1   ***Wolbachia* modifies thermal preference in *Drosophila melanogaster***

2

3   Amy M. Truitt[1], Martin Kapun[2], Rupinder Kaur[3], Wolfgang J. Miller[3*]

4

5   1.   Department of Environmental Science & Management, Portland State University,

6   Portland Oregon, USA, amtruitt@pdx.edu

7   2.   Department of Biology, Université de Fribourg, Switzerland, martin.kapun@unifr.ch

8   3.   Department of Cell & Developmental Biology, Medical University of Vienna, Vienna,

9   Austria, rupinder.kaur@meduniwien.ac.at, wolfgang.miller@meduniwien.ac.at

10

11

12

13   *Corresponding author: Wolfgang J. Miller, email: wolfgang.miller@meduniwien.ac.at, Tel:

14   +43 1 40160 37750, Fax: +43 1 40160 37790, address: Department of Cell & Developmental

15   Biology,   Center   of   Anatomy   and   Cell   Biology, Medical   University   of

16   Vienna, Schwarzspanierstrasse 17, A-1090 Vienna, Austria.

17

18

19

20

21   Running Title: *Wolbachia* and host thermal preferences

22 **ABSTRACT**

23

24 Environmental variation can have profound and direct effects on fitness, fecundity, and host-

25 symbiont interactions. Replication rates of microbes within arthropod hosts, for example, are

26 correlated with incubation temperature but less is known about the influence of host-symbiont

27 dynamics on environmental preference. Hence, we conducted thermal preference (T_p) assays

28 and tested if infection status and genetic variation in endosymbiont bacterium *Wolbachia*

29 affected temperature choice of *Drosophila melanogaster*. We demonstrate that isogenic flies

30 infected with *Wolbachia* preferred lower temperatures compared to uninfected *Drosophila*.

31 Moreover, T_p varied with respect to three investigated *Wolbachia* variants (*w*Mel, *w*MelCS

32 and *w*MelPop). While uninfected individuals preferred 24.4°C, we found significant shifts of -

33 1.2°C in *w*Mel- and -4°C in flies infected either with *w*MelCS or *w*MelPop. We, therefore,

34 postulate that *Wolbachia*-associated T_p variation within a host species might represent a

35 behavioral accommodation to host-symbiont interactions and trigger behavioral self-

36 medication and bacterial titer regulation by the host.

37

38   **INTRODUCTION**

39   Environmental variations through intrinsic (e.g. physiology, reproduction, metabolism) and

40   extrinsic (e.g. food sources, predation risk, immunity) factors impose a strong impact on the

41   fitness of all organisms (e.g., Levins 1968; Endler 1977; 1986; Fox *et al.* 2001). Temperature

42   is one of the most important environmental abiotic factors that affect the physiology and life

43   history traits in many organisms (Huey and Berrigan 2001; Hoffmann 2010; Bozinovic *et al.*

44   2011; Amarasekare and Savage 2012). Ectotherms, such as terrestrial insects, depend on

45   ambient conditions to maintain their body temperature within a thermoregulatory range

46   (Angilletta *et al.* 2004). For example, thermal preference (T_p) in *Drosophila melanogaster,* a

47   dipteran model species of world-wide distribution*,* varies with geography and elevation, and

48   is thus potentially shaped by selection (Martin and Huey 2008; Dillon *et al.* 2009; Garrity *et*

49   *al.* 2010; Hoffmann & Sgrò 2011; Huey *et al.* 2012; Rajpurohit and Schmidt 2016). In

50   addition, variation in temperature can have fundamental effects on ecological interactions

51   among organisms and their symbiotic microbes. Titers of endosymbiotic *Wolbachia* bacteria

52   are highly temperature-dependent in various arthropod hosts. For example, some *Wolbachia*

53   strains have increased replication rates at warmer temperatures (Clancy and Hoffmann 1998;

54   Hurst *et al.* 2000; Mouton *et al.* 2006; Correa and Ballard 2012; Strunov *et al.* 2013a), while

55   others are highly sensitive to heat stress (van Opijnen and Breeuwer 1999;

56   Wiwatanaratanabutr and Kittayapong 2009).

57   Endosymbionts of the genus *Wolbachia* are widespread and found in more than 50% of all

58   investigated terrestrial and some aquatic insects (Zug and Hammerstein 2012; Weinert *et al.*

59   2015; Sazama *et al.* 2017). *Wolbachia* have garnered extensive interest due to reproductive

60   manipulations they can inflict on their hosts, i.e., inducing parthenogenesis, male killing,

61   feminization, and cytoplasmic incompatibility (CI). By acting as reproductive parasites these

62   bacteria boost their own transmission (reviewed by Werren *et al.* 2008). However, *Wolbachia*

63   can also behave as facultative or obligate mutualists (reviewed by Zug and Hammerstein

64   2015) by enhancing host fecundity and fitness (Dedeine *et al.* 2001; Hosokawa *et al.* 2010;

65   Miller *et al.* 2010) and by providing protection against RNA viruses (Hedges *et al.* 2008;

66   Teixeira *et al.* 2008; Moreira *et al*. 2009; Osborne *et al*. 2009). Several closely related genetic

67   variants of *Wolbachia* have been isolated from natural and laboratory populations of *D.*

68   *melanogaster. w*Mel, *w*MelCS, and *w*MelPop, which represent three of the most well-studied

69   *Wolbachia* variants in *D. melanogaster* (Riegler *et al.* 2005), cause very weak, if any, CI in

70   their native host (Hoffmann 1988; Reynolds *et al*. 2003; Veneti *et al*. 2003; Fry *et al*. 2004;

71   Yamada *et al.* 2007), but provide virus protection to varying degrees (Chrostek *et al*. 2013;

72   Martinez *et al*. 2014). Both *w*Mel and *w*MelCS infect natural populations of *D. melanogaster.*

73   Historically, *w*MelCS existed globally at higher prevalence, but in the recent past *w*Mel has

74   almost completely replaced the more ancestral *w*MelCS strain in world-wide populations

75   (Riegler *et al*. 2005; Nunes *et al.* 2008; Richardson *et al*. 2012; Ilinsky 2013; Early and Clark

76   2014). In contrast, *w*MelPop was isolated from a laboratory stock of *D. melanogaster* during a

77   survey of genetic mutations and represents a pathogenic variant of *w*MelCS (Min and Benzer

78   1997; Richardson *et al.* 2012; Chrostek *et al.* 2013). Depending on rearing temperature,

79   *w*MelPop infections can lead to a strong reduction of host lifespan with respect to uninfected

80   controls (Min and Benzer 1997; McGraw *et al*. 2002; Reynolds *et al.* 2003; Chrostek *et al.*

81   2013). This detrimental effect is caused by over-proliferation in host tissues, such as the brain,

82   retina, and muscles (Min and Benzer 1997; Strunov *et al*. 2013b). Importantly, not only

83   *w*MelPop but also its natural predecessor *w*MelCS have significantly higher cellular densities

84   and growth rates than *w*Mel when assayed in the same fly genetic background at 25°C (**Table**

85   **1;** Chrostek *et al.* 2013). While high *Wolbachia* densities result in augmented antiviral

86   protection, they also have negative effects by reducing their host's lifespan. Accordingly, it

87   has been proposed that the higher titer - and hence more costly - *w*MelCS variant was

88   replaced by the low-titer *w*Mel variant in natural *D. melanogaster* populations (Chrostek *et al*.

