1 **Correlated evolution between heat tolerance and thermal performance**

2 **curves in *Drosophila subobscura***

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19 evolution; locomotor performance; thermal reaction norm; thermal stress.

20

21 **Abstract**

22   Global warming imposes important challenges for ectotherm organisms, which can avoid

23   the negative effects of thermal stress via evolutionary adaptation of their upper thermal

24   limits (CT_{max}). In this sense, the estimation of CT_{max} and its evolutionary capacity is crucial

25   to determine the vulnerability of natural populations to climate change. However, these

26   estimates depend on the thermal stress intensity and it is not completely clear whether this

27   thermal stress intensity can impact the evolutionary response of CT_{max} and thermal reaction

28   norms (i.e. thermal performance curve, TPC). Here we performed an evolutionary

29   experiment by selecting high heat tolerance using acute and chronic thermal stress in

30   *Drosophila subobscura*. After artificial selection, we found that knockdown temperatures (a

31   CT_{max} proxy) evolved in selected lines compared to control lines, whereas the realized

32   heritability and evolutionary rate change of heat tolerance did not differ between acute-

33   selected and chronic-selected lines. From TPC analysis, we found acute-selected lines

34   evolved a higher optimal performance temperature (T_{opt}) compared to acute-control lines,

35   whereas this TPC parameter was not different between chronic-selected and chronic-control

36   lines. The evolutionary response of T_{opt} caused a displacement of entire TPC to high

37   temperatures suggesting a shared genetic architecture between heat tolerance and high-

38   temperature performance, which only arose in the acute-selected lines. In conclusion,

39   thermal stress intensity has important effects on the evolution of thermal physiology in

40   ectotherms, indicating that different thermal scenarios conduce to similar evolutionary

41   responses of heat tolerance but do not for thermal performance. Therefore, thermal stress

42   intensity could have important consequences on the estimations of the vulnerability of

43   ectotherms to global warming.

44

Introduction

45

46 Environmental temperatures influence the organismic functions and fitness of ectotherm

47 animals (Huey & Stevenson, 1979; Huey & Kingsolver, 1993; Angilletta et al., 2009),

48 causing that distribution and abundance of these organisms to be mainly limited by their

49 thermal limits (Parmesan & Yohe, 2003; Beugrand et al., 2002; Lima et al., 2007; Pannetta

50 et al., 2018). Thus, the estimation of the evolutionary response of upper thermal limits

51 (CT_{max}) it is crucial to predict and understand the capability of ectotherms to respond to

52 increasing thermal challenges (Gunderson & Stillman, 2015). In this context, several

53 studies have revealed a limited evolutionary potential of CT_{max} in ectotherms, suggesting

54 that a high vulnerability of ectotherms to global warming (Sunday et al., 2011; Kellerman

55 et al., 2012; Kelly et al., 2012; Araujo et al., 2013; Hoffmann et al., 2013). However, some

56 experimental studies have reported a large evolutionary response for CT_{max} only after a few

57 generations under selection for increasing thermotolerance (Bubliy & Loeschcke, 2005;

58 Folk et al., 2006; Geerts et al, 2015; Hangartner & Hoffmann, 2016). Consequently, it is

59 mandatory to elucidate the factors that could explain this contrasting evidence about the

60 heat tolerance evolution.

61 Commonly, CT_{max} is estimated using dynamic assays, in which individuals are

62 exposed to non-stressful temperatures and then temperature is increased at a specific rate

63 until the organisms collapse (Cowles y Bogert, 1944; Hutchinson, 1961; Lutterschmidt y

64 Hutchison, 1997). However, several lines of evidence suggest that heat tolerance estimates

65 depend on the intensity of thermal stress employed during dynamic assays, showing that

66 organisms exposed to a chronic thermal stress (e.g. slow ramping assays) exhibit a lower

67 CT_{max} than those individuals exposed to an acute thermal stress (e.g. fast ramping assays)

68 (Terblanche et al., 2007; Chown et al., 2009; Peck et al., 2009; Mitchell & Hoffmann,

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69   2010; Ribeiro et al., 2012). This methodological impact on heat tolerance estimates has

70   been explained as the consequences of the physiological mechanisms related to heat stress

71   such as resource depletion, water loss and heat-induced cellular damage, which can be

72   more important during longer thermal assays (Rezende et al. 2011; Kingsolver &

73   Umbanhowar, 2018). Interestingly, the intensity of thermal stress also has effects on the

74   heritability estimates of CT_{max}: the longer the thermal assays, the lower the heritability

75   (Mitchell & Hoffmann, 2010; Blackburn et al., 2014; Castañeda et al. 2019). Therefore,

76   these resistance-associated mechanisms (e.g. resource depletion, water loss, cellular

77   damage) are expected to increase the environmental variance of CT_{max} assayed under

78   chronic thermal stress, leading to a reduced heritability estimates in comparison to

79   parameters estimated under acute thermal stress (Chown et al., 2009; Rezende et al. 2011).

80   However, to date the impact of the thermal stress intensity on the evolutionary response of

81   heat tolerance methodology has been only explored through computer simulations by

82   mimicking artificial selection experiments for increasing heat tolerance using different

83   ramping rates in *Drosophila melanogaster* (Santos et al., 2012). In this simulation, Santos

84   et al. (2012) found that not only a reduced increase of heat tolerance in slow-ramping

85   selected lines compared to fast-ramping selected lines was evident, but also that there was a

86   correlated physiological response (e.g. metabolic rate) in slow-ramping selected lines.

