Cross-tolerance evolution is driven by selection on heat tolerance in *Drosophila subobscura*

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ABSTRACT

The evolution of heat tolerance is a crucial mechanism for the adaptive response to global warming, but it depends on the genetic variance carried by populations and on the intensity of thermal stress in nature. Experimental selection studies have greatly benefited research into heat tolerance, providing valuable insights into its evolutionary process. However, the impact of varying levels of heat stress intensity on the associated changes in resistance traits has not yet been explored. Here, the correlated evolution of increasing knockdown temperature in *Drosophila subobscura* was evaluated on the knockdown time at different stress temperatures (35, 36, 37, and 38 ºC), thermal death time (TDT) curves, and desiccation and starvation resistance. The selection of heat tolerance was performed using different ramping temperatures to compare the impact of heat intensity selection on resistance traits. Correlated evolution was found for these four resistance traits in *D. subobscura*, indicating that the evolutionary response to tolerance of higher temperatures also confers the ability to tolerate other stresses such as desiccation and starvation. However, these correlated responses depended on the

intensity of thermal selection and sex, which may limit our ability to generalize these results to natural scenarios. Nevertheless, this study confirms the value of the experimental evolutionary approach for exploring and understanding the adaptive responses of natural populations to global warming.

Keywords: correlated evolution, global warming, heat stress intensity, stress resistance evolution, thermal tolerance landscape.

INTRODUCTION

Rising environmental temperatures are a major challenge for ectotherms (i.e., organisms whose body temperature depends on the ambient temperature) because their morphology, physiology, behavior, and performance depend on the thermal environment (Huey and Stevenson 1979; Cossins and Bowler 1987; Angilletta 2009). Furthermore, rising environmental temperatures increase the risk of extinction for many species living near their upper thermal limits (Deutsch et al. 2008; Huey et al. 2009; Hoffmann and Sgrò 2011). However, ectotherms can avoid the negative effects of heat through behavioral thermoregulation, evolutionary change, and/or phenotypic plasticity of the upper thermal limits (Visser 2008).

Evolutionary adaptation depends on the genetic variation exhibited by upper thermal limits; however, some studies have suggested that heat tolerance has a limited evolutionary potential to respond to increasing environmental temperatures (Chown et al. 2009; Mitchell and Hoffmann 2010; Kellermann et al. 2012). Yet, theoretical and empirical evidence suggests that heritability estimates for heat tolerance tend to be lower when heat tolerance is measured in longer assays (e.g., slow-ramping assays or static assays using sublethal temperatures) than in shorter assays (e.g., fast-ramping

assays or static assays using extremely high temperatures) (Chown et al. 2009; Mitchell and Hoffmann 2010; Rezende et al. 2011; Blackburn et al. 2014; Heerwaarden et al. 2016; Castañeda et al. 2019). Thus, the intensity of heat stress may influence our predictions regarding the evolutionary potential of heat tolerance, but how do populations respond to variable selection driven by heat stress? Selection under laboratory conditions has a long history of providing information on the adaptive evolution of specific selective agents (Lenski and Bennett 1993; Garland Jr 2003; Fuller et al. 2005; Gibbs and Gefen 2009). In particular, the experimental evolution of heat tolerance has been assessed in several species, including fish, corals, and insects (Baer and Travis 2000; Kelly et al. 2012; Geerts et al. 2015; Esperk et al. 2016). Experimental evolution of heat tolerance has also been studied in several *Drosophila* species, *including D. melanogaster* (Gilchrist and Huey 1999; Folk et al. 2006), *D. subobscura* (Quintana and Prevosti 1990; Mesas et al. 2021; Mesas and Castañeda 2023), and *D. buzzatti* (Krebs and Loeschcke 1996). Most of these studies reported the evolution of heat tolerance using fast ramping protocols, ranging from 0.4 ºC/min in Folk et al. (Folk et al. 2006) to 1 °C/min in Gilchrist and Huey (Gilchrist and Huey 1999) or static high-temperature assays (40 °C), as in Bubliy and Loeschcke (2005). Recently, Mesas et al. (Mesas et al. 2021) reported that selected lines of *D. subobscura* evolved higher heat tolerance, regardless of the heating rate used during the selection experiments (slow-ramping rate: 0.08 °C/min and fast-ramping rate: 0.4 °C/min).

Interestingly, several of these selection experiments on heat tolerance in *Drosophila* have found correlated responses in other traits such as starvation resistance, desiccation resistance, and heat shock proteins (Hoffmann et al. 1997; Feder et al. 2002; Bubliy and Loeschcke 2005). However, the intensity of thermal stress is expected to have important effects on the correlated responses of other traits to heat tolerance

selection (Fragata and Simões 2022). For example, fast-ramping selected lines have evolved thermal performance curves with higher optimum temperatures and narrower thermal breadths than slow-ramping selected lines (Mesas et al. 2021). In addition, Mesas and Castañeda (Mesas and Castañeda 2023) reported that the evolution of heat tolerance was associated with reduced activity of the enzymes involved in the glucose-6-phosphate branch point and increased performance of life-history traits in slow-ramping selected lines. However, they did not observe any changes in the metabolic rate of the selected lines, as predicted by Santos et al. (2012). In summary, there is evidence that heat stress intensity determines the magnitude of the evolutionary responses of performance, metabolic, and life-history traits to heat tolerance selection; however, the correlated evolution of resistance traits has not yet been tested. This information should explain how thermal stress intensity might determine the cross-tolerance evolution to stressful environmental conditions. Natural populations are regularly subjected to multiple environmental stressors, and it is well-established that enhanced tolerance to one stressor can enhance tolerance to another (Rodgers and Gomez Isaza 2023). Cross-tolerance induced by thermal stress has been widely studied in several arthropod species, increasing resistance to desiccation, insecticides, and pathogens (Kalra et al. 2017; Rodgers and Gomez Isaza 2021; Singh et al. 2022). However, the cross-tolerance patterns at the evolutionary level can be constrained or facilitated by genetic correlations among resistance traits depending on the environmental context (Lande and Arnold 1983; Bubliy and Loeschcke 2005; Gerken et al. 2016).

