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

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ABSTRACT

Evolution of heat tolerance is a crucial mechanism for the adaptive response to global warming, but it depends on the genetic variance harbored by populations and the thermal stress intensity in nature. Selection experiments on heat tolerance have been key to understand the heat tolerance evolution but the effect of variable heat stress intensity on the correlated responses of resistance traits has not been explored. Here, the effects of heat intensity selection (fast and slow ramping temperatures) for increasing knockdown temperature in *Drosophila subobscura* were evaluated on the knockdown time at different stressful temperatures (35, 36, 37, and 38 ºC), the thermal-death-time curves (TDT curves), the desiccation and starvation resistance. Correlated evolution was found for these four resistance traits in *D. subobscura*, which demonstrates that the evolutionary response to tolerate higher temperatures also confers the capacity to tolerate other stress such as desiccation and starvation. However, these correlated responses depended on the intensity of thermal selection and sex, which could limit our capacities to transfer these findings to natural scenarios. Nevertheless, this study

reaffirms the value of experimental evolutionary approach to explore and to understand the adaptive responses of natural populations to global warming.

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

INTRODUCTION

Increasing environmental temperatures represent an important challenge for ectotherms (i.e., organisms whose body temperature depends on the environmental temperature) because their morphology, physiology, behavior, and performance depend on the thermal environment [1–3]. Additionally, the rise in environmental temperatures increases the extinction risk of many species that live close to their upper thermal limits [4–6]. However, ectotherms can avoid the negative effects of heat via behavioral thermoregulation, evolutionary change, and/or phenotypic plasticity of the upper thermal limits [7].

Evolutionary adaptation depends on the genetic variation exhibited by upper thermal limits, but some studies have suggested that these traits have a limited evolutionary potential to respond to increasing environmental temperatures [8–10]. Nevertheless, theoretical and empirical evidence shows that heritability estimates of heat tolerance tend to be lower when heat tolerance is measured in long assays (e.g., slow ramping assays or static assays using sublethal temperatures) than in short assays (e.g., fast ramping assays or static assays using extremely high temperatures) [8,10–14]-. Therefore, heat stress intensity can influence our predictions regarding the evolutionary potential of heat tolerance; however, how do populations respond to variable selection driven by heat stress? Selection in laboratory conditions has a long

51 tradition and provides information about the adaptive evolution of specific selective
52 agents [15–18]. In particular, the experimental evolution of heat tolerance has been
53 explored in multiple species, including fishes, corals, and insects [19–22]. Experimental
54 evolution of heat tolerance has also been studied in several *Drosophila* species,
55 *including D. melanogaster* [23,24] and *D. subobscura* [25–27], and *D. buzzatti* [28,29].
56 Most of these studies reported the evolution of heat tolerance using fast ramping
57 protocols, ranging from 0.4 ºC/min in Folk et al. [24] to 1 °C/min in Gilchrist and Huey
58 [23], or high-temperature static assays (40 °C), as in Bubliy and Loeschcke (28).
59 Recently, Mesas et al. [26] reported that selected lines of *D. subobscura* evolved higher
60 heat tolerance, regardless of the warming rate used during the selection experiments
61 (slow ramping rate: 0.08 °C/min and fast ramping rate: 0.4 °C/min).
62 Interestingly, several of these selection experiments on heat tolerance in
63 *Drosophila* have found correlated responses in other traits such as starvation resistance,
64 desiccation resistance, and heat shock proteins [29–31]. However, it is expected that the
65 intensity of thermal stress has important effects on the correlated responses of other
66 traits to heat tolerance selection [32]. For instance, fast-ramping selected lines have
67 evolved thermal performance curves with higher optimal temperatures and narrower
68 thermal breadths than slow-ramping selected lines [26]. Additionally, Mesas and
69 Castañeda [27] reported that the evolution of heat tolerance was associated with reduced
70 activity of the enzymes involved in the glucose-6-phosphate branch point and increased
71 performance of life-history traits in slow-ramping selected lines. However, they did not
72 observe any changes in the metabolic rate of the selected lines, as predicted by Santos et
73 al. [33]. In summary, there is evidence that heat stress intensity determines the
74 magnitude of the evolutionary responses of performance, metabolic, and life-history
75 traits to heat tolerance selection; however, the correlated evolution of resistance traits

has not yet been tested. This information provides important clues regarding how the intensity of thermal stress could determine the evolution of cross-tolerance to stressful environmental conditions. Natural populations are exposed to multiple environmental stressors, and it is known that increased tolerance to one stressor can boost tolerance to another [34]. The cross-tolerance induced by thermal stress has been widely explored in several arthropod species and increasing traits such as desiccation, insecticide, pathogen resistances [35–37]. However, the cross-tolerance response at the evolutionary level can be constrained or facilitated by genetic correlations among resistance traits [38].