89   2013). Thereby, flies infected with the more recent *w*Mel variant have higher fitness due to

90   lower *Wolbachia* titers compared to flies infected with *w*MelCS. Alternatively, the highly

91   protective *w*MelCS variant may have been replaced by *w*Mel independent of the symbiont´s

92   capacity for virus resistance but because of better adaptation to viruses at the host level

93   (Martins *et al.* 2014). In line with this hypothesis, a recent study failed to find correlations

94   between RNA virus prevalence and *Wolbachia* frequency in natural populations of *D.*

95   *melanogaster* (Webster *et al.* 2015). However, the main causalities explaining the well-

96   documented global almost complete replacement of *w*MelCS by *w*Mel in worldwide

97   populations of *D. melanogaster* remains elusive.

98   Host-symbiont conflicts may arise from disparities between physiological requirements of

99   *Wolbachia* and those of their hosts. For example, some insects induce behavioral fever (Louis

100   *et al*. 1986) or behavioral chill (Fedorka *et al.* 2016) as an immune strategy to fight bacterial

101   pathogen infections. Conversely, some bacterial symbionts are known to alter their host's

102   thermal tolerance range in an adaptive manner (Russell and Moran 2006; Dunbar *et al*. 2007;

103   reviewed by Wernegreen 2012). We, therefore, speculate that additional ecological and

104   behavioral factors, such as host temperature preference, may play a pivotal role in

105   determining *Wolbachia* prevalence and the dynamics of their strain replacement in natural *D.*

106   *melanogaster* populations.

107   To test our hypothesis, we conducted laboratory-based temperature preference assays using

108   isogenic *D. melanogaster* w^{1118} strains that are either uninfected (*w*-) or infected with one of

109   the three common *Wolbachia* strains *w*Mel, *w*MelCS_b, and *w*MelPop (Teixeira *et al.* 2008;

110   Chrostek *et al.* 2013) and determined if *Wolbachia* affects the temperature preference of its

111   native host *D. melanogaster*. To this end, we built a custom thermal gradient apparatus and

112   determined the temperature preference of replicated fly populations with varying *Wolbachia*

113   infection statuses along the thermal gradient ranging from 17°C to 32°C. Our experiments

114   demonstrate that the temperature preference of *D. melanogaster* is neither sex- nor age-

115   dependent, but is highly dependent on the *Wolbachia* infection status and on the symbiont

116   genotype. Our results provide compelling evidence that *Wolbachia* infections can affect host

117   thermal preference behavior, at least under strict laboratory conditions in *D. melanogaster*

118   strains.

119

120   **RESULTS**

121   To determine whether T_p of adult *D. melanogaster* varies with *Wolbachia* infection status and

122   *Wolbachia* genotype, we conducted lab-based experiments using a custom-built temperature

123   gradient apparatus for assaying flies of the isogenic lab-strain w^{1118} that were either

124   uninfected (*w-*) or infected with one of the *Wolbachia* strains *w*Mel, *w*MelCS, or *w*MelPop

125   (**Supporting Information Fig. S1-4**). We first investigated whether age (3-4, 5-7 or 10-14

126   days post eclosion) and *Wolbachia* infections, or sex (males or females) and *Wolbachia*

127   infections had an influence on T_p by means of two-way mixed-effect Poisson regressions. We

128   neither found significant effects of age or sex nor significant interactions of either factor with

129   *Wolbachia* infections (see **Fig. 1A+B** and **Table 2A+B** and **Supporting Information Fig.**

130   **S5**; Poisson regression: $P > 0.05$ for factors age and sex and both interaction terms,

131   respectively). In contrast, both two-way regressions revealed highly significant effects of

132   *Wolbachia* infections on T_p (Poisson regression $P < 0.001$ for factor *Wolbachia* in both

133   analyses). Since both aforementioned analyses were carried out on different subsets of the

134   data which did not include all four infection types (*w-*, *w*Mel, *w*MelCS and *w*MelPop), we

135   further investigated all data jointly irrespective of sex and host age and evaluated the effect of

136   symbiont genetic variation on T_p by means of post-hoc pairwise comparisons based on

137   Tukey's honestly significant differences (HSD). We found that temperature preference of *D.*

138   *melanogaster* strongly depended on (1) the infection status of the flies and (2) on the

139   *Wolbachia* strain used for infections: Uninfected flies (*w-*) exhibited the highest mean T_p at

140   24.4°C (Median: 25°C; Mode: 26°C), while *w*Mel-infected flies preferred average

141   temperatures at 23.2°C (Median: 24°C; Mode: 24°C), which is 1.2°C lower than uninfected

142 (*w*-) flies. In contrast, flies infected with *w*MelCS or *w*MelPop showed highly similar thermal

143 preferences at 20.6°C and 20.5°C (Median: 19°C and Mode: 18°C for both) respectively,

144 which were both approximately 4°C lower than to *w*- (see **Fig. 1C, Table 2C** and **Table 3**).

145

146 **DISCUSSION**

147 In this study, we, for the first time, investigated the relationship between temperature

148 preference of *D. melanogaster* and *Wolbachia* infection under laboratory conditions. Using a

149 custom-built thermal gradient apparatus, we conducted temperature preference assays and

150 showed that the T_p of *D. melanogaster* is shifted to lower temperatures when flies are infected

151 with *Wolbachia*. Uninfected *D. melanogaster* flies preferred an average temperature of

152 24.4°C, whereas *w*Mel-infected flies preferred 23.2°C, and both *w*MelCS- and *w*MelPop-

153 infected flies preferred 20.6°C and 20.5°C respectively.

154 T_p can vary significantly between populations of the same species (Matute *et al*. 2009;

155 Rajpurohit and Schmidt 2016) and can have profound effects on immune function, fitness,

156 and fecundity (Huey and Berrigan 2001; Martin and Huey 2008; Hoffmann 2010). Recent

157 population analyses of *Wolbachia* and mitochondria from *D. melanogaster* have provided

158 evidence that over the past few thousand years, the *w*MelCS variant is being globally replaced

159 by the *w*Mel -variant (Riegler *et al*. 2005; Nunes *et al*. 2008; Richardson *et al*. 2012; Early

160 and Clark 2013). Rare cases of the *w*MelCS infection type were recently detected in the wild

161 (Nunes *et al.* 2008; Ilinsky 2013), thus replacement by *w*Mel is still incomplete. Although the

162 reason for the worldwide turn-over remains elusive, it has been hypothesized that *w*Mel,

163 which persists in hosts at significantly lower densities than *w*MelCS at 25°C (Chrostek *et al*.

164 2013), has better adapted to *D. melanogaster*. Accordingly, *w*Mel infections are less costly to

165 the host compared to the more ancestral *w*MelCS variant (Chrostek et al. 2013; reviewed by

166 Miller 2013).

167   Insects can actively reduce or avoid costs of potentially fitness-reducing symbionts or

168   parasites by behavioral adjustments such as changing egg deposition (Kacsoh *et al.* 2013) or

169   mating behavior (reviewed by Wedell 2013). We find compelling evidence for *Wolbachia*-

170   induced behavioral changes in host T_p, which may provide an alternative explanation for the

171   recent global replacement of *w*MelCS by *w*Mel independent of density costs or anti-viral

172   effects: we propose that *w*Mel is less costly for the host than *w*MelCS-infections because flies

173   harboring *w*Mel exhibit thermal preferences that are closer to uninfected flies under natural

174   conditions compared to flies infected with *w*MelCS. Since *Drosophila* development is strictly

175   temperature dependent (approximately 14 days of egg-to-adult development at 20°C and 9

176   days at 24°C; Ashburner 1989), flies infected with *w*Mel should have shorter generation times

177   and thereby produce more generations per year resulting in higher net fecundity compared to

178   *w*MelCS infected flies.