87   Then, these simulations suggest that the evolutionary response of heat tolerance and

88   correlated responses depend on the thermal stress intensity, which could be the result of the

89   how much precise is the estimation of CT_{max} (Castañeda et al. 2019). However, there is not

90   empirical evidence that supports this hypothesis to date and it is the main goal of the

91   present work.

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93   The correlated responses to the evolution of CT_{max} may be the result of the genetic

94 architecture underlying heat tolerance (Angilletta, 2009; Hangartner et al., 2016; Rolandi et

95 al., 2018), but the impact of the thermal stress intensity on the correlated evolutionary

96 response of other phenotypic traits related to CT_{max} is unknown. In their seminal work,

97 Huey & Kingsolver (1993) proposed different evolutionary consequences of the thermal

98 performance curve (TPC) as response to selection for increased heat tolerance. The TPC is

99 a reaction norm that describes the complex relationship between the organismic

100 performance (e.g. locomotion, growth, development, fecundity) and the environmental

101 temperature (Fig. 1a) (Huey & Stevenson 1979; Angilletta 2009). Specifically, Huey &

102 Kingsolver (1993) proposed four possible responses of TPC to selection on heat tolerance:

103 *i*) in absence of genetic correlations between performance at high and low temperatures, the

104 increase of CT_{max} might increase the TPC breadth (Fig. 1a); *ii*) if the thermal limits are

105 negatively correlated, the selection for a higher CT_{max} might reduce the performance at low

106 temperatures (e.g. lower thermal limit, CT_{min}), then moving the entire TPC to higher

107 temperatures (Fig. 1b); *iii*) if CT_{max} is positively correlated to the maximum performance,

108 then the selection to high heat tolerance should simultaneously increase the optimal

109 temperature and the maximum performance ("hotter is better", Fig. 1c); and finally *iv*) if

110 maximum performance is negatively correlated to TPC breadth, then selection for a higher

111 CT_{max} could boost the performance at low temperatures ("jack-of-all-temperatures is a

112 master of none", Fig. 1d). Previous evidence shows that acute thermal assays provide a

113 precise estimation of the heat tolerance and its genetic component (Rezende et al. 2011;

114 Santos et al. 2012; Castañeda et al. 2019). Thus, it is reasonable to expect that selection for

115 heat tolerance under different thermal stress intensities will result into different correlated

116 responses of TPC.

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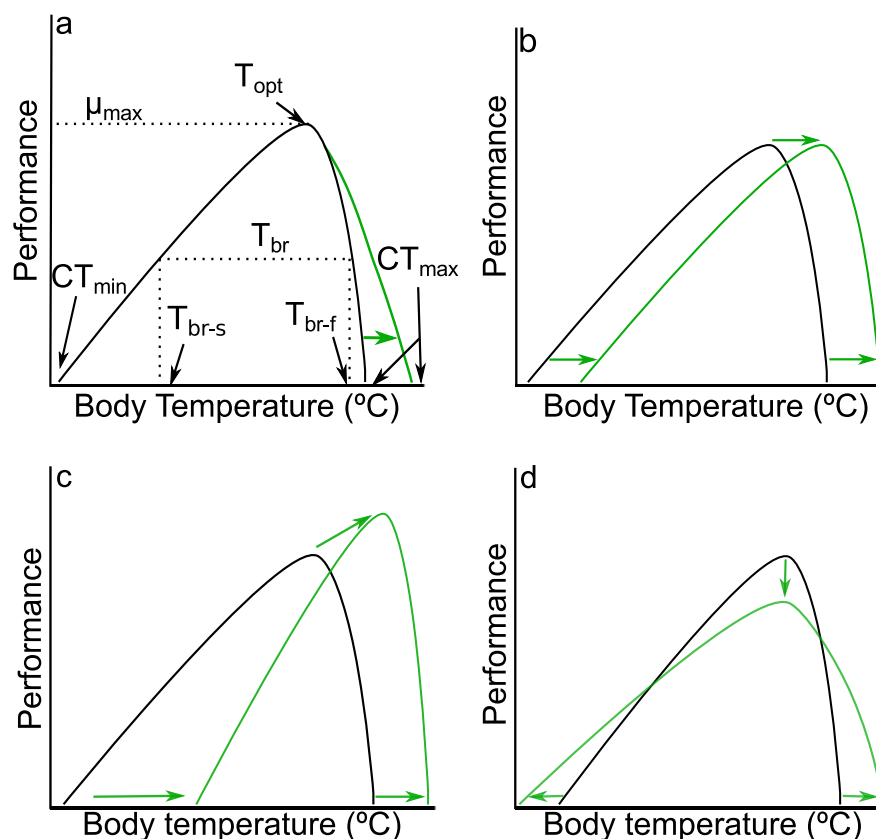
Figure 1. Thermal performance curve (TPC) and possible correlated responses to selection

for increased heat tolerance (modified from Huey & Kingsolver 1993). a) Parameters of

TPC and a hypothetical response of TPC when increased heat tolerance does not induce

changes in other TPC parameters, b) increased heat tolerance leads to a displacement of

TPC, c) increased heat tolerance increases μ_{max} and T_{opt} (hotter is better), and d) increased

heat tolerance reduces μ_{max}, but increases T_{br} (jack-of-all-temperatures is a master-of-none).