Here, effects of heat intensity selection for increasing knockdown temperature was explored on the cross-tolerance evolution of four different resistance traits in *D. subobscura*: knockdown time at different stressful temperatures, thermal-death-time curves (TDT), desiccation resistance, and starvation resistance. TDT curves represent an integrative approach for assessing how the probability of survival depends on the intensity and duration of heat stress [39]. This approach allows to estimate the critical thermal maxima (CT_{max}) and thermal sensitivity using the heat tolerance measurement obtained from different exposure temperatures. It is expected that fast-ramping selected lines would exhibit higher knockdown time at highly stressful temperatures and higher CT_{max} because fast-ramping protocols reduce the confounding effects on heat tolerance associated with the assay length. In contrast, slow-ramping selection lines should exhibit higher desiccation and starvation resistance, because individuals with higher starvation and desiccation resistance exhibit higher thermal tolerance during long assays (11,32).

Materials and Methods

Sampling and maintenance

101 D. subobscura females were collected in the spring of 2014 at the Botanical Garden of
102 Universidad Austral de Chile (Valdivia, Chile; 39º 48' S, 73º 14' W) using plastic
103 traps containing banana/yeast baits. A total of 200 females were collected and
104 individually placed in plastic vials with David's killed-yeast *Drosophila* medium to
105 establish isofemale lines. In the next generation, 100 isofemale lines were randomly
106 selected, and 10 females and 10 males per line were placed in an acrylic cage to set up
107 one large outbred population. In the next generation, the cages were split into three
108 replicate cages (R1, R2, and R3). After three generations, each replicate cage was
109 divided into four population cages. Thus, our experimental design had four different
110 artificial selection protocols in triplicate: fast-ramping selection, fast-ramping control,
111 slow-ramping selection, and slow-ramping control lines (see Mesas & Castañeda
112 [27]). During the selection experiments, population cages were maintained at 18 °C
113 (12:12 light-dark cycle) in a discrete generation, controlled larval density regime [40].
114 Each population cage had a population size of 1000-1500 breeding adults.
115
116 ***Heat tolerance selection***
117 For each replicated line, 120 four-day-old virgin females were randomly mated with
118 two males for two days, and then females were individually placed in a capped 5-mL
119 glass vial and males were discarded. Vials attached to a plastic rack were immersed in
120 a water tank with an initial temperature of 28 °C, controlled by a heating unit (Model
121 ED, Julabo Labortechnik, Seelbach, Germany). After an equilibration time of 10 min,
122 the temperature was increased to a rate of 0.08 ºC min^{-1} for the slow-ramping selection
123 protocol or 0.4 ºC min^{-1} for the fast-ramping selection protocol. The assays were
124 terminated when all flies collapsed. Each assay was video recorded using a high-
125 resolution camera (D5100, Nikon, Tokyo, Japan) and then visualized to score the

126 knockdown temperature for each fly, which was defined as the temperature at which
127 each fly ceased to move. Flies were ranked according to their knockdown temperature,
128 and four virgin females from the offspring of the 40 most tolerant females (upper 30%
129 of each assay) were selected to found the next generation. For the fast-control and
130 slow-control lines, the knockdown temperature was measured as mentioned above, but
131 the offspring were randomly chosen to found the next generation, regardless of the
132 knockdown temperature of their mother.
133 This artificial selection experiment was performed for 16 generations after which
134 flies from each selection treatment were dumped in separate acrylic cages and
135 maintained without selection at 18 °C (12:12 light-dark cycle). The present
136 experiments were performed in generation 23.
137
138 ***Knockdown time in static assays***
139 Eggs were collected from each population cage and transferred to vials at a density of
140 40 eggs/vial. At 4 days of age, ten females and ten males from each population cage
141 were assayed to measure their heat knockdown time at four different static
142 temperatures: 35, 36, 37, and 38 °C. This experimental setup allowed the measurement
143 of 960 flies in 16 assays (10 flies × 2 sexes × 4 static temperatures × 4 selection
144 treatments × 3 replicated lines). Static assays were similarly performed as knockdown
145 temperature assays, but static temperatures were used instead of using ramping
146 temperatures. A total of 240 flies were measured for each static temperature, except for
147 the assay at 35 °C (178 flies) because two flies died before the beginning of the assay,
148 and a video file of one assay was corrupted (60 flies). At 37 °C assay, four flies died
149 before the beginning of the assay, and the collapse time could not be measured for six