Previous research has examined the impact of varying levels of heat stress on the heat knockdown temperature of *D. subobscura*, as well as its associated impacts on thermal performance curves (Mesas et al. 2021), energy metabolism, and fitness-related traits (Mesas and Castañeda 2023). The evolutionary response of these traits was

evaluated using two thermal selection protocols that differed in the rate of temperature increase (hereafter, ramping rate) to measure the heat knockdown temperature: slow-ramping selection (0.08ºC min^{-1}) and fast-ramping selection (0.4ºC min^{-1}). The present study investigates the effects of heat intensity selection for increasing knockdown temperature on the cross-tolerance evolution of four different resistance traits in *D. subobscura*: knockdown time at different stress temperatures, thermal-death-time curves (TDT), desiccation resistance, and starvation resistance. In particular, TDT curves represent an integrative approach to assess how the probability of survival depends on the intensity and duration of heat stress, as they allow the estimation of the critical thermal maxima (CT_{max}) and thermal sensitivity using the thermal tolerance measurements obtained at different stress temperatures (Rezende et al. 2014). Here, it is expected that fast-ramping selected lines will exhibit higher knockdown time at highly stressful temperatures and higher CT_{max} because fast-ramping protocols reduce the confounding effects (e.g., hardening, rate of resource use) on heat tolerance associated with the assay length (see Rezende et al. 2011; Santos et al. 2012; Mesas et al. 2021). In contrast, slow-ramping selected lines should exhibit higher desiccation and starvation resistance because individuals with higher starvation and desiccation resistance exhibit higher thermal tolerance during long assays.

Materials and Methods

Sampling and maintenance

D. subobscura females were collected in the spring 2014 at the Botanical Garden of the Universidad Austral de Chile (Valdivia, Chile; 39º 48’ S, 73º 14’ W) using plastic traps containing banana/yeast baits. Two hundred females were collected and placed individually in plastic vials containing David’s killed-yeast *Drosophila* medium to

126 establish isofemale lines. In the next generation, 100 isofemale lines were randomly
127 selected, and 10 females and 10 males per line were placed in an acrylic cage to
128 establish a large, outbred population. In the next generation, the flies from this cage
129 were divided into three population cages (R1, R2, and R3), attempting to assign the
130 same number of flies to each cage. After three generations, the flies in each replicate
131 cage were divided into four population cages, trying to assign the same number of flies
132 to each cage. This procedure established 12 population cages assigned to each
133 artificial selection protocol in triplicate: fast-ramping selection, fast-ramping control,
134 slow-ramping selection, and slow-ramping control lines (Fig. S1). During the selection
135 experiments, population cages were maintained at 18 °C (12:12 light-dark cycle) in a
136 discrete generation, controlled larval density regime (Castañeda et al. 2015). Each
137 population cage had a population size of 1000-1500 breeding adults.
138
139 ***Heat tolerance selection***
140 For each replicate line, 120 four-day-old virgin females were randomly mated with
141 two males for two days, after which the females were individually placed in a capped
142 5-mL glass vial, and the males were discarded. The vials were attached to a plastic
143 rack and immersed in a water tank with an initial temperature of 28 °C, controlled by a
144 heating unit (model ED, Julabo Labortechnik, Seelbach, Germany). After an
145 equilibration period of 10 min, the temperature was increased to 0.08 ºC min^{-1} for the
146 slow-ramping selection protocol or 0.4 ºC min^{-1} for the fast-ramping selection
147 protocol. Assays were stopped when all flies collapsed. Each assay was recorded using
148 a high-resolution camera (model D5100, Nikon, Tokyo, Japan) and then visualized to
149 score the knockdown temperature for each fly, defined as the temperature at which
150 each fly ceased to move. Flies were ranked by knockdown temperature, and four

151 virgin females were selected from the progeny of the 40 flies with the highest
152 knockdown temperature (top 30% of each assay) to establish the next generation. For
153 the fast and slow control lines, the knockdown temperature was measured as described
154 above, but the progeny was randomly selected to establish the next generation,
155 regardless of the knockdown temperature of their mother.
156 This artificial selection experiment was performed for 16 generations, after which
157 flies from each selection treatment were placed in separate acrylic cages and
158 maintained without selection (e.g., relaxed selection) at 18 °C and a 12:12 light-dark
159 cycle.
160
161 ***Knockdown time in static assays***
162 Eggs were collected from each population cage and transferred to vials at a density of
163 40 eggs/vial. At 4 days of age, ten females and ten males from each population cage
164 were tested to measure their heat knockdown time at four different static temperatures:
165 35, 36, 37, and 38°C. This experimental design allowed the measurement of 960 flies
166 (10 flies × 2 sexes × 4 static temperatures × 4 selection treatments × 3 replicated lines).
167 Static assays were performed similarly to knockdown temperature assays, but static
168 temperatures were used instead of ramping temperatures. A total of 240 flies were
169 measured for each static temperature, except for the assay at 35°C (178 flies) because
170 two flies died before the start of the assay, and a video file of one assay was corrupted
171 (data for 60 flies were lost). For the 37°C assay, four flies died before the assay began,
172 and the collapse time could not be measured for six flies. Finally, for the 38ºC assay,
173 three flies died before the start of the assay and the collapse time could not be measured
174 for five flies. Heat knockdown assays were performed in generation 23 (Fig. S1).
175

176 ***Desiccation and starvation resistance***

177 Eggs from each replicate cage were collected and maintained in vials at a density of 40
178 eggs/vial. Only fast control lines were measured as control lines. This decision was
179 based on logistical reasons (i.e., the high number of vials) and statistical support
180 because fast and slow control lines did not differ in their knockdown times and CT_{max}
181 values (see *the Results* section).