179   Small fluctuations in temperature can cause considerable modifications to host-symbiont

180   interactions (Blanford and Thomas 1999). Pathogenicity of *w*MelPop is attributed to its active

181   proliferation in host tissues at temperatures ≥ 19°C. The increase of *w*MelPop density confers

182   strong anti-viral protection but leads to a significant reduction in host lifespan at 25°C

183   (Chrostek *et al.* 2013). However, at temperatures < 19°C, pathogenicity of *w*MelPop is

184   eliminated (Reynolds *et al.* 2003). Similarly, but less dramatically *w*MelCS, the progenitor of

185   *w*MelPop, is also costly by reducing host lifespan due to high symbiont densities at 25°C

186   (Chrostek *et al.* 2013). We, therefore, speculate that the adjustment of lower temperature

187   preference in *D. melanogaster* as a response to the *w*MelCS and *w*MelPop infections

188   represents a physiological self-medicating behavior or behavioral chill (Fedorka *et al.* 2016)

189   to attenuate the fitness costs associated with deleterious effects of *Wolbachia* over-

190   proliferation and high cell densities (Chrostek *et al.* 2013; Strunov *et al.* 2013a; Strunov *et al.*

191   2013b).

192   *Wolbachia*'s ability to provide anti-viral protection to their hosts has emerged as the most

193   promising approach to combatting insect-vector borne pathogens that pose serious health risks

194   to humans, such as dengue fever and Zika (Moreira *et al.* 2009; Iturbe-Ormaetxe *et al.* 2011;

195   Dutra *et al.* 2016). However, because the strength of anti-viral protection is associated with

196   higher *Wolbachia* densities (Chrostek *et al.* 2013; Martinez *et al.* 2014) and bacterial titers are

197   a temperature sensitive trait (Hoffmann *et al.* 1990; Reynolds *et al.* 2003; Mouton *et al.* 2006;

198   Mouton *et al.* 2007; Bordenstein & Bordenstein 2011; Correa and Ballard 2012; Chrostek *et*

199   *al.* 2013; Strunov *et al.* 2013a; Strunov *et al.* 2013b; Murdock *et al.* 2014; Versace *et al.*

200   2014), it is feasible that under certain thermal conditions such as lower environmental

201   temperatures, *Wolbachia*-induced virus protection could be attenuated or absent (Chrostek

202   2014). Furthermore, our findings, as demonstrated in a highly inbred lab strain of *D.*

203   *melanogaster,* need to be tested first in different host backgrounds, which are naturally or

204   artificially infected with the endosymbiont.

205   In conclusion, we present experimental support for a potential ecological conflict between

206   host and symbiont that may have profound effects on host physiology. Our results provide a

207   novel conceptual platform from which to further investigate host temperature preference, or

208   behavioral chill, in other *Wolbachia*-infected insect hosts. Future studies should examine if

209   host temperature preference has a direct impact on *Wolbachia* density regulation.

210   Additionally, it is important to determine any effects that host T_p has on the strength of anti-

211   viral protection that *Wolbachia* provide to some hosts.

212

213   **EXPERIMENTAL PROCEDURES**

214   **Fly Lines**

215   For all assays, we used *D. melanogaster* without *Wolbachia* (*w*-) as well as flies infected

216   with one of three genetic variants of the *Wolbachia* *w*Mel-strain; *w*Mel, *w*MelCS_b, and

217 *w*MelPop all set in the DrosDel w^{1118} isogenic background, which were kindly provided by

218 Luis Teixeira and previously described by Teixeira *et al.* (2008) and Chrostek *et al.* (2013).

219   We used biological replicates of approximately 30 flies per vial, independently rearing

220 each vial of flies at 25°C, in a 12:12 light - dark cycle with constant 45% humidity. Flies were

221 raised on *Drosophila* Formula 4-24® Instant Medium (Carolina®, NC) that was

222 supplemented with fresh yeast. Approximately equal numbers of male and female flies were

223 used in each assay except for assays that explicitly tested sex-class T_p differences (see

224 **Supporting Information Table S1 and Supporting Information File 1**). In addition to

225 testing for sex-class T_p differences, we performed assays to test for age-specific T_p

226 differences, thus all fly lines were segregated into three age-classes – 3-4 days, 5-7 days, and

227 10-14 days post eclosion. Due to fitness costs to the host associated with infection by

228 *w*MelPop at 25°C, possibly due to the onset of the life reducing phenotype (Min and Benzer

229 1997) or increase in copy numbers of the Octomom repeat (Chrostek and Teixeira 2015), our

230 *w*MelPop-infected fly line did not produce enough flies to conduct all three age-class assays.

231 Therefore, we excluded *w*MelPop from the statistical analyses of age-specific effects (see

232 **Supporting Information Table S1** and the description of statistical analyses).

233

234 **Genotyping of *Wolbachia* strains**

235   Genome sections that contain hypervariable loci or hypervariable regions covering tandem

236 repeats were used as genetic markers to differentiate *Wolbachia* strains and strain variants

237 (O'Neill *et al.* 1992; Werren *et al.* 1995; Zhou *et al.* 1998; Riegler *et al.* 2012). To confirm

238 *Wolbachia*-infection status, we performed diagnostic PCR amplification using primers for a

239 gene that encodes the *Wolbachia* surface protein, *wsp* (Jeyaprakash and Hoy 2000), and for an

240 intergenic region with 141bp tandem repeats, VNTR-141 loci (Riegler *et al.* 2005). The PCR

241 reactions for *wsp* amplification were carried out in a total volume of 10µl containing 2µl

242 Promega 5x Green GoTaq buffer, 4mM Promega MgCl$_2$, 0.8µM of forward and reverse

243   primers, 35µM of each dNTP, 0.04 U Promega GoTaq DNA Polymerase, and 1µl of genomic

244   DNA template. Diagnostic VNTR-141 PCR reactions were each a total of 10µl comprised of

245   the following: 2µl Promega 5x Green GoTaq buffer, 1.5mM Promega $MgCl_2$, 0.3µM of

246   forward and reverse primers, 35µM of each dNTP, 0.04 U Promega GoTaq DNA Polymerase,

247   and 1µl of genomic DNA template. PCR products were visualized on a 1% agarose gel.

248   Presence/absence of the *wsp* signal and the size of the diagnostic VNTR-141 locus confirmed

249   their respective infection type (Riegler *et al.* 2012). The proper infection status of the

250   *w*MelPop isoline was verified by assaying flies for early mortality at 29°C.

251

252   **Thermal gradient apparatus**

253   Temperature preference assays were performed using a custom made thermal gradient

254   apparatus that allowed the flies to move in a three-dimensional space (adapted from

255   Rajpurohit and Schmidt 2016; **Supporting Information Fig. S2**). An aluminum rod (length

256   74.93cm, diameter 3.02cm; Part #R31-316 Metals Depot, Winchester, KY) was encased

257   within a 58.76cm long and 6.35cm inside diameter polycarbonate tube, creating an enclosed

258   chamber allowing for three-dimensional movement. Constant voltage was applied to Peltier

259   devices on each end of the aluminum rod to create a temperature gradient inside the thermal

260   preference chamber. Temperatures along the gradient were measured at seven points that

261   were 8.39cm apart using K-type thermocouples and two four-channel thermocouple recorders.

262   We recorded temperatures on the aluminum rod and inside polycarbonate tube surfaces

263   (bottom, top, and mid-point between the top and bottom surfaces; **Supporting Information**

264   **Fig. S3**). The average temperatures from each thermocouple point on all surfaces from 57

265   different assays are depicted in **Supporting Information Fig. S1**. Mean temperatures

266   increased linearly and ranged from 12°C at the coldest point to 40°C at the hottest point of the

267   aluminum rod, 58.76 cm distance (**Supporting Information Fig. S4**). Along the aluminum

268   rod, for every 4.2cm from cold to hot, the temperature increased by 2°C. Temperatures along

269   each of the measured polycarbonate tube surfaces (bottom, mid-point, and top) increased 1°C

270   every 4.2cm from cold to hot. The gradient reached thermal stability after approximately 20

271   minutes and remained stable for at least 3 hours. Assays were conducted once the device had

272   attained thermal stability.