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125   In the present work, we studied the impact of the thermal stress intensity on the

126   evolutionary response of heat tolerance and the correlated response of TPC in *D.*

127   *subobscura* (Collin). To accomplish this: (1) we compared the evolutionary response of

128   heat tolerance to the artificial selection under two thermal intensity treatments: chronic

129   (slow ramping selection, 0.08 ºC min^{-1}) and acute thermal stress (fast ramping selection, 0.4

130   ºC min^{-1}); and (2) we compared the correlated response of TPC to heat tolerance selection

131   between different thermal intensity treatments (selected versus control lines), where TPC

132   was estimated as the relationship between locomotor performance (i.e. climbing velocity)

133   and environmental temperature. Because acute thermal assays provide a more precise

134   estimation of heat tolerance and its genetic component (Rezende et al. 2011; Santos et al.

135   2012; Castañeda et al. 2019), we hypothesize that (1) the evolutionary response and

136   realized heritability of the heat tolerance should be higher in selected lines under acute

137   thermal stress than chronic thermal stress, and (2) as correlated responses should depend on

138   the intensity of the thermal stress, we expect a correlated response of TPC only for selected

139   lines under acute thermal stress compared to control lines and selected lines under chronic

140   thermal stress. We used *D. subobscura* as a model organism because this species shows

141   evidence of thermal adaptation in several phenotypic traits. For instance, latitudinal

142   variation has been reported for body size, chromosomal inversion polymorphisms,

143   desiccation resistance, thermal preference, heat tolerance and fertility (Budnik et al. 1991;

144   Huey et al. 2000; Castañeda et al. 2013, 2015; Gilchrist et al. 2008; Porcelli et al. 2017). In

145   addition, laboratory selection studies have demonstrated that populations of *D. subobscura*

146   exposed for three years to different rearing temperatures show evolutionary responses in

147   several traits such as chromosomal inversion polymorphisms, transcriptomic profiles, wing

148   size, wing shape, and development and life-history traits (Santos et al. 2004, 2005, 2006;

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149   Laayouni et al. 2007;). Therefore, *D. subobscura* is a suitable model to evaluate the

150   evolutionary responses of heat tolerance and TPC to high temperature selection.

151

Methodology

Fly maintenance

154   A mass-bred population of *D. subobscura* was established from the offspring of 100

155   isofemale lines derived from inseminated females collected in Valdivia, Chile (39.8 ºS 73.2

156   ºW). We dumped 10 females and 10 males from each isofemale line into an acrylic cage

157   ($27 \times 21 \times 16$ cm^3) to setup one large outbred population (>1500 breeding adults), which

158   was maintained at 21 ºC (12:12 light:dark cycle) and feed with David's killed-yeast

159   *Drosophila* medium in Petri dishes (David 1962). At the next generation, a total of 150

160   eggs collected from Petri dishes were placed into 150-mL bottles with food, and a total of

161   45 bottles were maintained at 21 ºC. Emerged flies from 15 bottles were dumped into one

162   acrylic cage, resulting in a total of three population cages: R1, R2 and R3. After three

163   generations, with a larger population size and the environmental effects removed, each

164   replicated cage (R1, R2 and R3) was split into four acrylic cages with more 1500

165   individuals per cage. The resulting four cages for each replicated cage were assigned to

166   different treatments of our artificial selection experiment: acute-selected and acute-control

167   lines, and chronic-selected and chronic-control lines. Thus, we established a total of 12

168   experimental lines, designated to four selection treatments where each treatment was

169   replicated three times. All population cages were maintained on a nonoverlapping

170   generation cycle and with controlled larval density as described in Castañeda et al. (2013,

171   2015).

172

Selection on heat knockdown temperature

After three generations of the founding of experimental lines, three Petri dishes with

David's medium and extra dry yeast were placed into each experimental cage to collect

eggs, which were transferred to vials with a density of 40 eggs per vial. Then, a total of 160

virgin females per experimental line were randomly chosen, individually placed into vials

with fly medium, and mated with two non-related males from the same experimental line.

After two days, males were discarded, and vials were checked for positive oviposition. This

procedure was done before of the thermal selection assays because heat stress might cause

mortality and sterilization in *Drosophila* (David et al., 2005). For each experimental line,

120 females were randomly chosen to evaluate the heat knockdown temperature as a proxy

of CT_{max}. Each female fly was individually placed in a capped 5-mL glass vial, which was

attached to a rack with capacity to attach 60-capped vials (4 rows × 15 columns). Each rack

was immersed in a water tank with an initial temperature of 28 ºC, which was controlled by

a heating unit (Model ED, Julabo Labortechnik, Seelbach, Germany). After an equilibration

time of 10 min, temperature was increased with a heating rate of 0.08 ºC min^{-1} for the

chronic-selected lines or 0.4 ºC min^{-1} for the acute-selected lines. Each assay was

photographed every 3 s with a high-resolution camera (D5100, Nikon, Tokyo, Japan). All

photos for each assay were collated into a single file that was visualized to score the

knockdown temperature defined as the temperature at which each fly ceased to move.

Knockdown temperatures were ranked, and we selected vials containing offspring of the 40

most tolerant female flies (upper 33% of each assay) to establish the next generation. From

each one of these vials, four virgin females were individually placed in new vials with

David medium and mated with two unrelated males. This procedure reestablished the

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196   original number of 160 female flies (4 females per vial × 40 vials = 160 females) before

197   each heat thermal assay.

198   We performed the same procedure described above for control lines, except that

199   founding flies were randomly chosen in each line. Briefly, the knockdown temperature of

200   40 females flies for each control line were assayed using a heating rate of 0.08 ºC min^{-1} for

201   the chronic-control lines or 0.4 ºC min^{-1} for the acute-control lines. After measuring

202   knockdown temperature for each female fly, we randomly selected 10 of them and we used

203   their offspring to found the next generation of control lines.