182 For desiccation resistance assays, five flies from each sex were separately placed
183 in a vial containing five desiccant droplets (Drierite) and sealed with parafilm (flies had
184 no access to food or water during the assay). For starvation resistance assays, five flies
185 from each sex were separately placed in a vial containing agar only (flies had access to
186 water but no food). For both desiccation and starvation resistance assays, the number of
187 live flies was counted every 3 h until all the flies were dead. Desiccation and starvation
188 resistance were measured in 126 vials containing 10 flies each, respectively (7 vials × 2
189 sexes × 3 selection treatments × 3 replicate lines). These experiments were conducted at
190 18 °C using flies from generation 24 (Fig. S1).

191

192 **Statistical analysis**

193 Normality and homoscedasticity were tested for all variables, and the knockdown times
194 were squared root transformed to meet the parametric assumptions. All analyses were
195 performed with R software (R Development Core Team 2011).

196

197 ***Heat tolerance***

198 For the knockdown temperature, control and selection lines were compared separately
199 for the fast- and slow-ramping selection because it is well known that the knockdown
200 temperature is higher in fast-ramping than in slow-ramping assays (Chown et al. 2009;

201 see Mesas et al. 2021). For the knockdown time analysis, a mixed linear model with
202 ramping selection (fixed effect with fast-control, slow-control, fast-selection, and slow-
203 selection lines as levels), sex (fixed effect with females and males as levels), and
204 replicate lines nested within the thermal selection (random effect with replicates 1, 2
205 and 3 as levels) was performed using the library *lme4* package for R (Bates et al. 2015).
206 Fixed effects were tested by a type III ANOVA and the random effect was tested by a
207 likelihood ratio test comparing the model with and without the replicate lines. Both tests
208 were performed using the library *lmerTest* package for R (Kuznetsova et al. 2017). If
209 the selection effect was significant, *a posteriori* comparisons were performed using
210 false discovery rate adjustment implemented in the *emmeans* package for R (Lenth et al.
211 2018).
212 Knockdown times were also used to plot the survival curves based on the
213 Kaplan-Meier formula using the *survfit* function implemented in the *survival* package
214 for R (Therneau 2023).
215
216 ***Thermal death time curves (TDT)***
217 Average knockdown times were calculated for each sex, replicate lines, and selection
218 treatment combination (Table S1). These values were regressed against the assay
219 temperatures according to Equation 1 (Rezende et al. 2014):
220
221 $$log_{10}t = \frac{CT_{max}-T}{z} \qquad \text{eqn. 1}$$
222
223 , where T is the assay static temperature (ºC), $CT_{\max}$ is the upper thermal limit (ºC), t is
224 the knockdown time (min), and z is the thermal sensitivity. These curves allowed the
225 estimation of $CT_{\max}$ as the extrapolated temperature that would result in a knockdown

226 time of $\log_{10} t = 0$ (i.e., knockdown time at 1 min) and the estimation of the thermal
227 sensitivity ($z = -1/\text{slope}$), where the lower z values, the higher the thermal sensitivity.
228 Using equation 1, 24 TDT curves (2 sexes × 3 replicate lines × 4 selection
229 protocols) were fitted, from which CT_{max} and z values were estimated as described
230 above. A linear model with ramping selection treatment (levels: fast-control, slow-
231 control, fast-selection, and slow-selection lines), sex (levels: females and males), and
232 their interaction was performed to evaluate their effects on CT_{max} and z values. TDT
233 curve analysis did not include replicate lines as a random effect because only one CT_{max}
234 and z value was estimated by each replicate line. Additionally, a linear mixed model
235 with ramping selection (fixed effect with fast-control, slow-control, fast-selection, and
236 slow-selection lines as levels), sex (fixed effect with females and males as levels), and
237 replicate lines nested within the thermal selection (random effect with replicates 1, 2
238 and 3 as levels), and assay temperatures (as covariate) was fitted on the knockdown
239 time using the *lmer* package for R.
240
241 ***Desiccation and starvation resistance***
242 To determine the lethal time at which 50% of flies of each vial were dead (LT_{50}), a
243 generalized linear model following a binomial distribution was fitted with the
244 proportion of flies alive as the dependent variable and time as the predictor variable.
245 The generalized linear model was run using the g*lm* function of the *lme4* package for R
246 (Bates et al. 2015). The LT_{50} of each vial was then estimated using the function *dose.p*
247 from the *MASS* package for R (Venables and Ripley 2002).
248 To estimate the median LT_{50} and the 95% confidence intervals for each selection
249 treatment and sex, each LT_{50} was transformed into a survival object using the *Surv* and
250 *survfit* functions of the *survival* package for R (Therneau 2023). This procedure also

allowed to estimate the survival curves in each vial. Finally, to test the effect of selection treatment (levels: control, fast-selection, and slow-selection lines) and sex (levels: females and males) on desiccation and starvation resistance, a Cox proportional regression model was fitted with LT_{50} as the dependent variable, and selection protocol and sex as predictor variables. The Cox model was run using the *coxph* function of the *survival* package (Therneau 2023).