150 flies. Finally, for the assay at 38 ºC assay, three flies died before at the beginning of the
151 assay and the collapse time could not be measured for five flies.
152
153 ***Desiccation and starvation resistance***
154 Eggs from each replicate cage were collected and maintained in vials at a density of 40
155 eggs/vial. Only fast control lines were measured as control lines. This decision was
156 based on logistic reasons (i.e., the high number of vials) and statistical support because
157 fast-control and slow-control lines did not differ in their knockdown times and CT_{max}
158 values (see *the Results* section).
159 For desiccation resistance assays, five flies were placed in a vial containing five
160 desiccant droplets (Drierite), which were sealed with parafilm (flies had no access to
161 food or water during the assay). For starvation resistance assays, five flies were placed
162 in a vial containing agar only (flies had access to water but not food). For both
163 desiccation and starvation resistance assays, the number of live flies was recorded every
164 3 h until all the flies were dead. The experiments were performed at 18 °C.
165
166 **Statistical analysis**
167 Normality and homoscedasticity were tested for all variables, and the knockdown times
168 were log_{10}-transformed to meet the parametric assumptions. All analyses were
169 performed using R software [41].
170
171 ***Heat tolerance***
172 Analysis to evaluate the evolutionary response of the knockdown temperature to
173 ramping selection was separately performed for the fast and slow ramping selection
174 because it is widely known that knockdown temperature is higher in fast ramping than

175 slow ramping assays [8,26]. Mixed linear models (full model) with ramping selection
176 (fixed effect), sex (fixed effect), and replicated lines nested within thermal selection
177 (random effect) were performed on knockdown temperature and knockdown time using
178 the library *lme4* package for R [42].Fixed effects were tested through a type-III
179 ANOVA using the library *lmerTest* package for R [43], whereas the random effect was
180 tested using a likelihood-ratio test comparing the model with versus without the
181 replicated lines. When the selection effect was significant, posteriori comparisons were
182 performed using Tukey tests implemented in the *emmeans* package for R [44].
183 Knockdown times were also employed to plot the survival curves based on the
184 Kaplan-Meier formula using the function *survfit* implemented in the *survival* package
185 for R [45].
186
187 ***Thermal death time curves (TDT)***
188 Average knockdown times were calculated for each sex, replicated lines, and selection
189 treatment combination (Table S1). These values were regressed against the assayed
190 temperatures according to eqn. 1 (Fig. 1; Rezende et al. [39]):
191
192 $$log_{10}t = \frac{CT_{max}-T}{Z} \qquad \text{eqn. 1}$$
193
194 , where T is the assayed static temperature (ºC), CT_{max} is the upper thermal limit (ºC), t
195 is knockdown time (min), and z is the thermal sensitivity. These curves allowed the
196 estimation of CT_{max} as the extrapolated temperature that would result in a knockdown
197 time of $\log_{10} t = 0$ (i.e., knockdown time at 1 min) and the estimation of the thermal
198 sensitivity ($z = -1/\text{slope}$), where the lower z-values, the higher thermal sensitivity.