273

274   **Thermal preference assays**

275   All assays were conducted in a room with a constant temperature of 24°C and constant

276   40% humidity. During several trial runs, we established that 75-100 flies for each assay

277   resulted in distributions along the thermal gradient that avoided over-crowding in preferred

278   temperature ranges, eliminating potential counting errors during analysis. Flies were

279   introduced by aspiration into the thermal gradient chamber through a small hole located

280   halfway along the top of the polycarbonate tube, where the temperature consistently averaged

281   25°C. Flies used for thermal preference assays were never anesthetized because of the strong

282   effects from CO_2 treatment on *Drosophila* behavior (Barron 2000). Each assay was conducted

283   for thirty minutes. Between assays, the temperature gradient chamber was taken apart and

284   thoroughly cleaned to avoid contamination from any pheromone particles. All aluminum parts

285   were cleaned using 95% ethanol. Because ethanol and polycarbonate are chemically

286   incompatible, the polycarbonate tube and end caps were cleaned using hot water and soap,

287   followed by a four-minute rinse with hot water to ensure that surfaces were free of soap

288   residue.

289

290   **Data collection**

291   Using three GoPro HERO3+ cameras, we collected data for each assay in the form of

292   digital images. To capture images of the entire thermal gradient and the flies within it, we

293   mounted the cameras above, lateral to, and below the apparatus, capturing images every 30

294   seconds for the duration of each treatment (30 minutes). Images were analyzed using Adobe

295   Photoshop CS6. All 60 images from each assay were reviewed, from which we determined

296   that A) the flies were highly active, retaining the ability to relocate as necessary, for the entire

297   assay, and B) after being introduced to the thermal gradient, actively flew around for up to 15

298   mins before they settled on either the aluminum rod or polycarbonate tube surfaces.

299   Therefore, we selected images for analysis of fly distribution at the 20-minute time point as

300   representative of the 30-minute experiment. For each assay, we manually counted flies and

301   marked the location of flies on a custom grid that delineated gradient surfaces and surface

302   temperatures.

303

304   **Statistical analyses**

305   We calculated generalized linear mixed models (GLMM) with a Poisson error structure

306   using the *R* (R Development Core Team 2009) package *lme4* (Bates *et al.* 2015) to account

307   for the statistical properties of count data from flies observed at different temperatures. To test

308   for significance of a given predictor variable, we compared the full model including all

309   factors to a reduced model excluding the given factor by analysis of deviance with χ^2 tests

310   using the *R* function *anova* (see **Supporting Information File 1** for full *R* code).

311   At first, we excluded flies infected with *w*MelPop, since we failed to obtain sufficient flies

312   to test for age-specific T_p at all three age-classes (3-4 days, 5-7 days and 10-14 days post

313   eclosion; **Supporting Information Table S1**) and tested for age- and *Wolbachia*-specific

314   differences in thermal preference with a two-way GLMM of the form: $T_i = wol + age + wol \times$

315    $age + Rep + \varepsilon_i$. Here, *T* is the continuous response variable "Temperature", *age* is a nominal

316   fixed factor with three levels each (*age*: 3-4 days, 5-7 days and 10-14 days post eclosion), *wol*

317   is a nominal fixed factor "*Wolbachia*" with three levels (un-infected, *w*Mel and *w*MelCS), *wol*

318    $\times$ *age* is the interaction term, *Rep* is a nominal random factor "Replicate" for replicate trials

319   and ε_i is the error (**Table 2A, Fig. 1A**). In a complementary analysis, we removed all flies of

320   the age class 3-4 days and repeated the abovementioned analysis including all *Wolbachia*

321   strains on two age classes (5-7 days and 10-14 days post eclosion) only. This latter analysis

322   yielded qualitatively similar results to the former analysis including all age classes without

323   *w*MelPop (**Supporting Information Table S2**).

324   Next, we censored flies with undetermined sex status and excluded uninfected flies (*w*-),

325   since we failed to obtain sufficient replication to test for male-specific T_p for uninfected flies

326   (**Supporting Information Table S1**). We then tested for sex- and *Wolbachia*-specific

327   differences in thermal preference with a two-way GLMM of the form: $T_i = wol + sex + wol \times$

328     $sex + Rep + \varepsilon_I$ Here, *T* is the continuous response variable "Temperature", *sex* is a nominal

329   fixed factor with two levels (male and female), *wol* is a nominal fixed factor "*Wolbachia*"

330   with three levels (*w*Mel, *w*MelCS, and *w*MelPop), *wol × age* is the interaction term, *Rep* is a

331   nominal random factor "Replicate" for replicate trials and ε_I is the error (**Table 2B; Fig. 1B**).

332   Finally, we included all flies, irrespective of age and sex status, and tested for the effect of

333   infection status and *Wolbachia* strain variation on thermal preference with a GLMM of the

334   form: $T_i = wol + Rep + \varepsilon_i$, where *T* is the continuous response variable "Temperature", *wol* is

335   a nominal fixed factor "*Wolbachia*" with four levels (un-infected, *w*Mel, *w*MelCS, and

336   *w*MelPop), *Rep* is the nominal random factor "Replicate" and ε_i is the error (**Table 2C; Fig.**

337   **1C**). Here, we further tested for significant pair-wise comparisons among the level of the

338   factor "*Wolbachia*" with Tukey's honestly significant difference (HSD) post-hoc tests using

339   the *R* package *multcomp* (**Table 3**). We conservatively applied Bonferroni corrections to the *α*

340   threshold (*α'*= 0.05/3 = 0.017) to account for multiple testing.

341

346

Author contributions:

348   A.T. and W.J.M. conceived and planned the study, A.T. performed the experiment, A.T.,

349   R.K., and M.K. analyzed the data and A.T., W.J.M., and M.K. wrote the paper.

350

Funding Source

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.org10.1086/662551

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

381   Chrostek, E.A. (2014) Genomic and environmental factors influence *Wolbachia-Drosophila*

382   symbiosis. Chapter 5: Temperature dependence of *Wolbachia*-conferred antiviral

383   protection. Dissertation. Instituto de Tecnologia Química e Biológica.

384   https://run.unl.pt/handle/10362/14860

385   Chrostek, E. and Teixeira, L. (2015) Mutualism Breakdown by Amplification of *Wolbachia*

386   Genes. *PLoS Biol* **13:** e1002065. http://doi.org/10.1371/journal.pbio.1002065

387   Clancy, D.J. and Hoffmann, A.A. (1998) Environmental effects on cytoplasmic

388   incompatibility and bacterial load in *Wolbachia* infected *Drosophila simulans*. *Entomol*

389   *Exp Appl* **86:** 13–24. http://doi.org10.1046/j.1570-7458.1998.00261.x

390   Correa, C.C. and Ballard, J.W.O. (2012) *Wolbachia* gonadal density in female and male

391   *Drosophila* vary with laboratory adaptation and respond differently to physiological and

392   environmental challenges. *J Invertebr Pathol* **111**: 197–204.