RESULTS

Knockdown temperature evolution

Knockdown temperature evolved in response to artificial selection for increased heat tolerance, regardless of the ramping assay protocol: the knockdown temperature was significantly higher in fast-ramping selected lines than in fast-ramping control lines (mean fast-ramping selected lines [95% CI] = 37.71 ºC [37.63 – 37.78] and mean fast-ramping control lines [95% CI] = 37.23 ºC [37.0 – 37.38]; $F_{1,4} = 32.0$, $P = 0.005$); and the knockdown temperature in slow-ramping selected lines was significantly higher than in slow-ramping control lines (mean slow-ramping selected lines [95% CI] = 35.48ºC [35.41 – 35.55] and mean fast-ramping control lines [95% CI] = 34.97 ºC [34.82 – 35.12]; $F_{1,4} = 41.7$, $P = 0.003$). These results were previously reported by Mesas et al. (2021) and are reported here to show that selected lines used in this study evolved higher thermal tolerance compared to control lines.

Knockdown time evolution

As expected, the knockdown time decreased significantly as the assay temperatures increased ($F_{1,877} = 649.1$, $P < 2\times10^{-16}$). The mean knockdown time and 95% CI for each

275 static assay are as follows: 35º C = 33.77 min [32.1 – 35.5]; 36º C = 16.98 min [16.1
276 – 17.9]; 37º C = 8.84 min [8.4 – 9.3]; and 38º C = 6.78 min [6.3 – 7.0].
277 Knockdown times differed significantly between selection treatments when flies
278 were assayed at 36 and 37ºC (Table 1; Table S2; Fig. 1). At these temperatures, slow
279 and fast selected lines showed higher heat tolerance than slow and fast control lines
280 (Table S1- S3; Fig. 1C, E). Also, fast-selected lines showed a higher heat tolerance than
281 slow-selected lines in flies assayed at 37ºC but not at 36ºC (Table S1-S3; Fig. 1C, E),
282 whereas fast and slow control lines did not differ (Table S3; Fig 1). On the other hand,
283 replicate lines had no significant effect on knockdown time, indicating consistent
284 evolutionary responses within each selection and control treatment (Table S2).
285 Concerning sex, females showed a higher thermal tolerance than males but only when
286 flies were assayed at 35 and 38ºC (Table 1; Fig. 1B, H). Finally, non-significant
287 interactions between selection and sex were found for all assay temperatures (Table 1).
288
289 ***TDT curves evolution***
290 Linear regressions between $\log_{10}(LT_{50})$ and assay temperatures enabled the estimation
291 of 24 TDT curves (4 selection treatments × 3 replicate lines × 2 sexes) with high
292 coefficients of determination (mean R^2 = 0.946, range: 0.820 – 0.989; Table S4),
293 confirming that heat knockdown time is linearly related to stressful sublethal
294 temperatures. From these TDT curves, the mean CT_{max} [95% CI] was 41.21ºC [41.02
295 – 41.41], and the mean *z* [95% CI] was 4.18ºC [4.03 – 4.32]. CT_{max} were significantly
296 different between selection treatments ($F_{3,20}$ = 4.46, P = 0.015; Fig. 2A). A post hoc
297 analysis showed that fast-ramping selected and slow-ramping control lines were
298 significantly different in their CT_{max} values (t_{20} = 3.195, P = 0.02). In contrast, fast and
299 slow control lines had similar CT_{max} values (t_{20} = 0.911, P = 0.80). Thus, when control

300 lines are pooled, CT_{max} still differs between selection treatments ($F_{2,18} = 6.69$, $P =$
301 0.007), with fast-ramping (mean CT_{max} [95% CI] = 41.55 ºC [41.2 – 41.9]) and slow-
302 ramping selected lines (mean CT_{max} [95% CI] = 41.43 ºC [41.1 – 41.8]) had higher
303 CT_{max} than control lines (mean CT_{max} [95% CI] = 40.94 ºC [40.7 – 41.2]) ($t_{18} = 3.27$, P
304 $= 0.01$ and $t_{18} = 2.64$, $P = 0.04$, respectively). CT_{max} was not different between the
305 selected lines ($t_{18} = 0.54$, $P = 0.85$). On the other hand, sex and the interaction between
306 selection treatments and sex had no significant effect on CT_{max} ($F_{1,18} = 0.004$, $P = 0.95$
307 and $F_{3,18} = 2.11$, $P = 0.15$, respectively). Regarding z (i.e., thermal sensitivity), it shows
308 no significant effects of selection treatments ($F_{3,16} = 0.91$, $P = 0.46$; Fig. 2), sex ($F_{1,16} =$
309 1.30, $P = 0.27$), nor the interaction between selection treatments and sex ($F_{3,16} = 2.23$, P
310 $= 0.12$). In summary, the evolution of a higher CT_{max} is not associated with an
311 evolutionary change in thermal sensitivity (Fig. 2B). Indeed, the relationship between
312 CT_{max} and z did not change with the evolution of increasing thermal tolerance ($r_{\text{control-lines}}$
313 $= 0.979$ and $r_{\text{selected-lines}} = 0.929$; Z-test = 0.76, $P = 0.45$). Additionally, using a linear
314 mixed model with the assay temperature as a covariate, this result was corroborated by a
315 non-significant interaction between selection treatment and assay temperature ($F_{3,865} =$
316 0.30, $P = 0.82$).
317
318 ***Desiccation resistance evolution***
319 Survival analysis showed a significant effect of sex and selection treatment on
320 desiccation resistance, but not for the interaction of the two effects (Table S5). Males
321 showed a higher risk of desiccation than female flies (hazard ratio = 7.11, $P < 2\times10^{-7}$;
322 Fig. 3). Females showed a significant difference between selected and control lines
323 (LTR: $\chi^2_2 = 6.72$, $P = 0.03$; Fig. 3A). Specifically, females of the slow-ramping
324 selection lines showed a higher desiccation resistance than females of the control lines