199 Using eqn. 1, 18 TDT curves (2 sexes × 3 replicated lines × 3 selection
200 protocols) were fitted, from which CT_{max} and z values were estimated as explained
201 above. A linear model with ramping selection treatment, sex, and their interaction was
202 performed to evaluate their effects on CT_{max} and z values.
203
204 ***Desiccation and starvation resistance***
205 To determine the lethal time at 50% of flies of each vial were dead (LT_{50}), a generalized
206 linear model following a binomial distribution was performed with the proportion of
207 alive flies as a dependent variable and time as a predictor variable. The generalized
208 linear model was run using the function g*lm* of the *lme4* package for R [42]. Then, the
209 LT_{50} of each vial was estimated using the function *dose.p* of the package *MASS* for R
210 [46].
211 To estimate the median LT_{50} and the 95% confidence intervals for each selection
212 treatment and sex, each LT_{50} was transformed into a survival object using the functions
213 *Surv* and *survfit* of the package *survival* for R . This procedure also allowed to create the
214 survival curves. Finally, to test the effect of selection treatment and sex on desiccation
215 and starvation resistances, a Cox proportional regression model was fitted with LT_{50} as
216 a dependent variable, and selection protocol and sex as predictor variables. The Cox
217 model was run using the function *coxph* of the *survival* package.
218
219 **RESULTS**
220 **Knockdown temperature evolution**
221 Knockdown temperature evolved in response to artificial selection for increasing heat
222 tolerance, regardless of ramping assay protocol: knockdown temperature was
223 significantly higher in fast-ramping selected lines compared to fast-ramping control

224 lines ($mean_{fast\text{-}ramping}$ ± SD = 37.71 ± 0.68 ºC and $mean_{fast\text{-}control}$ ± SD = 37.23 ± 0.79 ºC;
225 $F_{1,4}$ = 32.0, P = 0.005); and knockdown temperature in slow-ramping selected lines
226 was significantly higher than in slow-ramping control lines ($mean_{slow\text{-}ramping}$ ± SD =
227 35.48 ± 0.66 ºC and $mean_{slow\text{-}control}$ ± SD = 34.97 ± 0.72 ºC; $F_{1,4}$ = 41.7, P = 0.003).
228 These findings have been previously reported by Mesas et al. [25] and reported here to
229 contextualize the following results.
230
231 ***Knockdown time evolution***
232 As expected, knockdown time significantly decreased towards higher assayed
233 temperatures ($F_{1,876}$ = 647.8, P < 0.0001): static assay at 35º C = 33.77 ± 11.63 min;
234 static assay at 36º C = 16.98 ± 6.87 min; static assay at 37º C = 8.84 ± 3.56 min; and
235 static assay at 38º C = 6.78 ± 2.68 min.
236 Knockdown times significantly differed between selection treatments when flies
237 were assayed at 36 and 37 ºC (Table 1; Fig. 1). At these temperatures, slow-selected and
238 fast-selected lines exhibited higher heat tolerance than slow- and fast-control lines
239 (Table S1, S2; Fig. 1C, E). Also, fast-selected lines showed a higher heat tolerance than
240 slow-selected lines in flies assayed at 37 ºC but not at 36 º C (Table S1, S2; Fig. 1C, E),
241 whereas fast and slow control lines did not differ between them (Table S2; Fig 1). On
242 the other hand, replicate lines had not a significant effect on knockdown time, indicating
243 that replicate lines showed a consistent response within each selection and control
244 treatment (χ^2_1 = 0 and P =1 for all static assays). Regarding sex, females showed a
245 higher thermal tolerance than males but only when flies were assayed at 35 and 38 ºC
246 (Table 1; Fig. 1B, H). Finally, non-significant interactions between selection and sex
247 were found for any assayed temperatures (Table 1).
248