204   Artificial selection for heat tolerance was performed for 16 generations, after which

205   flies from each experimental line were dumped into an acrylic cage and maintained

206   without selection at 21ºC (12:12 light-dark cycle), until the measurements of locomotor

207   performance were performed at the generation 25.

208

209   **Locomotor performance**

210   Locomotor performance was evaluated as the climbing velocity (cm s^{-1}) in four days-old

211   virgin females from generation 25. The locomotor performance for selected and control

212   lines was measured at 5, 15, 20, 25, 30 and 35 ºC. For the experimental temperatures below

213   to room temperature (~ 21 ºC), bath water was cooled using a cooling unit (Model FP 50-

214   HP, Julabo Labortechnik, Seelbach, Germany), whereas for experimental temperatures

215   above room temperature, bath water was heated using a heating unit (Model ED, Julabo

216   Labortechnik, Seelbach, Germany). At each temperature, we evaluated 15 females from

217   each experimental line, which were individually placed in 5 mL glass pipettes. The sealed

218   pipettes were attached to a rack with a capacity of 15 pipettes, and the rack was immersed

219   in a 40 L water tank setup to a specific experimental temperature. After 30 min in dark

220   conditions, we lifted the rack 5 cm and let it fall to allow the flies dropped to the bottom of

221   the pipettes, and then they climbed inside the pipettes. In addition, we turned on a light bulb

222   placed over the water tank to induce the climbing taking advantage of positive

223   phototropism and negative geotropism of flies. Each assay was video-recorded with a high-

224   resolution camera (D5100, Nikon, Tokyo, Japan), and the video files were visualized to

225   score the climbing velocity (cm s^{-1}) for each assayed female.

226

227   **Statistical analysis**

228   **Evolutionary response of upper thermal limit**

229   We evaluated the evolutionary response of the knockdown temperatures by comparing the

230   knockdown temperatures between selected and control lines at generation 16. We used a

231   mixed linear model including the thermal selection as fixed factor and the replicated lines

232   nested within thermal selection as random factor. This analysis was performed using the

233   *lme* function of the *nlme* package for R (Pinheiro et al., 2018). As expected, we found that

234   knockdown temperature was significant higher in acute-selected lines compared to chronic-

235   selected lines ($F_{1,4} = 1952.5$, $P = 1.6$ x 10^{-9}). Similar result was found for the comparison

236   between control lines ($F_{1,4} = 242.5$, $P = 8.1$ x 10^{-7}). Then, analyses were performed

237   separately for the acute-selected and chronic-selected lines since it is known that acute

238   stress assays estimate higher thermal tolerance than chronic stress assays (Chown et al.

239   2009; Castañeda et al. 2015, 2019).

240   We estimated the rate of evolutionary change for knockdown temperature in

241   selected lines by calculating the Haldane index (*h*) for synchronic comparisons between

242   selected and their respective control lines (see details in Hendry & Kinnison, 1999):

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$$243 \qquad h_{s,c} = ((\bar{X}_s\,\sigma_p{}^{-1}) - (\bar{X}_c\,\sigma_p{}^{-1}))\; g^{-1} \qquad \text{(eq. 1)},$$

244   ,where $\bar{X}_s$ represents the mean knockdown temperature for each selected line and $\bar{X}_c$

245   represents the pooled mean knockdown temperature between control lines, σ_p is the pooled

246   standard deviation of selected and control lines, and g is the number of generations of

247   thermal selection ($g = 16$ for the present study). For each thermal selection regime, we

248   calculated the Haldane values for each replicated line (n = 3), and we evaluated if these

249   values were significantly different from zero using a one sample t-test. Additionally, we

250   compared whether the evolutionary rate of heat tolerance was different between both

251   thermal selection treatments using a Kruskal-Wallis test.

252   The realized heritability ($h^2{}_n$) of the knockdown temperatures was calculated by

253   regressing the cumulative response to selection against the cumulative selection

254   differential. Because thermal selection was only performed on females and sires were

255   randomly chosen, it is expected that the selection differential is only half. First, we

256   estimated the cumulative response to selection on the cumulative selection differential for

257   each replicated line, and these values were averaged within each selection treatment. Then,

258   we regressed the averaged cumulative response to selection against the averaged

259   cumulative selection differential forcing the regression through the origin, and the

260   estimated slope was equated to the $h^2{}_n$ for each selection treatment (Walsh & Lynch, 2018).

261   Finally, we compared the $h^2{}_n$ between selection treatments using a slope comparison

262   approach: we used a linear model with the averaged cumulative response to selection as

263   response variable and the interaction between the averaged cumulative selection differential

264   and selection treatment as predictor variables. Differences of $h^2{}_n$, between selection

265   treatments were considered when the interaction was significant.

266

267   **Locomotor performance**

268   We measured the climbing velocity for the acute- and chronic-selected lines but because

269   logistic reasons, we employed as control lines those lines belonging to the acute thermal

270   stress control lines (Fig. S1). We estimated the TPC parameters for each replicated line (n =

271   9) by fitting four different models: the Lactin model (Lactin et al. 1995); the Briere model

272   (Briere et al. 1999); the Performance model (Shi et al., 2011; Wang et al., 2013); and a

273   modified Gaussian model. We found that the Lactin model showed the lowest Akaike

274   Information Criteria (AIC) compared to the other three model (Table S1), so we use this

275   model to estimate the TPC parameters: the optimal performance temperature (T_{opt}), the

276   maximum performance (μ_{max}), the thermal breadth for the 50% and 80% of upper

277   performance (T_{br-50} and T_{br-80}, respectively), and the starting and ending temperatures of

278   these thermal breadths (T_{br-50s}, T_{br-50f}, T_{br-80s} and T_{br-80f}). Prior to TPC comparisons,

279   normality and homoscedasticity of data were evaluated for all TPC parameters using the

280   Lilliefors and Levene test, respectively (Table S2). Differences among TPC parameters

281   were evaluated between acute-selected, chronic-selected and control lines using an one-way

282   ANOVA. A posteriori differences among groups were evaluated using a HSD Tukey's test.

283   Only the T_{br-80} data were analyzed using the Kruskal-Wallis tests due to normality

284   assumption violation. The TPC fitting was performed using the ThermPerf package for R

285   (https://github.com/mdjbru-R packages/thermPerf).