325 (hazard ratio = 0.42, $P = 0.009$), whereas females of the fast-ramping selection and
326 control lines showed similar desiccation risk (hazard ratio = 0.56, $P = 0.072$). On the
327 other hand, males showed no differences in desiccation resistance between selected and
328 control lines (LTR: $\chi^2_2 = 1.88$, $P = 0.4$; Fig. 3B).
329
330 ***Starvation resistance evolution***
331 A significant effect of sex, selection treatment, and the interaction between the two
332 effects on starvation resistance was found in the survival analysis (Table S6). Males had
333 a higher risk of starvation than female flies (hazard ratio = 22.75, $P < 1\times10^{-16}$; Fig. 4).
334 In female flies (Fig. 4A), fast-ramping selection and slow-ramping selection lines
335 showed a higher starvation risk than control lines (hazard ratio = 2.37, $P = 0.009$; and
336 hazard ratio = 2.20, $P = 0.014$, respectively). In contrast, male flies had an opposite
337 pattern (Fig. 4B): slow-ramping selection lines had a lower starvation risk than control
338 lines (hazard ratio = 0.50, $P = 0.03$), but nonsignificant differences were found between
339 fast-ramping selection and control lines (hazard ratio = 0.64, $P = 0.16$).
340
341 **Discussion**
342 Studying the evolutionary responses of thermal limits is key to understanding the
343 adaptive responses and evolutionary constraints to global warming. Cross-tolerance
344 studies can provide valuable information on the evolutionary response to multiple
345 environmental stressors. Cross-tolerance evolution has been reported among different
346 resistance traits (Hoffmann and Parsons 1993; Bubliy and Loeschcke 2005; Stazione et
347 al. 2020; Singh et al. 2022), but the magnitude of the evolutionary response could be
348 explained by the trait under direct selection or the stress intensity (Gerken et al. 2016).
349 Here, artificial selection for heat tolerance (i.e., knockdown temperature) resulted in

350 correlated responses in heat knockdown time, the thermal tolerance landscape (TDT
351 curves), desiccation resistance, and starvation resistance. However, these responses
352 depended on the intensity of thermal selection and sex, suggesting that the evolutionary
353 response to tolerate higher temperatures also confers partial tolerance to other stresses
354 such as desiccation and starvation.

355 Different approaches to measuring the upper thermal limit of ectotherms
356 produce different genetic and phenotypic estimates. Fast-ramping assays generally
357 estimate higher upper thermal limits and higher heritabilities than slow-ramping assays
358 (Chown et al. 2009; Rezende et al. 2011). For instance, the heritability of thermal
359 tolerance was 0.13 for fast assays and 0.08 for slow assays in *D. subobscura* (Castañeda
360 et al. 2019). Because heritability is commonly used as a predictor of the evolutionary
361 response of a trait to natural or artificial selection, the evolutionary response of heat
362 tolerance would be expected to depend on the ramping rate used during selection.
363 However, previous work did not support this prediction for *D. subobscura*, finding that
364 the evolution of heat tolerance was independent of the ramping rate (Mesas et al. 2021),
365 but the correlated responses of the thermal performance curves or the energy
366 metabolism depended on the intensity of the thermal selection (Mesas et al. 2021;
367 Mesas and Castañeda 2023). In the present study, the evolution of knockdown
368 temperature (e.g., heat tolerance measured in dynamic assays) induced a correlated
369 response on the heat knockdown time (e.g., heat tolerance measured in static assays)
370 when it was assayed at intermediate temperatures (36 and 37ºC), but not at less or more
371 extreme assay temperatures (35 and 38ºC). These findings can be explained because
372 stress tolerance at 35ºC should depend on the physiological state of the organism during
373 prolonged thermal assays (e.g., availability of energy resources; see Rezende et al.
374 2011, but also see Overgaard et al. 2012) and not only on heat tolerance, whereas heat

375 tolerance at 38ºC could be limited by physical properties of ectotherms (e.g., protein
376 denaturation, membrane permeability). However, a previous study found a clinal pattern
377 for heat tolerance in *D. subobscura* only for flies assayed in static assays (specifically at
378 38ºC), but this clinal pattern was not detected using ramping assays (Castañeda et al.
379 2015). Differences between these two studies could be explained by the number of
380 generations under thermal selection, which could result in a different evolutionary
381 response of heat tolerance. According to Begon (1976), *D. subobscura* can have
382 between 4 and 6 generations per year, which makes it possible to estimate about 125
383 generations of selection from the introduction of *D. subobcura* in Chile until the study
384 by Castañeda et al. (2015). On the other hand, the type of selection is completely
385 different between the two studies (e.g., natural versus artificial selection), which could
386 lead to various evolutionary outcomes. In any case, beyond these results from specific
387 thermal assays, these findings support the idea that (1) the use of a single static
388 temperature would miss genetic or phenotypic effects on heat tolerance, and (2)
389 unifying several knockdown time estimates into a single approach (TDT curves) should
390 be necessary to elucidate genetic and phenotypic patterns of heat tolerance in
391 ectotherms (Rezende et al. 2014; Jørgensen et al. 2021).

392 TDT curves evolved in response to heat tolerance selection in *D. subobscura*.
393 TDT curves showed that fast- and slow-ramping selected lines evolved higher CT_{max}
394 than control lines (ΔCT_{max} = 0.49 ºC). This differential CT_{max} value is slightly lower
395 than the population differences (0.9ºC) observed between the lowest and highest latitude
396 populations (~8 latitudinal degrees) of *D. subobscura* studied by Castañeda et al. (2015)
397 and even lower than the CT_{max} variation reported among *Drosophila* species (Jørgensen
398 et al. 2019; Alruiz et al. 2022). On the other hand, although CT_{max} and z (i.e., thermal
399 sensitivity) are phenotypically correlated (see Castañeda et al. 2015; Molina et al.