249 ***TDT curves evolution***

250 Linear regressions between $\log_{10}(LT_{50})$ and assayed temperatures allowed to estimate 24
251 TDT curves (4 selection treatments × 3 replicated lines × 2 sexes) with high coefficients
252 of determination (average R^2 = 0.946, range: 0.820 – 0.989; Table S3), corroborating
253 that heat knockdown time is linearly associated to stressful sublethal temperatures.
254 From these TDT curves, the average CT_{max} was 41.21ºC (SD = 0.46ºC) and the average
255 *z* was 4.18ºC (SD = 0.34ºC). CT_{max} were significantly different between selection
256 treatments ($F_{3,20}$ = 4.46, P = 0.015; Fig. 2A). *A posteriori* analysis showed that only fast-
257 ramping selected and slow-control lines significantly differed in their CT_{max} values (t_{20}
258 = 3.195, P = 0.02), whereas fast-control and slow-control showed similar CT_{max} values
259 (t_{20} = –0.911, P = 0.80). Thus, if control lines are pooled, CT_{max} still differs between
260 selection treatments ($F_{2,18}$ = 6.69, P = 0.007), with fast-ramping (CT_{max} = 41.55 ± 0.36
261 ºC) and slow-ramping selected lines (CT_{max} = 41.43 ± 0.35 ºC) showing higher CT_{max}
262 than control lines (CT_{max} = 40.94 ± 0.41 ºC) (t_{18} = 3.27, P = 0.01 and t_{18} = 2.64, P =
263 0.04, respectively). CT_{max} between selected lines was not different between them (t_{18} =
264 0.54, P = 0.85). On the other hand, sex and the interaction between selection treatments
265 and sex had no significant effects on CT_{max} ($F_{1,18}$ = 0.004, P = 0.95 and $F_{3,18}$ = 2.11, P =
266 0.15, respectively). Regarding *z* (i.e., thermal sensitivity), it did not exhibit significant
267 effects of selection treatments ($F_{3,16}$ = 0.91, P = 0.46; Fig. 2), sex ($F_{1,16}$ = 1.30, P =
268 0.27), nor the interaction between selection treatments and sex ($F_{3,16}$ = 2.23, P = 0.12).
269 Summarizing, the evolution of a higher CT_{max} is not related to an evolutionary change
270 of the thermal sensitivity (Fig. 2B). Indeed, the relationship between CT_{max} and *z* did
271 not change with the evolution of increasing heat tolerance ($r_{\text{control-lines}}$ = 0.979 and
272 $r_{\text{selected-lines}}$ = 0.929; Z-test = 0.76, P = 0.45).

273

274 ***Desiccation resistance evolution***

275 Survival analysis showed a significant interaction between selection treatments and sex
276 on desiccation resistance ($LTR_5 = 83.55$, $P < 2\times10^{-16}$). Males showed a higher
277 desiccation risk than female flies (Hazard ratio = 7.11, $P < 2\times10^{-7}$; Fig. 3). Females
278 showed a significant difference between selected and control lines (LTR: $\chi^2_2 = 6.72$, P
279 = 0.03; Fig. 3A). Specifically, females of the slow-ramping selection lines showed a
280 higher desiccation resistance than females of the control lines (Hazard ratio = 0.42, P =
281 0.009), whereas females of the fast-ramping selection and control lines showed similar
282 desiccation risk (Hazard ratio = 0.56, P = 0.072). On the other hand, males showed no
283 differences in desiccation resistance between selected and control lines (LTR: χ^2_2 =
284 1.88, P = 0.4; Fig. 3B). Results of the desiccation survival analysis testing the effect of
285 selection protocol, sex, and its interaction are reported in the Table S4.

286

287 ***Starvation resistance evolution***

288 Survival analysis showed a significant interaction between selection treatments and sex
289 on desiccation resistance ($LTR_5 = 94.89$, P-value $< 2\times10^{-16}$). Males showed a higher
290 starvation risk than female flies (Hazard ratio = 22.75, $P < 1\times10^{-16}$; Fig. 4). For female
291 flies (Fig. 4A), fast-ramping selection and slow-ramping selection lines showed a higher
292 starvation risk than control lines (Hazard ratio = 2.37, P-value = 0.009 and Hazard ratio
293 = 2.20, P-value = 0.014, respectively). Whereas male flies showed an oppositive pattern
294 (Fig. 4B): slow-ramping selection lines showed a lower starvation risk than control lines
295 (Hazard ratio = 0.50, P-value = 0.03), but non-significant differences were found
296 between fast-ramping selection and control lines (Hazard ratio = 0.64, P-value = 0.16).
297 Results of the starvation survival analysis testing the effect of selection protocol, sex,
298 and its interaction are reported in the Table S5.

299

300 **Discussion**

301 Studying the evolutionary responses of thermal limits is key to understanding the
302 adaptive responses and evolutionary constraints to global warming. Then, cross-
303 tolerance studies can provide valuable information about the evolutionary response to
304 multiple environmental stressors. Cross-tolerance evolution has been reported among
305 different resistance traits [29,35,47,48], but the magnitude of the evolutionary response
306 could be explained by the trait under direct selection or the stress intensity [38]. Here,
307 an artificial selection experiment was performed on knockdown temperature to explore
308 whether the intensity of thermal selection influences the cross-tolerance evolution of
309 resistance traits in *D. subobscura*. Briefly, a direct response heat knockdown
310 temperature to selection was found (see also 25,26), which resulted into a correlated
311 responses on heat knockdown time (static assays), the thermal tolerance landscape
312 (TDT curves), desiccation resistance, and starvation resistance. These findings
313 demonstrate that the evolutionary response to tolerate higher temperatures also confers
314 the capacity to tolerate other stress such as desiccation and starvation. Interestingly,
315 these correlated responses depend on the intensity of thermal selection and sex (Table
316 2).