286

287   **Results**

288   **Evolutionary response of the upper thermal limit**

289   Artificial selection for increasing heat tolerance resulted that the knockdown temperature

290   evolved in both thermal selection treatments. We found that knockdown temperature was

291   significant higher for the acute-selected lines in comparison to control lines ($\bar{X}_{acute} \pm SD =$

292   37.71 ± 0.68 ºC and $\bar{X}_{control} \pm SD = 37.19 \pm 0.90$ ºC; $F_{1,4} = 36.2$, $P = 0.004$; Fig 2). Similarly,

293   chronic-selected lines showed a significant increase of knockdown temperature in

294   comparison to control lines ($\bar{X}_{chronic} \pm SD = 35.48 \pm 0.66$ ºC and $\bar{X}_{control} \pm SD = 34.97 \pm 0.72$

295   ºC; $F_{1,4} = 41.7$, $P = 0.003$; Fig. 2). On the other hand, we found no significant differences

296   on the knockdown temperatures among replicated lines for acute thermal stress ($\chi^2_2 = 1.9$ x

297   10^{-7}, $P = 0.99$) neither for chronic thermal stress ($\chi^2_2 = 3.3$ x 10^{-7}, $P = 0.99$).

298   We found that the heat tolerance showed a significant different evolutionary rate

299   from zero, both for acute-selected ($h_{acute,control} \pm SD = 0.061 \pm 0.008$, $t_2 = 13.30$, $P = 0.006$)

300   and chronic-selected lines ($h_{chronic-control} \pm SD = 0.060 \pm 0.004$, $t_2 = 26.99$, $P = 0.001$).

301   However, evolutionary rates were similar between both thermal selection treatments

302   (Kruskal-Wallis, $\chi^2_2 = 2.0$, $P = 0.37$). Additionally, the realized heritabilities of the

303   knockdown temperatures were significantly different from zero for acute-selected ($h^2_{pooled-}$

304    $_{replicates} = 0.180 \pm 0.013$, $t_1 = 13.75$, $P < 0.0001$) and chronic-selected lines ($h^2_{pooled-replicates} =$

305   0.226 ± 0.020, $t_1 = 11.46$, $P < 0.0001$). Despite the realized heritability was higher for

306   chronic-selected lines, it was not significantly different from those estimated for acute-

307   selected lines ($F_{1,20} = 0.28$, $P = 0.60$).

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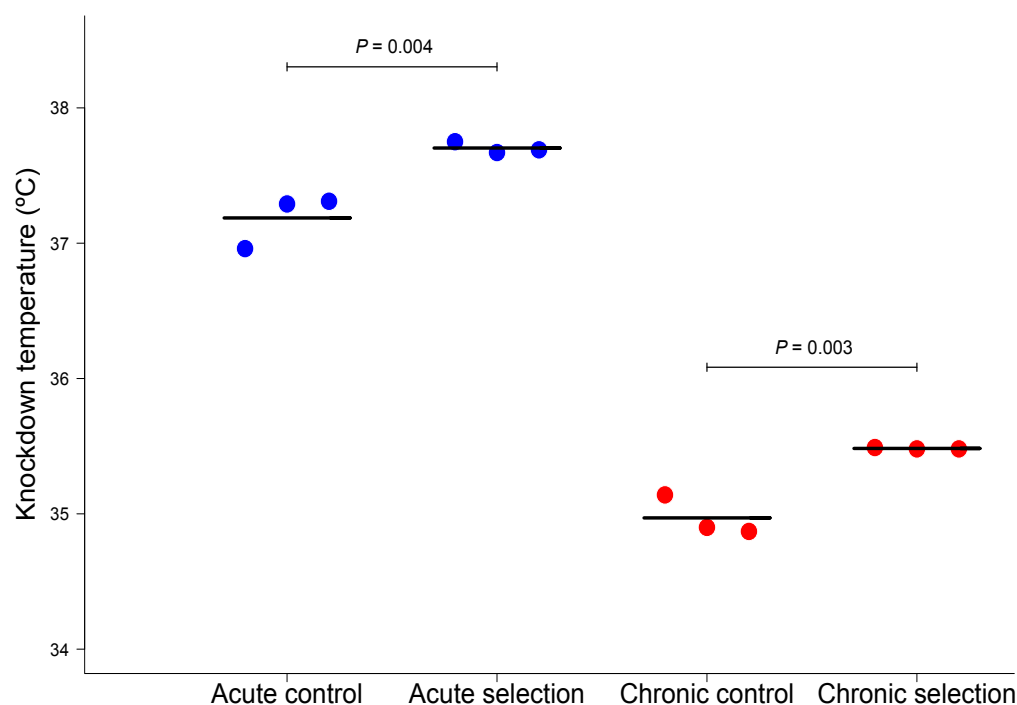
Figure 2. Knockdown temperature (°C) of acute-control and acute-selected lines (blue) and chronic-control and chronic-selected lines (red) after 16 generations of artificial selection for increasing heat tolerance in *Drosophila subobscura*. Dots represent the mean value for each replicated line, and black lines represent the overall mean of each selection or control treatment. *P*-values were estimated using a mixed-linear model with selection treatment as fixed effect and replicated line as random effects nested within selection treatment.