400 2023), the evolutionary increase in CT_{max} was not associated with a correlated response
401 in thermal sensitivity (z). This result suggests that both thermal parameters are not
402 genetically constrained, but further evidence from quantitative genetic studies is needed
403 to assess the genetic association between CT_{max} and z. A caveat for this finding could be
404 related to the fact that thermal selection for heat tolerance was carried out over 16
405 generations, followed by 7 generations of relaxed selection (i.e., no selection).
406 However, previous evidence suggests that differences in heat tolerance between control
407 and selected lines were consistent between generations 16 and 25 (Mesas et al. 2021).
408 Indeed, Passananti et al. (2004) also reported that phenotypic values did not change
409 after 35 generations of relaxed selection in desiccation-selected populations of *D.*
410 *melanogaster*.
411 It was expected that flies selected for higher heat tolerance using slow-ramping
412 rate protocols would exhibit greater desiccation and starvation resistance than flies
413 selected using fast-ramping selection protocols. This is because flies assayed for heat
414 tolerance in long assays are also exposed to desiccation and starvation stress (Santos et
415 al. 2012). This study provides partial support for this hypothesis. First, slow-ramping
416 selected lines evolved a higher desiccation resistance than control and fast-ramping
417 selected lines. However, this was only observed in female flies, while males of the
418 different selection treatments did not show any difference in desiccation resistance. On
419 the other hand, starvation resistance evolved in opposite directions depending on sex:
420 females of the fast-ramping and slow-ramping selected lines showed lower starvation
421 resistance than females of the control lines, whereas males of the slow-ramping selected
422 lines showed higher starvation resistance than males of the control and fast-ramping
423 selected lines. Differential evolutionary responses between the sexes could be due to
424 heat thermal selection only being applied to females, which could have exacerbated the

425 evolutionary responses of female flies. However, previous studies that artificially
426 selected exaggerated male traits also found fitness consequences in females (Harano et
427 al. 2010). Differential evolutionary responses between females and males can then be
428 explained by sexually antagonistic selection on genetically correlated traits (Eyer et al.
429 2019; Fanara et al. 2023). Kwan et al. (2008) reported that desiccation-selected females
430 had higher desiccation resistance than desiccation-selected males (see also Chippindale
431 et al. 2004), which can be explained by males using resources at a faster rate than
432 females (e.g., males lose weight, water, and metabolites faster than females). Sexual
433 dimorphism in stress resistance traits has been mainly explained by differences in
434 cuticular composition, resource storage, and energy conservation between the sexes
435 (Schwasinger-Schmidt et al. 2012; Rusuwa et al. 2022). Although energy content was
436 not measured here, Mesas and Castañeda (2023) found that body mass and metabolic
437 rate were similar between control and heat-tolerance selected lines of *D. subobscura*,
438 suggesting that neither resource storage nor energy conservation explains the sex-
439 dependent correlated response for stress resistance traits. However, the same study
440 found that heat-tolerance selected lines had higher fecundity than control lines, whereas
441 previous studies have found negative associations between fecundity and starvation
442 resistance in *D. melanogaster* (Bubliy and Loeschcke 2005; Kalra et al. 2017). Then,
443 the decrease in starvation resistance in females of the heat-selected lines could be
444 related to increased fecundity, which is consistent with the reported trade-off between
445 stress resistance traits and life-history traits (van Noordwijk and de Jong 1986; Rion and
446 Kawecki 2007).

447 In conclusion, the present study shows that heat tolerance evolution is associated
448 with evolutionary responses in other stress resistance traits, which could be explained
449 by pleiotropic effects or linkage disequilibrium among the traits evaluated. However,

further evidence (e.g., quantitative genetic or genome-wide analysis studies) is needed to elucidate the genetic basis of the cross-tolerance evolution in *D. subobscura*. In addition, this study provides evidence for rapid evolutionary responses in ectotherms mediated by thermal selection, but the evolutionary outcomes depend on the intensity of the thermal stress (Mesas and Castañeda 2023) and sex (Rogell et al. 2014; Rusuwa et al. 2022). This study also highlights the importance of *D. subobscura* as a suitable model to study thermal adaptation mediated by natural selection (Huey 2000; Gilchrist et al. 2008; Castañeda et al. 2013, 2015), and laboratory selection (Santos et al. 2005, Santos et al. 2021; Simões et al. 2017; Mesas et al. 2021; Mesas and Castañeda 2023). In addition, this study highlights the relevance of experimental evolutionary studies for understanding the adaptive responses to climate change (Mitchell and Whitney 2018; Brennan et al. 2022; Kelly 2022). Finally, these results suggest that ectotherms may evolve in response to climate warming, but evolutionary responses may differ between sexes and/or the warming rates experienced by natural populations, which may make it difficult to propose general trends in the fate of ectotherms in a changing world where temperature is not the only driver of climate change, but species are also expected to be exposed to changes in precipitation patterns and food availability.

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Data availability

Data and scripts are available at https://doi.org/10.6084/m9.figshare.24085107.v5

Conflict of interest disclosure

The author declares that he complies with the PCI rule of having no financial conflicts in relation to the content of the article.

