W., Newton, I.L.G., Hawley, R.S., et al.
529 (2015) Dynamics of Wolbachia pipientis Gene Expression Across the Drosophila
530 melanogaster Life Cycle. *G3 (Bethesda)* 5: 2843–2856.
531 Hague, M.T.J., Caldwell, C.N., and Cooper, B.S. (2020) Pervasive Effects of Wolbachia on
532 Host Temperature Preference. *mBio* 11: e01768-20.
533 Hamada, F.N., Rosenzweig, M., Kang, K., Pulver, S.R., Ghezzi, A., Jegla, T.J., and Garrity,
534 P.A. (2008) An internal thermal sensor controlling temperature preference in
535 Drosophila. *Nature* 454: 217–220.
536 Hart, B.L. (1988) Biological basis of the behavior of sick animals. *Neurosci Biobehav Rev* 12:
537 123–137.
538 Head, L.M., Tang, X., Hayley, S.E., Goda, T., Umezaki, Y., Chang, E.C., et al. (2015) The
539 Influence of Light on Temperature Preference in Drosophila. *Current Biology* 25:
540 1063–1068.
541 Hoffmann, A.A. (2010) Physiological climatic limits in Drosophila: patterns and implications.
542 *Journal of Experimental Biology* 213: 870–880.
543 Hong, S.-T., Bang, S., Paik, D., Kang, J., Hwang, S., Jeon, K., et al. (2006) Histamine and
544 Its Receptors Modulate Temperature-Preference Behaviors in Drosophila. *J*
545 *Neurosci* 26: 7245–7256.
546 Huey, R.B. and Berrigan, D. (2001) Temperature, Demography, and Ectotherm Fitness. *The*
547 *American Naturalist* 158: 204–210.
548 Inglis, G.D., Johnson, D.L., and Goettel, M.S. (1996) Effects of Temperature and
549 Thermoregulation on Mycosis byBeauveria bassianain Grasshoppers. *Biological*
550 *Control* 7: 131–139.
551 Kain, J.S., Zhang, S., Akhund-Zade, J., Samuel, A.D.T., Klein, M., and de Bivort, B.L. (2015)
552 Variability in thermal and phototactic preferences in Drosophila may reflect an
553 adaptive bet-hedging strategy. *Evolution* 69: 3171–3185.

554 Komsta, L., & Novomestky, F. (2022). *moments: Moments, Cumulants, Skewness, Kurtosis*
555 *and Related Tests* (0.14.1). https://CRAN.R-project.org/package=moments
556 MacLean, H.J., Overgaard, J., Kristensen, T.N., Lyster, C., Hessner, L., Olsvig, E., and
557 Sørensen, J.G. (2019) Temperature preference across life stages and acclimation
558 temperatures investigated in four species of Drosophila. *Journal of Thermal Biology*
559 86: 102428.
560 Min, K.-T. and Benzer, S. (1997) Wolbachia, normally a symbiont of Drosophila, can be
561 virulent, causing degeneration and early death. *Proceedings of the National*
562 *Academy of Sciences* 94: 10792–10796.
563 Mirth, C.K., Saunders, T.E., and Amourda, C. (2021) Growing Up in a Changing World:
564 Environmental Regulation of Development in Insects. *Annual Review of*
565 *Entomology* 66: 81–99.
566 Osborne, S.E., Leong, Y.S., O'Neill, S.L., and Johnson, K.N. (2009) Variation in Antiviral
567 Protection Mediated by Different Wolbachia Strains in Drosophila simulans. *PLOS*
568 *Pathogens* 5: e1000656.
569 Pontier, S.M. and Schweisguth, F. (2015) A Wolbachia-Sensitive Communication between
570 Male and Female Pupae Controls Gamete Compatibility in Drosophila. *Curr Biol* 25:
571 2339–2348.
572 Ponton, F., Wilson, K., Holmes, A., Raubenheimer, D., Robinson, K.L., and Simpson, S.J.
573 (2015a) Macronutrients mediate the functional relationship between Drosophila and
574 Wolbachia. *Proceedings of the Royal Society B: Biological Sciences* 282:
575 20142029.
576 Ponton, F., Wilson, K., Holmes, A., Raubenheimer, D., Robinson, K.L., and Simpson, S.J.
577 (2015b) Macronutrients mediate the functional relationship between Drosophila and
578 Wolbachia. *Proceedings of the Royal Society B: Biological Sciences* 282:
579 20142029.
580 Poulin, R. (2010) Chapter 5 - Parasite Manipulation of Host Behavior: An Update and
581 Frequently Asked Questions. In *Advances in the Study of Behavior*. Brockmann,
582 H.J., Roper, T.J., Naguib, M., Wynne-Edwards, K.E., Mitani, J.C., and Simmons,
583 L.W. (eds). Academic Press, pp. 151–186.
584 Pulgar, J.M., Aldana, M., Bozinovic, F., and Ojeda, F.P. (2003) Does food quality influence
585 thermoregulatory behavior in the intertidal fish Girella laevifrons? *Journal of*
586 *Thermal Biology* 28: 539–544.
587 R Core Team (2021). R: A language and environment for statistical computing. R
588 Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-
589 project.org/.