317 Different approaches used to measure the upper thermal limit of ectotherms
318 generate different genetic and phenotypic estimates. In general, fast ramping assays
319 estimate higher upper thermal limits and higher heritabilities than slow ramping assays
320 [8,12]. As heritability is commonly used as a predictor of the evolutionary response of a
321 trait to natural or artificial selection, it is expected that the evolutionary response of heat
322 tolerance depends on the ramping rate used during selection. However, previous work
323 did not support this prediction for *D. subobscura*, finding that the evolution of the

knockdown temperature was independent of the ramping rate [26], but the correlated responses of the thermal performance curves or the energy metabolism depended on the intensity of the thermal selection [26,27]. Here, the evolution of knockdown temperature induced a correlated response on heat knockdown time when it was assayed at intermediate temperatures (36 and 37 ºC), but not at less or more extreme assayed temperatures (35 and 38ºC). These findings can be explained because stress tolerance at 35ºC should depend on the physiological status of the organism during the thermal assay (e.g., availability of energy resources) rather than only heat tolerance, whereas heat tolerance at 38ºC could be constrained to physical properties of ectotherms (e.g., protein denaturation, membrane permeability). A previous study compared *D. subobscura* populations distributed along a latitudinal gradient and found a clinal pattern for heat tolerance only for flies assayed in static assays (specifically at 38ºC), but this clinal pattern was not detected using ramping assays [40]. The difference between both studies could be explained by the number of generations under thermal selection, which could result in a difference in the evolutionary response of heat tolerance. According to Begon [49], *D. subobscura* has between 4 to 6 generations per year. Thus, it is feasible to estimate almost 125 generations since the *D. subobscura* population studied by Castañeda et al. [40] was introduced in Chile, whereas the present artificial selection experiment lasted 16 generations. Anyway, beyond these results from specific thermal assays, these findings support the idea that (1) the use of a single static temperature would miss genetic or phenotypic effects on heat tolerance, and (2) unifying several knockdown time estimates into a single approach (TDT curves) should be necessary to elucidate genetic and phenotypic patterns of heat tolerance in ectotherms [39,50].

348 TDT curves evolved in response to the selection of heat tolerance in *D.*
349 *subobscura*. TDT curves revealed that fast- and slow-ramping selected lines evolved
350 higher CT_{max} than control lines (ΔCT_{max} = 0.49 ºC). This differential CT_{max} value is
351 slightly lower than population differences (0.9ºC) observed between the lowest- and
352 highest-latitude populations (~8 latitudinal degrees) of *D. subobscura* studied by
353 Castañeda et al. (2015), and even lower than the CT_{max} variation reported among
354 *Drosophila* species [51,52]. On the other hand, despite CT_{max} and z (i.e., thermal
355 sensitivity) are phenotypically correlated (see 40,53), the evolutionary increase in CT_{max}
356 was not associated with a correlated response on thermal sensitivity (z). This result
357 suggests that both thermal parameters are not genetically constrained, but further
358 evidence from quantitative genetic studies is needed to evaluate the genetic correlation
359 between CT_{max} and z. A caveat for this finding could be related to the fact that thermal
360 selection on heat tolerance was performed by 16 generations followed by 7 generations
361 of relaxed selection (i.e., no selection). However, previous evidence indicates that
362 differences in heat tolerance between control and selected lines were consistent between
363 generations 16 and 25 [26]. Passananti et al. [54] also reported that phenotypic values
364 did not change after 35 generations of relaxed selection in desiccation-selected
365 populations of *D. melanogaster*.
366 According to our expectation, flies selected for higher heat tolerance using slow
367 ramping rate protocols should exhibit greater desiccation and starvation resistance than
368 flies selected using fast-ramping selection protocols (see Santos et al. [33]). However,
369 both desiccation resistance as well as starvation resistance evolved in a context-
370 dependent manner. For instance, females evolved higher desiccation resistance in the
371 slow-ramping selected lines, whereas males showed no correlated response. On the
372 other hand, males evolved for a higher starvation resistance in the slow-ramping