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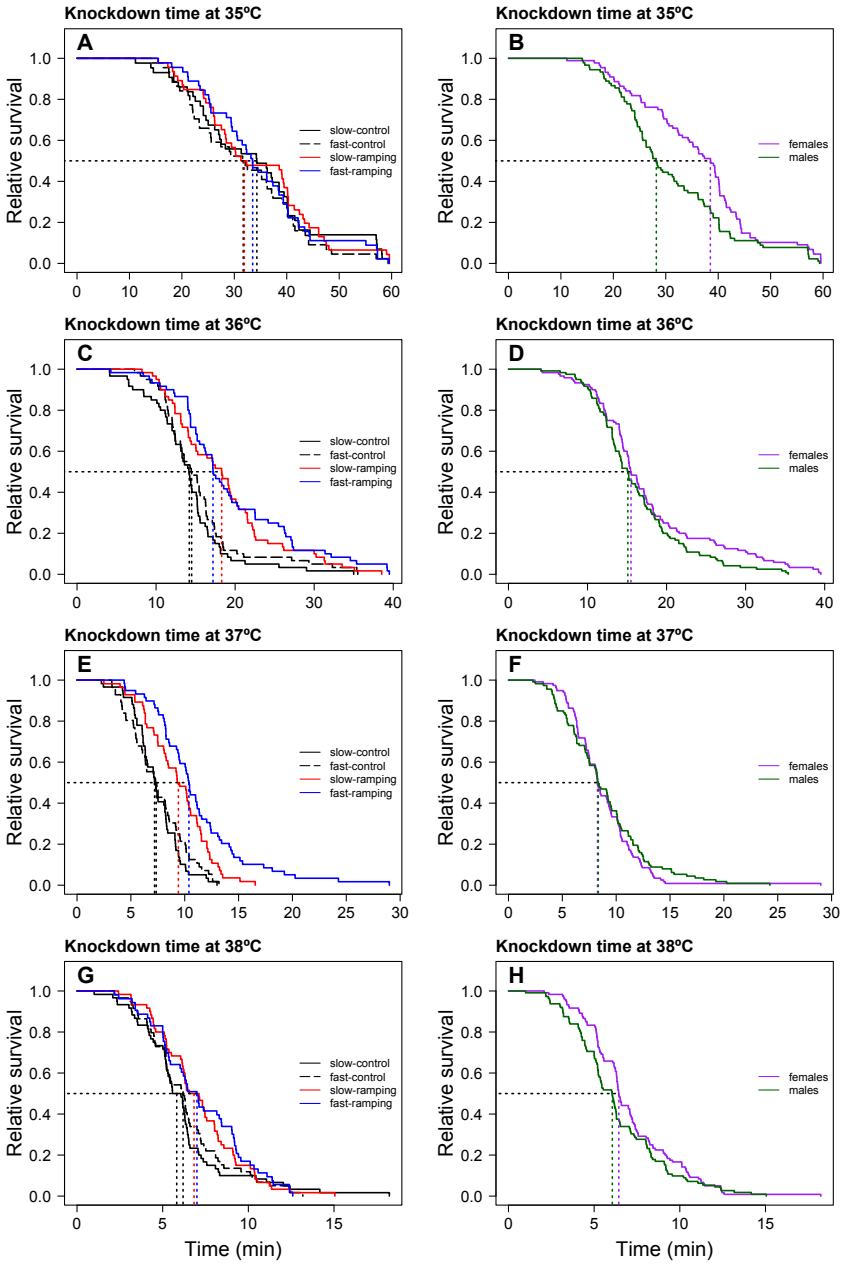
702 **Table 1**. Mixed linear effect model for the knockdown time of *Drosophila subobscura*
703 assayed at four static temperature assays. For simplicity, results for the random effect
704 (replicate lines) are not shown because they were not statistically significant (see
705 Materials and Methods). Significant effects (P values < 0.05) are indicated in boldface
706 type.
707

Knockdown time	**Selection**	**Sex**	**Selection × Sex**
Static assay at 35ºC	$F_{3,170} = 0.62$ $P = 0.60$	$F_{1,170} = 8.64$ $\boldsymbol{P = 0.004}$	$F_{3,170} = 0.64$ $P = 0.59$
Static assay at 36ºC	$F_{3,232} = 9.86$ $\boldsymbol{P = 3.8\times10^{-6}}$	$F_{1,232} = 2.65$ $P = 0.10$	$F_{3,232} = 0.74$ $P = 0.53$
Static assay at 37ºC	$F_{3,222} = 18.39$ $\boldsymbol{P = 1.1\times10^{-10}}$	$F_{1,222} = 0.001$ $P = 0.97$	$F_{3,222} = 2.05$ $P = 0.11$
Static assay at 38ºC	$F_{3,224} = 1.93$ $P = 0.13$	$F_{1,224} = 4.63$ $\boldsymbol{P = 0.032}$	$F_{3,224} = 2.44$ $P = 0.07$