393   http://doi.org/10.1016/j.jip.2012.08.003

394   Dedeine, F., Vavre, F., Fleury, F., Loppin, B., Hochberg, M.E. and Boulétreau, M. (2001)

395   Removing symbiotic *Wolbachia* bacteria specifically inhibits oogenesis in a parasitic

396   wasp. *Proc Natl Acad Sci U S A* **98:** 6247–6252. http://doi.org/10.1073/pnas.101304298

397   Dillon, M.E., Wang, G., Garrity, P.A., and Huey, R.B. (2009) Thermal preference in

398   Drosophila. *J Therm Biol* **34:** 109 – 119. http://doi.org/10.1016/j.jtherbio.2008.11.007

399   Dunbar, H.E., Wilson, A.C.C., Ferguson, N.R., and Moran, N.A. (2007) Aphid thermal

400   tolerance is governed by a point mutation in bacterial symbionts. *PLoS Biol* **5:** *e96.*

401   http://doi.org/10.1371/journal.pbio.0050096

402   Dutra, H.L., Rocha, M.N., Dias, F.B., Mansur, S.B., Caragata, E.P., and Moreira, L.A. (2016)

403   *Wolbachia* blocks currently circulating Zika virus isolates in Brazilian *Aedes aegypti*

404   mosquitoes. *Cell Host Microbe* **19:** 771-774. doi: 10.1016/j.chom.2016.04.021

405   Early, A.M. and Clark, A.G. (2013) Monophyly of *Wolbachia pipientis* genomes within

406   *Drosophila melanogaster*: geographic structuring, titre variation, and host effects across

407   five populations. *Molecular Ecology*, **22:** 5765–5778. https://doi.org/10.1111/mec.12530

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

409   Press.

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

411   Fedorka, K.M., Kutch, I.C., Collins, L., and Musto, E. (2016) Cold temperature preference in

412   bacterially infected *Drosophila melanogaster* improves survival but is remarkably

413   suboptimal.   *J   Insect   Physiol   **93–94:**   36–41.

414   http://doi.org/10.1016/j.jinsphys.2016.08.005

415   Fox, C.W., Roff, D.A., and Fairbairn, D.J. (2001) *Evolutionary Ecology*. Oxford University

416   Press.

417   Fry, A.J., Palmer, M.R., and Rand, D.M. (2004) Variable fitness effects of *Wolbachia*

418   infection   in   *Drosophila   melanogaster.   Heredity*   **93:**   379–389.

419   http://doi.org/10.1038/sj.hdy.6800514

420   Garrity, P., Goodman, M., Samuel, A., and Sengupta, P. (2010) Running hot and cold:

421   behavioral strategies, neural circuits, and the molecular machinery for thermotaxis in *C.*

422   *elegans* and *Drosophila*. *Genes Dev* **24:** 2365–2382. http://doi.org/10.1101/gad.1953710

423   Hedges, L.M., Brownlie, J.C., O'Neill, S.L., and Johnson, K.N. (2008) *Wolbachia* and virus

424   protection in insects. *Science* **322:** 702. http://doi.org/10.1126/science.1162418

425   Hoffmann, A.A. (1988) Partial cytoplasmic incompatibility between two Australian

426   populations of *Drosophila melanogaster*. *Entomol Exp Appl 48*(1):61–67.

427   Hoffmann, A.A. (2010) Physiological climatic limits in *Drosophila*: patterns   and

428   implications. *J Exp Biol* **213:** 870–80. http://doi.org/10.1242/jeb.037630

429   Hoffmann, A.A., Turelli, M., and Harshman, L.G. (1990) Factors affecting the distribution of

430   cytoplasmic incompatibility in *Drosophila simulans*. *Genetics* **126:** 933–948.

431   Hoffmann, A.A. and Sgró, C.M. (2011) Climate change and evolutionary adaptation. *Nature*

432   **470:** 479–85. http://doi.org/10.1038/nature09670

433   Hosokawa, T., Koga, R., Kikuchi, Y., Meng, X., and Fukatsu, T. (2010) *Wolbachia* as a

434   bacteriocyte-associated nutritional mutualist. *Proc Natl Acad Sci U S A* **107:** 769-774.

435   http://doi.org/10.1073/pnas.0911476107

436   Huey, R.B., and Berrigan, D. (2001) Temperature, demography, and ectotherm fitness. *Am*

437   *Nat* **158:** 204–210. http://doi.org/10.1086/321314

438   Huey, R.B., Kearney, M.R., Krockenberger, A., Holtum, J.A.M., Jess, M., and Williams, S.E.

439   (2012) Predicting organismal vulnerability to climate warming: roles of behaviour,

440   physiology and adaptation. *Philos Trans R Soc Lond B Biol Sci* **367:** 1665–1679.

441   http://doi.org/10.1098/rstb.2012.0005

442   Hurst, G. D, Johnson, A.P., Schulenburg, J.H., and Fuyama, Y. (2000) Male-killing

443   *Wolbachia* in *Drosophila*: a temperature-sensitive trait with a threshold bacterial density.

444   *Genetics* **156:** 699–709.

445   Ilinsky, Y. (2013) Coevolution of *Drosophila melanogaster* mtDNA and *Wolbachia*

446   genotypes. *PLoS ONE* **8:** e54373. https://doi.org/10.1371/journal.pone.0054373

447   Iturbe-Ormaetxe, I., Walker, T., and O' Neill, S.L. (2011) *Wolbachia* and the biological

448   control   of   mosquito-borne   disease.   *EMBO   Rep*   **12:**   508–518.

449   http://doi.org/10.1038/embor.2011.84

450   Jeyaprakash, A., and Hoy, M.A. (2000) Long PCR improves *Wolbachia* DNA amplification:

451   *wsp* sequences found in 76% of sixty-three arthropod species. *Insect Mol Biol* **9:** 393–

452   405. http://doi.org/10.1046/j.1365-2583.2000.00203.x

453   Kacsoh, B.Z., Lynch, Z.R., Mortimer, N.T., and Schlenke, T.A. (2013) Fruit flies medicate

454   offspring   after   seeing   parasites.   *Science*   **339:**   947-950.

455   http://doi.org/10.1126/science.1229625.

456   Levins, R. (1968) *Evolution in Changing Environments*. Princeton University Press.

457   Louis, C., Jourdan, M., and Cabanac, M. (1986) Behavioral fever and therapy in a *rickettsia-*

458   infected *Orthoptera*. *Am J Physiol* **250:** R991–R995.

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

460   Martin, T.L., and Huey, R.B. (2008) Why "suboptimal" is optimal: Jensen's inequality and

461   ectotherm thermal preferences. *Am Nat* **171:** 102–118. http://doi.org/10.1086/527502

462   Martinez, J., Longdon, B., Bauer, S., Chan, Y.S., Miller, W.J., Bourtzis, K., *et al.* (2014)

463   Symbionts commonly provide broad spectrum resistance to viruses in insects: A

464   comparative analysis of *Wolbachia* strains. *PLoS Pathog* **10:** e1004369.

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

466   Martins, N.E., Faria, V.G., Nolte, V., Schlötterer, C., Teixeira, L., Sucena, É., and Magalhães,

467   S. (2014) Host adaptation to viruses relies on few genes with different cross-resistance

468   properties. *Proc Natl Acad Sci U S A* **111:** 5938–5943.

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

470   Matute, D.R., Novak, C.J., and Coyne, J.A. (2009) Temperature-based extrinsic reproductive

471   isolation in two species of *Drosophila*. *Evolution* **63:** 595–612.

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

473   McGraw, E.A., Merritt, D.J., Droller, J.N., and O'Neill, S.L. (2002) *Wolbachia* density and

474   virulence attenuation after transfer into a novel host. *Proc Natl Acad Sci U S A* **99:** 2918-

475   2923. http://doi.org/10.1073/pnas.052466499

476   Miller, W.J. (2013) Bugs in transition: The dynamic world of *Wolbachia* in insects. *PLoS*

477   *Genet* **9:** e1004069. http://doi.org/10.1371/journal.pgen.1004069

478   Miller, W.J., Ehrman, L., and Schneider, D. (2010) Infectious speciation revisited: Impact of

479   symbiont-depletion on female fitness and mating behavior of *Drosophila paulistorum*.