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TPC correlated evolution

Because locomotor performance was evaluated in at generation 25 (after nine generations without thermal selection on heat tolerance), we evaluated the knockdown temperature in all experimental lines before the climbing assays with the same protocol previously applied. Similarly to our findings found at generation 16, we found that knockdown temperatures of the acute-selected lines were significantly higher than those exhibited for the control lines ($\bar{X}_{acute} \pm$ S.D. $= 38.59 \pm 0.98$ ºC and $\bar{X}_{control} \pm$ S.D. $= 37.61 \pm 1.45$ ºC; $F_{1,4} = 56.4$, $P = 0.002$), and chronic-selected lines also showed a higher knockdown temperature than control lines ($\bar{X}_{chronic} \pm$ S.D. $= 35.92 \pm 0.67$ ºC and $\bar{X}_{control} \pm$ S.D. $= 35.14 \pm 0.81$ ºC; $F_{1,4} = 83.5$, $P = 0.001$).

We found that some TPC parameters were significantly influenced by the thermal selection regime (Table 1 for parameter estimated using the Lactin model. Table S3 and S4 show the same parameter estimated using the Performance and modified Gaussian models, respectively). For instance, T_{opt} was significantly influenced by thermal selection ($F_{2,6} = 6.9$, $P = 0.02$). Interestingly, acute-selected lines showed a higher T_{opt} than control lines (Table 1; Fig. 3; HSD Tukey: $P = 0.02$), chronic-selected lines exhibited a similar T_{opt} than control lines (Table 1; Fig. 3; HSD Tukey: $P = 0.269$), and acute-selected and chronic-selected lines did not differ between them (Table 1; Fig. 3; HSD Tukey: $P = 0.197$). Additionally, temperatures delimiting the thermal breadth for the 50% and 80% of upper performance (T_{br-50} and T_{br-80}, respectively) were significantly affected by thermal selection Table 1; Fig. 3). Specifically, initial and final temperatures of T_{br-50} (Table 1) were significantly different between experimental lines (T_{br-50s}: $F_{2,6} = 9.89$, $P = 0.01$; and T_{br-50f}: $F_{2,6} = 10.1$, $P = 0.01$, respectively), similarly to initial and final temperature of T_{br-80} (T_{br-80s}: $F_{2,6} = 10.01$, $P = 0.01$; and T_{br-80f}: $F_{2,6} = 10.02$, $P = 0.01$). We found that delimiting

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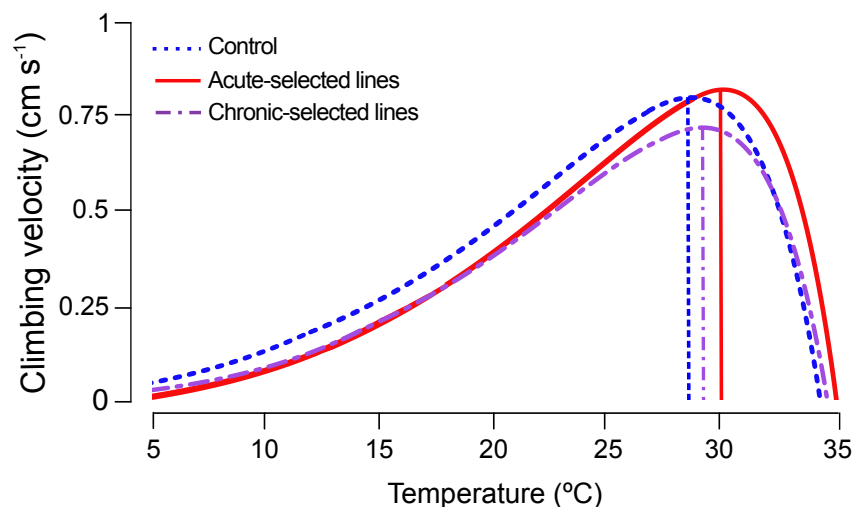
Figure 3. Thermal performance curves estimated from the climbing velocity measured at 6 temperatures (5, 15, 20, 25, 30 and 35 ºC) for acute-selected (red), chronic-selected (purple) and control lines (blue) from an artificial selection experiment for increasing heat tolerance in *Drosophila subobscura*. Vertical lines represent the averaged optimal temperature (n = 3) for each selection treatment. Climbing velocity for each replicated line of each selection treatments can be visualized in the Fig. S1.

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354

355   **Table 1**. Parameters (mean ± S.E.) of the thermal performance curve (estimated as

356   climbing velocity) estimated with the Lactin model in acute-selected, chronic-selected and

357   control lines in an artificial selection experiment for increasing heat tolerance in *Drosophila*

358   *subobscura*.