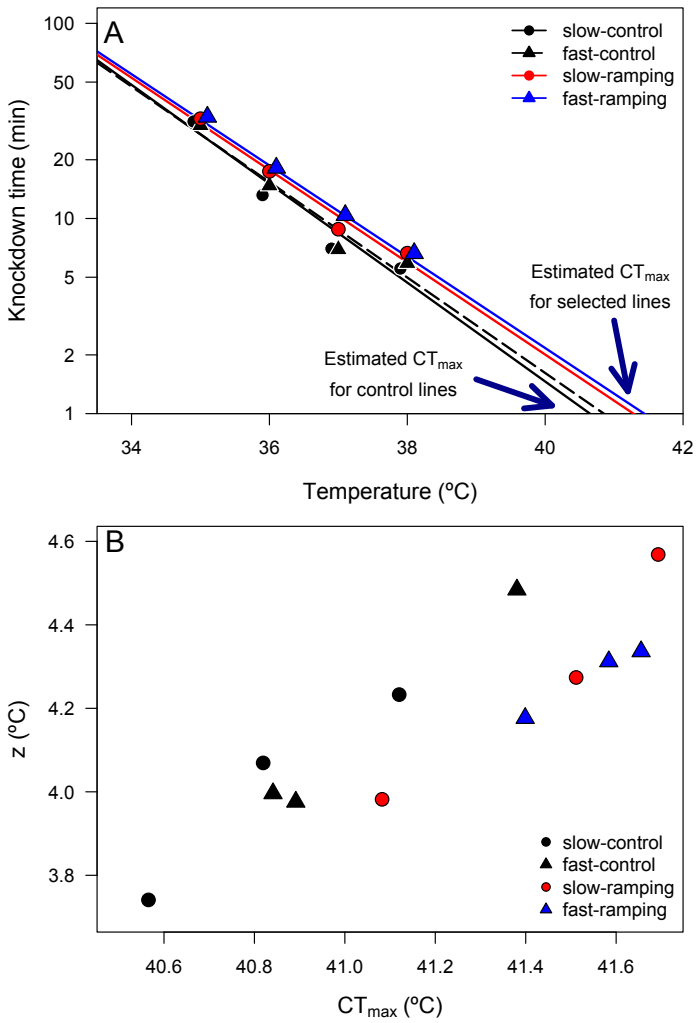
708
709

710 **Figure 1**. Heat-induced mortality in *Drosophila subobscura* flies assayed at four static
711 temperatures. Left panels show the heat knockdown time of slow-ramping control (solid
712 black line), fast-ramping control (dashed black line), slow-ramping selection (red line),
713 and fast-ramping selection lines (blue lines). The right panels show the heat knockdown
714 time of female (purple line) and male (green line) flies. Dotted lines indicate the median
715 knockdown time for each selection protocol (left panels) and sex (right panels).



716

717 **Figure 2**. (A) Thermal death curves for control (black solid and dashed lines) and
718 selected (red and blue lines) lines for increasing heat tolerance in *Drosophila*
719 *subobscura*. Symbols represent the average knockdown time at the different assay
720 temperatures. Each symbol represents the average knockdown time for each replicate
721 line for each thermal regime: slow-control (black circle), fast-control (black triangle),
722 slow-ramping (red circle), and fast-ramping (blue triangle). (B) Relationship between
723 CT_{max} and z for slow-ramping control (solid black line), fast-ramping control (dashed
724 black line), slow-ramping selection (red line), and fast-ramping selection lines (blue
725 lines). Each symbol represents the CT_{max} and z estimated for each replicate line.
726



727

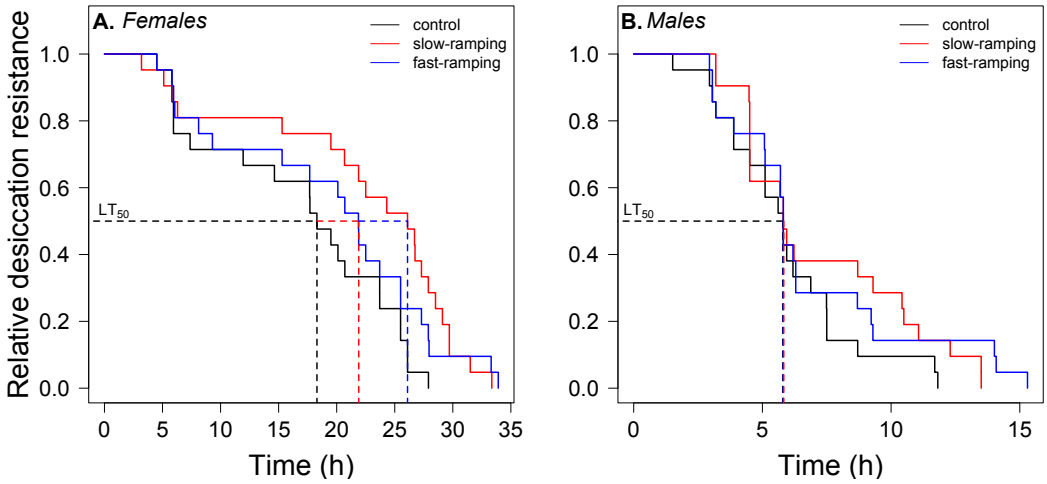
728 **Figure 3**. Desiccation survival curves of (A) females and (B) males from control (black

729 line), slow-ramping selection (red line), and fast-ramping selection lines (blue lines) of

730 *Drosophila subobscura*. Dashed lines indicate the median mortality time for each

731 selection protocol (pooled replicate cages).

732



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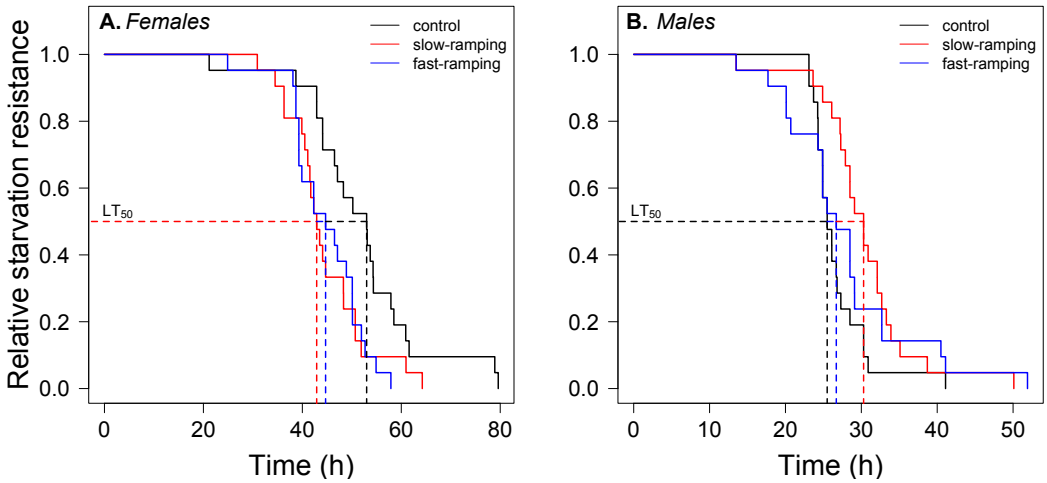
734

735 **Figure 4**. Starvation survival curves of (A) females and (B) males from control (black

736 line), slow-ramping selection (red line), and fast-ramping selection lines (blue lines) of

737 *Drosophila subobscura*. Dashed lines indicate the median mortality time for each

738 selection protocol (pooled replicate cages).



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