480   *PLoS Pathog* **6:** e1001214 . http://doi.org/10.1371/journal.ppat.1001214

481   Min, K.T., and Benzer, S. (1997) *Wolbachia*, normally a symbiont of *Drosophila*, can be

482   virulent, causing degeneration and early death. *Proc Natl Acad Sci U S A* **94:** 10792–

483   10796.

484   Moreira, L.A., Iturbe-Ormaetxe, I., Jeffery, J.A., Lu, G., Pyke, A.T., Hedges, L.M., *et al.*

485   (2009) A *Wolbachia* symbiont in *Aedes aegypti* limits infection with dengue,

486   Chikungunya, and Plasmodium. *Cell* **139:** 1268-1278.

487   https://doi.org/10.1016/j.cell.2009.11.042

488   Mouton, L., Henri, H., Bouletreau, M., and Vavre, F. (2006) Effect of temperature on

489   *Wolbachia* density and impact on cytoplasmic incompatibility. *Parasitology* **132:** 49–56.

490   http://doi.org/10.1017/S0031182005008723

491   Mouton, L., Henri, H., Charif, D., Boulétreau, M., and Vavre, F. (2007) Interaction between

492   host genotype and environmental conditions affects bacterial density in *Wolbachia*

493   symbiosis. *Biol Lett* **3:** 210–213. http://doi.org/10.1098/rsbl.2006.0590

494   Murdock, C.C., Blanford, S., Hughes, G.L., Rasgon, J.L., & Thomas, M.B. (2014)

495   Temperature alters *Plasmodium* blocking by *Wolbachia*. *Sci Rep* **4:** 3932.

496   http://doi.org/10.1038/srep03932

497   Nunes, M.D.S., Nolte, V., and Schlötterer, C. (2008) Nonrandom *Wolbachia* infection status

498   of *Drosophila melanogaste*r strains with different mtDNA haplotypes. *Mol Biol Evol* **25:**

499   2493–2498. http://doi.org/10.1093/molbev/msn199.

500   O'Neill, S.L., Giordano, R., Colbert, A.M., Karr, T.L., and Robertson, H.M. (1992) 16S

501   rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic

502   incompatibility in insects. *Proc Natl Acad Sci U S A* **89:** 2699–2702.

503   Osborne, S.E., Leong, Y.S., O'Neill, S.L., and Johnson, K.N. (2009) Variation in antiviral

504   protection mediated by different *Wolbachia* strains in *Drosophila simulans. PLoS*

505   *Pathog* **5:** e1000656. http://doi.org/10.1371/journal.ppat.1000656

506   R Development Core Team (2009) R: A Language and Environment for Statistical

507   Computing. *R-project.org.*

508   Rajpurohit, S., and Schmidt, P.S. (2016) Measuring thermal behavior in smaller insects: a

509 case study in *Drosophila melanogaster* demonstrates effects of sex, geographic origin,

510 and rearing temperature on adult behavior. *Fly* **10:** 149-161

511 http://doi.org/10.1080/19336934.2016.1194145

512 Reynolds, K.T., Thomson, L.J., and Hoffmann, A.A. (2003) The effects of host age, host

513 nuclear background and temperature on phenotypic effects of the virulent *Wolbachia*

514 strain popcorn in *Drosophila melanogaster*. *Genetics* **164:** 1027–1034.

515 Richardson, M.F., Weinert, L.A., Welch, J.J., Linheiro, R.S., Magwire, M.M., Jiggins, F.M.,

516 and Bergman, C.M. (2012) Population genomics of the *Wolbachia* endosymbiont in

517 *Drosophila melanogaster*. *PLoS Genet* **8:** e1003129.

518 http://doi.org/10.1371/journal.pgen.1003129

519 Riegler, M., Iturbe-Ormaetxe, I., Woolfit, M., Miller, W.J., and O'Neill, S.L. (2012) Tandem

520 repeat markers as novel diagnostic tools for high resolution fingerprinting of *Wolbachia*.

521 *BMC Microbiol* **12 Suppl 1:** S12. http://doi.org/10.1186/1471-2180-12-S1-S12

522 Riegler, M., Sidhu, M., Miller, W.J., and O'Neill, S.L. (2005) Evidence for a global

523 *Wolbachia* replacement in *Drosophila melanogaster*. *Curr Biol* **15:** 1428–1433.

524 http://doi.org/10.1016/j.cub.2005.06.069

525 Russell, J.A., and Moran, N.A. (2006) Costs and benefits of symbiont infection in aphids:

526 variation among symbionts and across temperatures. *Proc Biol Sci* **273:** 603–610.

527 http://doi.org/10.1098/rspb.2005.3348

528 Sazama, E.J., Bosch, M.J., Shouldis, C.S., Ouellette, S.P., and Wesner, J. S. (2017) Incidence

529 of *Wolbachia* in aquatic insects. *Ecol Evol* **7:** 1165–1169.

530 http://doi.org/10.1002/ece3.2742

531 Strunov, A. A., Ilinskii, Y. Y., Zakharov, I. K., & Kiseleva, E. V. (2013a). Effect of high

532 temperature on survival of *Drosophila melanogaster* infected with pathogenic strain of

533 *Wolbachia* bacteria. *Russ J Genet Appl Res* **3:** 435–443.

534 http://doi.org/10.1134/S2079059713060099

535   Strunov, A., Kiseleva, E., and Gottlieb, Y. (2013b) Spatial and temporal distribution of

536   pathogenic *Wolbachia* strain *w*MelPop in *Drosophila melanogaster* central nervous

537   system under different temperature conditions. *J Invertebr Pathol* **114:** 22–30.

538   http://doi.org/10.1016/j.jip.2013.05.001

539   Teixeira, L., Ferreira, Å., and Ashburner, M. (2008) The bacterial symbiont *Wolbachia*

540   induces resistance to RNA viral infections in *Drosophila melanogaster. PLoS Biol* **6:** e2.

541   http://doi.org/10.1371/journal.pbio.1000002

542   van Opijnen, T.V. and Breeuwer, J.A.J. (1999) High temperatures eliminate *Wolbachia,* a

543   cytoplasmic incompatibility inducing endosymbiont, from the two-spotted spider mite.

544   *Exp Appl Acarol* **23:** 871–881.

545   Veneti, Z., Clark, M.E., Zabalou, S., Karr, T.L., Savakis, C., and Bourtzis, K. (2003)

546   Cytoplasmic incompatibility and sperm cyst infection in different *Drosophila-Wolbachia*

547   associations. *Genetics* **164:** 545–552.

548   Versace, E., Nolte, V., Pandey, R.V., Tobler, R., and Schlötterer, C. (2014) Experimental

549   evolution reveals habitat-specific fitness dynamics among *Wolbachia* clades in

550   *Drosophila melanogaster. Mol Ecol* **23:** 802–814. http://doi.org/10.1111/mec.12643

551   Webster, C.L., Waldron, F.M., Robertson, S., Crowson, D., Ferrari, G., Quintana, J.F., *et al.*

552   (2015) The discovery, distribution, and evolution of viruses associated with *Drosophila*

553   *melanogaster*. *PLoS Biol* **13:** e1002210. http://doi.org/10.1371/journal.pbio.1002210

554   Wedell, N. (2013) The dynamic relationship between polyandry and selfish genetic elements.

555   *Philos   Trans   R   Soc   Lond   B   Biol   Sci*   **368:**   20120049.