	Acute-selected lines	Chronic-selected lines	Control lines
T_{opt} (°C)	30.08 ± 0.73	29.22 ± 0.52	28.46 ± 0.24
μ_{max} (cm s^{-1})	0.80 ± 0.08	0.68 ± 0.07	0.80 ± 0.12
T_{br-50} (°C)	14.99 ± 0.01	14.99 ± 0.01	14.98 ± 0.02
T_{br-50s} (°C)	19.12 ± 0.15	18.78 ± 0.31	18.32 ± 0.16
T_{br-50f} (°C)	34.11 ± 0.15	33.77 ± 0.31	33.30 ± 0.16
T_{br-80} (°C)	5.99 ± 0.01	5.99 ± 0.01	5.99 ± 0.01
T_{br-80s} (°C)	26.25 ± 0.26	25.71 ± 0.48	25.01 ± 0.22
T_{br-80f} (°C)	32.24 ± 0.26	31.70 ± 0.48	30.99 ± 0.22

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360   temperatures were significantly higher for acute-selected lines than for control lines (HSD

361   Tukey: T_{br-50s}: $P = 0.01$; T_{br-50f}: $P = 0.01$; T_{br-80s}: $P = 0.01$; T_{br-80f}: $P = 0.01$). Whereas,

362   these parameters were similar between chronic-selected and control lines (HSD Tukey: T_{br-}

363    $_{50s}$: $P = 0.231$; T_{br-50f}: $P = 0.23$; T_{br-80s}: $P = 0.204$; T_{br-80f}: $P = 0.205$), and they also were

364   similar between both thermal selected lines (HSD Tukey: T_{br-50s}: $P = 0.093$; T_{br-50f}: $P =$

365   0.088; T_{br-80s}: $P = 0.101$; T_{br-80f}: $P = 0.1$). Finally, the maximum performance (μ_{max}) was not

366   significantly affected by the thermal selection ($F_{2,6} = 1.77$, $P = 0.25$), similarly to the

367   response exhibited by the thermal breadth for the 50% ($F_{2,6} = 0.64$, $P = 0.56$) and 80% of

368   upper performance ($F_{2,6} = 0.80$, $P = 0.49$). Summarizing, our artificial selection experiment

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369   for increasing heat tolerance of *D. subobscura* resulted in a correlated response on the TPC,

370   but only for the acute-selected lines. Therefore, an evolutionary increase of the heat

371   tolerance induced a displacement of TPC to higher temperatures without changes on its

372   topology (Fig. 3).

373

374   Discussion

375   When evaluating the effects of global warming on distributions and survival of ectotherm

376   organisms becomes essential to contrast and predict the evolutionary responses of the

377   thermal limits across populations (Panetta et al., 2018). In the present study, we evaluated

378   the evolutionary response of the upper thermal limit in *D. subobscura* populations selected

379   for an increasing heat tolerance at different thermal stress intensities. We demonstrated that

380   upper thermal limits of *D. subobscura* evolved independently of thermal stress intensity,

381   suggesting that thermal tolerance to high temperatures has enough genetic variation to

382   respond to an increase in temperatures due to global warming. Our results are particularly

383   interesting because the arrival of *D. subobscura* to South America from Europe was

384   characterized by a strong founder effect due to reduced number of colonizing individuals,

385   resulting in a reduced genetic variation before the expansion along the colonized

386   geographical range in Chile (Pascual et al., 2007). Despite that, *D. subobscura* exhibits

387   signals of thermal adaption only a few decades after of its introduction in South America

388   (Brncic et al. 1981; Ayala et al., 1989; Huey et al., 2000; Castañeda et al., 2013, 2015).

389   Additionally, the evolutionary response of heat tolerance resulted after artificial thermal

390   selection performed only in females, which should reduce the selection effectiveness near

391   to 50% (Huey & Kingsolver, 1993).

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392   The evolution of the upper thermal limit reported in this work agrees with previous

393   increases of CT_{max} in experiments of artificial selection in *D. melanogaster* and *D. buzzatii*

394   (Bubliy and Loeschcke, 2005; Folk et al., 2006; Sambucetti et al., 2010; Hangartner and

395   Hoffmann et al., 2016), but contrasts with the multiple evidences of a limited evolutionary

396   potential to heat tolerance in the *Drosophila* clade (Chown et al., 2009; Mitchell and

397   Hoffmann, 2012; Kellerman et al., 2012; Blackburn et al., 2014; van Heerwaarden et al.,

398   2015; MacLean et al., 2019). In this sense, unlike studies of artificial selection,

399   comparatives studies provide with a wide vision of thermal evolution in the *Drosophila*,

400   suggesting that the evolution of heat tolerance is severely constrained (Kellerman et al.,

401   2012; MacLean et al., 2019). However, it has been widely demonstrated that the capacity

402   for detecting evolutionary patterns of CT_{max} in intra- and interspecific studies can be

403   hindered by uncontrolled factors as the intensity of thermal stress because chronic thermal

404   stress decreases the accuracy to detect these patterns (Rezende et al., 2014; Chown et al.,

405   2009; Mitchell and Hoffmann 2010; Blackburn et al., 2014; Kingsolver and Umbanhowar,

406   2018; Sunday et al., 2019; Castañeda et al., 2019; Kovacevic et al., 2019). In fact, genetic

407   quantitative studies in *Drosophila* have demonstrated that long thermal assays (e.g. chronic

408   stress or slow ramping assays) provide a lower estimations of the evolutionary potential for

409   heat tolerance compared to short thermal assays (e.g. acute stress or fast ramping assays).