590 Rajpurohit, S. and Schmidt, P.S. (2016) Measuring thermal behavior in smaller insects: A
591 case study in Drosophila melanogaster demonstrates effects of sex, geographic
592 origin, and rearing temperature on adult behavior. *Fly (Austin)* 10: 149–161.
593 Riegler, M., Iturbe-Ormaetxe, I., Woolfit, M., Miller, W.J., and O'Neill, S.L. (2012) Tandem
594 repeat markers as novel diagnostic tools for high resolution fingerprinting of
595 Wolbachia. *BMC Microbiol* 12: S12.
596 de Roode, J.C. and Lefèvre, T. (2012) Behavioral Immunity in Insects. *Insects* 3: 789–820.
597 Russell V. Lenth (2021). emmeans: Estimated Marginal Means, aka Least-Squares Means.
598 R package version 1.6.2-1. https://CRAN.R-project.org/package=emmeans.
599 Sayeed, O. and Benzer, S. (1996) Behavioral genetics of thermosensation and
600 hygrosensation in Drosophila. *Proc Natl Acad Sci U S A* 93: 6079–6084.
601 Serbus, L.R., White, P.M., Silva, J.P., Rabe, A., Teixeira, L., Albertson, R., and Sullivan, W.
602 (2015) The Impact of Host Diet on Wolbachia Titer in Drosophila. *PLOS Pathogens*
603 11: e1004777.
604 Shih, H.-W., Wu, C.-L., Chang, S.-W., Liu, T.-H., Sih-Yu Lai, J., Fu, T.-F., et al. (2015)
605 Parallel circuits control temperature preference in Drosophila during ageing. *Nat*
606 *Commun* 6: 7775.
607 Sokabe, T., Chen, H.-C., Luo, J., and Montell, C. (2016) A Switch in Thermal Preference in
608 Drosophila Larvae Depends on Multiple Rhodopsins. *Cell Reports* 17: 336–344.
609 Sokolowski, M.B., Kent, C., and Wong, J. (1984) Drosophila larval foraging behaviour:
610 Developmental stages. *Animal Behaviour* 32: 645–651.
611 Stevenson, R.D. (1985) The Relative Importance of Behavioral and Physiological
612 Adjustments Controlling Body Temperature in Terrestrial Ectotherms. *The American*
613 *Naturalist* 126: 362–386.
614 Strunov, A., Kiseleva, E., and Gottlieb, Y. (2013) Spatial and temporal distribution of
615 pathogenic Wolbachia strain wMelPop in Drosophila melanogaster central nervous
616 system under different temperature conditions. *Journal of Invertebrate Pathology*
617 114: 22–30.
618 Strunov, A., Lerch, S., Blanckenhorn, W.U., Miller, W.J., and Kapun, M. (2022) Complex
619 effects of environment and Wolbachia infections on the life history of Drosophila
620 melanogaster hosts. *Journal of Evolutionary Biology* 35: 788–802.
621 Strunov, A.A., Ilinskii, Yu.Yu., Zakharov, I.K., and Kiseleva, E.V. (2013) Effect of high
622 temperature on survival of Drosophila melanogaster infected with pathogenic strain
623 of Wolbachia bacteria. *Russ J Genet Appl Res* 3: 435–443.
624 Teixeira, L., Ferreira, Á., and Ashburner, M. (2008) The Bacterial Symbiont Wolbachia
625 Induces Resistance to RNA Viral Infections in Drosophila melanogaster. *PLOS*
626 *Biology* 6: e1000002.

627 Thomson, R.C.M. (1938) The Reactions of Mosquitoes to Temperature and Humidity.
628 *Bulletin of Entomological Research* 29: 125–140.
629 Truitt, A.M., Kapun, M., Kaur, R., and Miller, W.J. (2019) Wolbachia modifies thermal
630 preference in Drosophila melanogaster. *Environmental Microbiology* 21: 3259–
631 3268.
632 Ueno, T., Tomita, J., Kume, S., and Kume, K. (2012) Dopamine Modulates Metabolic Rate
633 and Temperature Sensitivity in Drosophila melanogaster. *PLoS One* 7: e31513.
634 Watson, D.W., Mullens, B.A., and Petersen, J.J. (1993) Behavioral Fever Response of
635 Musca domestica (Diptera: Muscidae) to Infection by Entomophthora muscae
636 (Zygomycetes: Entomophthorales). *Journal of Invertebrate Pathology* 61: 10–16.
637 Yamamoto, A. and Ohba, S. (1984) Temperature preferences of 11 drosophila species from
638 japan the relationship between preferred temperature and some ecological
639 characteristics in their natural habitats. *Zoological Science* 1: 631–640.
640 Zhou, W., Rousset, F., and O'Neill, S. (1998) Phylogeny and PCR–based classification of
641 Wolbachia strains using wsp gene sequences. *Proceedings of the Royal Society of*
642 *London Series B: Biological Sciences* 265: 509–515.
643

Supplementary Material

Supp. Table S1. *Tp* of *D. melanogaster* under the influence of various environmental factors. Significant results are highlighted in bold.

Supp. Table S2. Observations of T_p from literature with detailed information about physical parameters and environmental conditions used in a study.

Supp. raw data table. Strunov_etal_WolbTP_2023_RawData.xlsx

Supp. *R* code. Strunov_etal_WolbTP_2023_Rcode.zip