556   http://doi.org/10.1098/rstb.2012.0049

557   Weinert, L.A., Araujo-jnr, E.V, Ahmed, M.Z., Welch, J.J., and Welch, J.J. (2015) The

558   incidence of bacterial endosymbionts in terrestrial arthropods. *Proc Biol Sci* **282:**

559   *20150249*. http://dx.doi.org/10.1098/rspb.2015.0249

560   Wernegreen, J.J. (2012) Mutualism meltdown in insects: Bacteria constrain thermal

561   adaptation. *Curr Opin Microbiol* *15*(3), 255–262.

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

563   Werren, J. H., Baldo, L., and Clark, M.E. (2008) *Wolbachia*: master manipulators of

564   invertebrate biology. *Nat Rev Microbiol* **6**: 741–751. http://doi.org/10.1038/nrmicro1969

565   Werren, J.H., Zhang, W., and Guo, L.R. (1995) Evolution and phylogeny of *Wolbachia*:

566   reproductive parasites of arthropods. *Proc Biol Sci* **281**: 55-63.

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

568   Wiwatanaratanabutr, I. and Kittayapong, P. (2009) Effects of crowding and temperature on

569   *Wolbachia* infection density among life cycle stages of *Aedes albopictus, J Invertebr*

570   *Pathol* **102:** 220–224. http://doi.org/10.1016/j.jip.2009.08.009

571   Yamada, R., Floate, K.D., Riegler, M., and Neill, S.L.O. (2007) Male development time

572   influences the strength of *Wolbachia*-induced cytoplasmic incompatibility expression in

573   *Drosophila melanogaste*r. *Genetics* **177:** 801–808.

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

575   Zhou, W., Rousset, F., and O'Neil, S. (1998) Phylogeny and PCR-based classification of

576   *Wolbachia* strains using *wsp* gene sequences. *Proc Biol Sci* **265:** 509–515.

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

578   Zug, R. and Hammerstein, P. (2012) Still a host of hosts for *Wolbachia*: analysis of recent

579   data suggests that 40% of terrestrial arthropod species are infected. *PloS One*, **7:** e38544.

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

581   Zug R. and Hammerstein P. (2015) Bad guys turned nice? A critical assessment of *Wolbachia*

582   mutualisms in arthropod hosts. *Biol Rev Camb Philos Soc* **90:** 89-111. doi:

583   10.1111/brv.12098

584

585

586   **TABLE AND FIGURE LEGENDS**

587

588   **Table 1.** Comparison of strain type titer levels, growth rates, and effects on host's lifespan at

589   25°C.

590

591   ***Table 2:*** Table showing the results of three analyses based on generalized linear mixed

592   models with a Poisson error structure to account for the statistical properties of count data.

593   The columns show ID's for the different analyses (A-C), the models, the individual factors

594   and interactions tested, the samples size, the degrees of freedom for the χ^2 test of the analysis

595   of deviance, the χ^2 value and the corresponding *P*-value. Note that analyses with significant

596   effects after Bonferroni correction (adjusted $\alpha = 0.017$) are highlighted in bold.

597

598   ***Table 3:*** Table showing z-values from post-hoc pairwise comparisons with Tukey's HSD for

599   the factor Wolbachia (Analysis C; see Experimental Procedures) with four levels (non-

600   infected, wMel, wMelCS, and wMelPop). Bold type indicates significance after Bonferroni

601   correction (adjusted $\alpha' = 0.017$). * p < 0.05; ** p < 0.01; *** p < 0.001.

602

603   ***Figure 1*: Thermal preference of *Drosophila* with and without *Wolbachia* infections.**

604   Panels A and B show average T_p (blue diamonds) with respect to age (3-4, 5-7 or 10-14 days

605   post eclosion, n=4370 excluding flies infected with wMelPop) and sex (male or female;

606   n=1718, excluding uninfected flies), respectively. Each symbol represents the average T_p for a

607   replicate at a given factor level of either age (circle: 3-4 days, triangle: 5-8 days and square:

608   10-14 days) or sex (circle: females, triangle: males). Panel C shows line plots with relative

609   proportions of flies observed at a given temperature. Each line represents the average

610   proportion of flies which were either uninfected (*w*-; black), or infected with wMel (red),

611   wMelCS (blue) or wMelPop (green). The error bars represent standard errors for average

612   frequencies at a given temperature across all replicated experiments carried out for each