410   This is probably because long assays increase the environmental variance of heat tolerance

411   and then, heritability estimates become lower (Chown et al., 2009; Mitchell and Hoffmann

412   2010; Blackburn et al., 2014; Castañeda et al. 2019). According to this, the evolutionary

413   capacity of heat tolerance should depends on the intensity of thermal stress thus we

414   expected a reduced response of the heat tolerance when the artificial selection was

415   performed using a chronic thermal stress. Despite these predictions, we did not find

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416   significant differences in the evolutionary change rate or realized heritability of heat

417   tolerance between thermal selection treatments (averaged $h^2_n = 0.18$ for acute-selected

418   lines, and averaged $h^2_n = 0.23$ for chronic-selected lines). These realized heritabilities are

419   closer to the mean heritability reported for terrestrial ectotherms ($h^2 = 0.28$; Diamond,

420   2017), whereas the heritability estimated for chronic-selected lines was even higher than

421   those previously reported in studies of thermal selection or using quantitative genetics

422   (Gilchrist et al., 1999; Mitchell & Hoffmann, 2010; Blackburn et al., 2014). In a previous

423   work, our group estimated the narrow-sense heritability (h^2) for heat tolerance in *D.*

424   *subobscura* using static (38 ºC) and ramping assays (0.1 ºC min^{-1}) (Castañeda et al., 2019).

425   We found than h^2 using static assay was higher ($h^2 = 0.134$) that those estimated using a

426   slow ramping assay ($h^2 = 0.084$), supporting the hypothesis that acute thermal assays

427   provide a more precise estimation of the genetic component of heat tolerance in comparison

428   to chronic thermal assays.

429   We performed artificial selection on heat tolerance for 16 generations and for

430   logistic reasons, we were able to perform the climbing assays nine generations later. This

431   means that selected lines experience relaxed thermal selection during nine generations.

432   Hence, we checked for differences of heat tolerance between selected and control lines

433   before performing the climbing assays. A plausible result of this analysis could have been

434   that relaxed selection allows a reversion of the evolutionary increase of heat tolerance in

435   selected populations because the high energy costs associated with an increase of

436   thermotolerance (Callahan et al., 2008; Lahti et al., 2009). Previous evidence indicates that

437   selection on heat tolerance results into an increase in heat-shock protein (HSP) levels,

438   which is a well-known thermoprotective mechanism that maintain the cellular homeostasis

439   (Sørensen et al., 1999). However, the increase of the HSP production leads to an increase of

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440   the metabolic demand (Hoekstra & Montooth, 2013) and reduction of the reproductive

441   output (Krebs & Loeschcke, 1994; Krebs & Feder, 1997). Thus, if thermal selection would

442   have led to elevated energy costs associated with an increase of thermotolerance, a

443   reversion of the upper thermal limit should be expected after the relaxation of thermal

444   selection. However, we found that the evolutionary response of the upper thermal limit was

445   maintained after nine generations without thermal selection, suggesting that the

446   evolutionary increase of the heat tolerance in *D. subobscura* did not involve an increase of

447   maintenance costs. These results agree with previous studies in *D. melanogaster* (Williams

448   et al., 2012) and in the copepod *Trigriopus californicus* (Kelly et al., 2013), in which the

449   evolution of heat tolerance was principally limited by the presence of genetic variation,

450   showing no tradeoff or cost that limit their evolutionary responses. Moreover, we also

451   found that metabolic rate did not differ between selected and control lines and moreover,

452   fecundity increased in the selected lines compared to control lines (A. Mesas, unpublished

453   results).

454   Evolutionary theory predicts that thermal performance of ectotherms should evolve

455   in response to environmental temperatures (Huey and Kingsolver, 1989; Angilletta, 2009).

456   High environmental temperatures are expected to impose selective pressures on natural

457   populations, resulting in evolution of heat tolerance and TPC (Huey & Kingsolver, 1993).

458   To our knowledge, the present work shows for first time the evolution of TPC as response

459   to artificial selection on CT_{max}, suggesting that performance at low and high temperatures

460   could be negatively correlated. Additionally, the evolutionary response of TPC depended

461   on thermal stress intensity employed during thermal selection. Specifically, we found a

462   displacement of the TPC to higher temperatures only in the acute-selected lines, suggesting

463   that associations between different TPC parameters are more likely to be detected using

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464   acute thermal assays, especially for heritability estimates (Mitchell and Hoffmann, 2010;

465   Blackburn et al., 2014; van Heerwaarden & Sgrò, 2014; Castañeda et al., 2019). This TPC

466   displacement to high temperatures is according with one of the scenarios proposed by Huey

467   and Kingsolver (1993), in contrast to other two well-supported scenarios such as "hotter is

468   better" (Angilletta et al., 2010; Nati et al., 2016) and "jack-of-all-temperatures is a master-

469   of-none" (Huey & Hertz 1984; Gilchrist, 1995). Our results also agree with previous results

470   of thermal selection experiments in *Drosophila* species (Huey & Kingsolver, 1993; Bubliy

471   & Loeschcke, 2005; Mori & Kimura, 2008; Diamond et al., 2017) and in ectotherm

472   vertebrates as *Anolis sagrei* (Logan et al., 2014), suggesting that the natural selection

473   mediated by high temperatures is strong enough to produce changes in thermal performance

474   of ectotherm species (but see Gilbert & Miles, 2017).

475   In conclusion, the intensity of thermal stress has important effects on the evolution

476   of thermal physiology in ectotherms, indicating that different thermal scenarios might yield

477   a similar evolution for heat tolerance but having contrasting consequence on thermal

478   performance. Therefore, the intensity of thermal stress could also have important

479   consequences on the estimations of vulnerability of ectotherms to global warming.

480

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488

Author contribution

490 AM designed experiments and conducted experiments and statistical analyses, and wrote

491 the manuscript. AJ maintained the experimental animals, performed experiments and

492 approved the final version of the manuscript. LEC conceived the original idea, designed

493 experiments, conducted statistical analyses, provided funds for all experiments, and heavily

494 edited and wrote the manuscript.

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