613   infection type. We found that infected flies exhibit significantly lower thermal preference
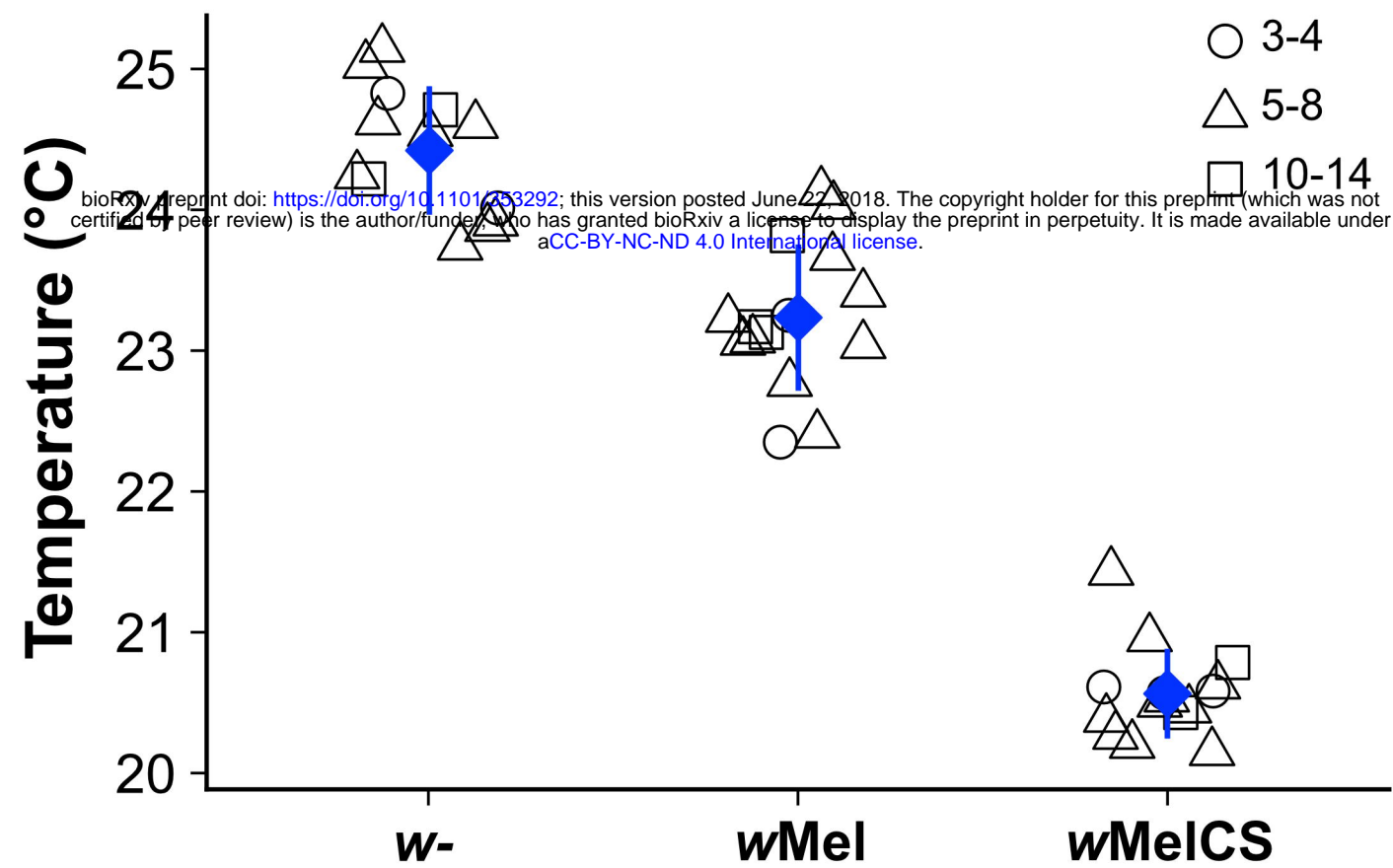
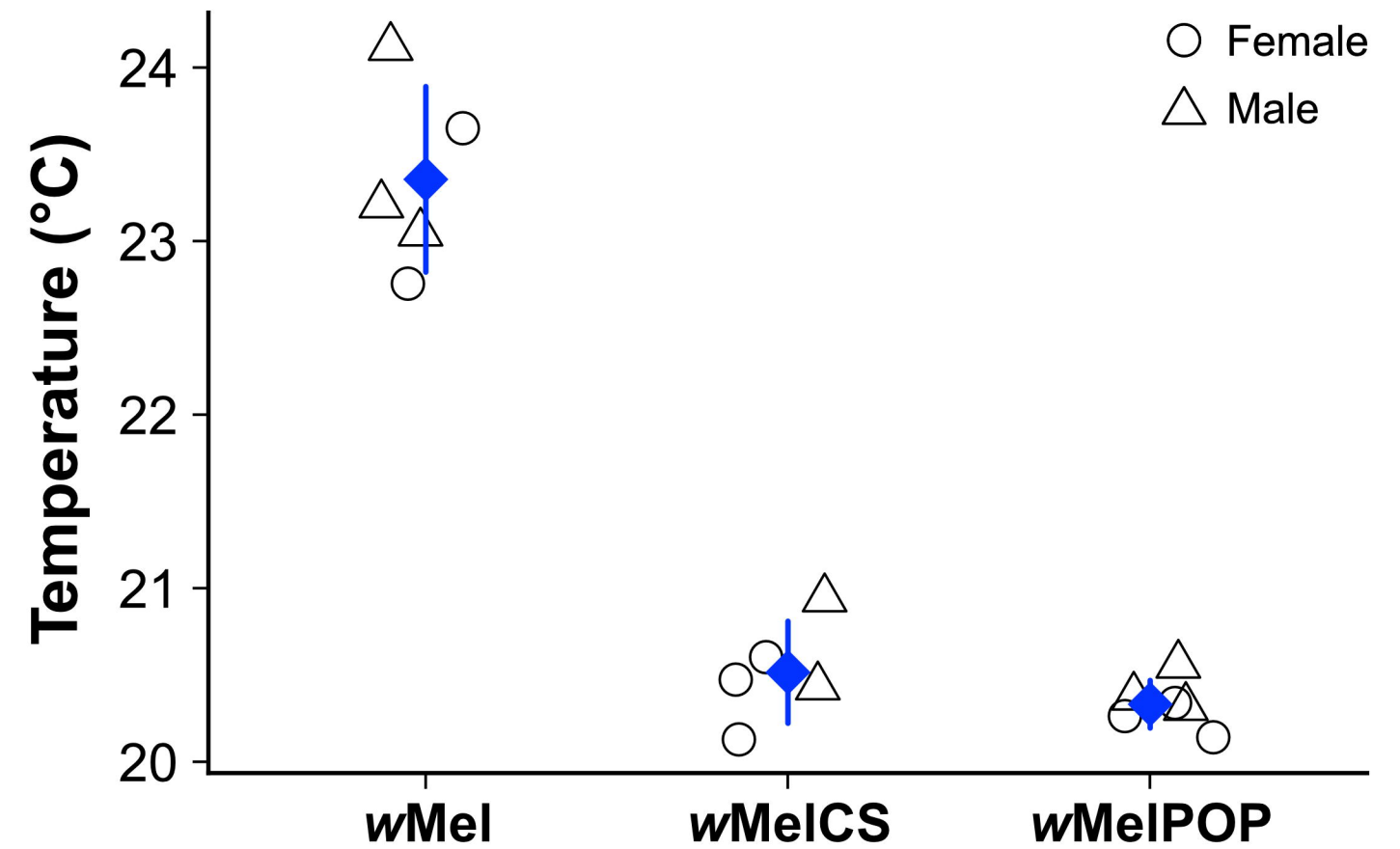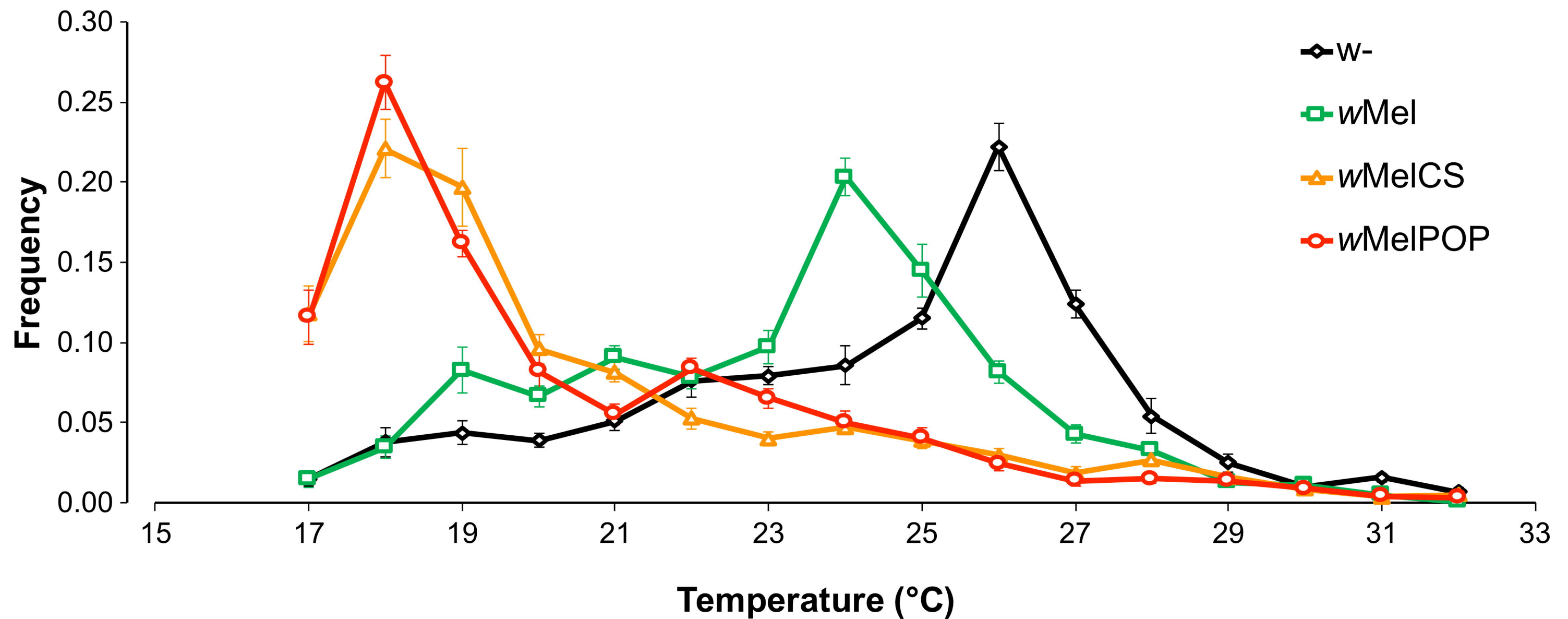614   compared to uninfected flies.

Table 1.

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

Note: Information on titer levels, growth rate, host's lifespan effects for *w*Mel and *w*MelCS from Chrostek *et al.* 2013, information on *w*MelPop's effects on host's lifespan from Reynolds *et al.* 2003.

Table 2

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

1

2

3 **Table 3**

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

4