373 selected lines, whereas females evolved for a lower starvation resistance regardless of
374 the thermal intensity selection. Differential evolutionary responses between sexes could
375 be explained because heat thermal selection was only performed on females, which
376 could have exacerbated the evolutionary responses of female flies. However, previous
377 studies performing artificial selection on exaggerated male traits found fitness
378 consequences in females [55]. Then, dimorphic correlated responses between females
379 and males can be explained by sexually antagonistic selection on genetically correlated
380 traits [56,57]. Kwan et al. [58] reported that desiccation-selected females evolved for a
381 higher desiccation resistance than males (see also 59), which is explained because males
382 lose weight, water, and metabolites at a faster rate than female flies. Indeed, sexual
383 dimorphism in stress resistance traits has been mainly explained by differences in
384 cuticular composition, resource storage, and energy conservation between sexes [60,61].
385 However, here the energy content was not measured in the different control and selected
386 lines, which could have provided additional information about the evolutionary
387 response in metabolite storage. However, body mass and metabolic rate were similar
388 between control and selected lines [27], suggesting that neither difference in resource
389 storage nor energy conservation explain sex-dependent correlated response for stress
390 resistance traits. However, direct evaluation of the underlying mechanisms related to
391 desiccation and starvation resistance should be explored to provide a better idea of the
392 physiological basis of the evolution of stress resistance traits.
393 In conclusion, heat tolerance evolution induced evolutionary responses in other
394 stress resistance traits, which could be explained by pleiotropic effects or linkage
395 disequilibrium among evaluated traits. However, further evidence is needed (e.g.,
396 quantitative genetic or genome-wide analysis studies) to elucidate the genetic basis of
397 the cross-tolerance evolution in *D. subobscura*. In addition, this study provides evidence

of rapid evolutionary responses in fruit flies mediated by thermal selection, even the evolutionary outcomes depend on the intensity of the thermal stress as has been reported by Mesas & Castañeda [27]. Interestingly, insect species can evolve in response to selection for higher thermal resistance, which should mean an adaptive advantage in the global warming context. However, it is necessary to know if these evolutionary responses will be fast enough to adjust to the accelerated rate of climate change.

Acknowledgments

I thank Andres Mesas, Angélica Jaramillo, Julio Figueroa and Jaiber Solano for their help with fly maintance, experiments, and data management. I also thank Enrico Rezende and Mauro Santos for their valuable suggestions and advices at the moment of design the artificial selection experiment.

Funding

This work was supported by the Fondo Nacional de Investigación Científica y Tecnológica (FONDECYT) [grant number 1140066] – Chile.

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602 **Table 1**. Mixed linear effect model for the knockdown time of *Drosophila subobscura*
603 assayed in four static temperature assays. For simplicity, results for the random effect
604 (replicate lines) are not shown because they were not statistically significant (see
605 Materials and Methods). Significant P-values (< 0.05) are indicated in boldface type.
606

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

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608

609 **Table 2**. Summary of the correlated responses of different stress resistance traits to

610 selection on heat tolerance in *D. subobscura*. Note: ↑ symbol indicates an increased

611 resistance, and ↓ symbol indicates a decreased resistance.

Trait	**Female response to heat tolerance selection**	**Male response to heat tolerance selection**
Knockdown time at 35 ºC	No correlated response	No correlated response
Knockdown time at 36 ºC	↑ fast-ramping selection	↑ fast-ramping selection
	↑ slow-ramping selection	↑ slow-ramping selection
Knockdown time at 37 ºC	↑ fast-ramping selection	↑ fast-ramping selection
	↑ slow-ramping selection	↑ slow-ramping selection
Knockdown time at 38 ºC	No correlated response	No correlated response
CT_{max}	↑ fast-ramping selection	↑ fast-ramping selection
	↑ slow-ramping selection	↑ slow-ramping selection
Thermal sensitivity (z)	No correlated response	No correlated response
Desiccation resistance	↑ slow-ramping selection	No correlated response
Starvation resistance	↓ fast-ramping selection	↑ slow-ramping selection
	↓ slow-ramping selection	

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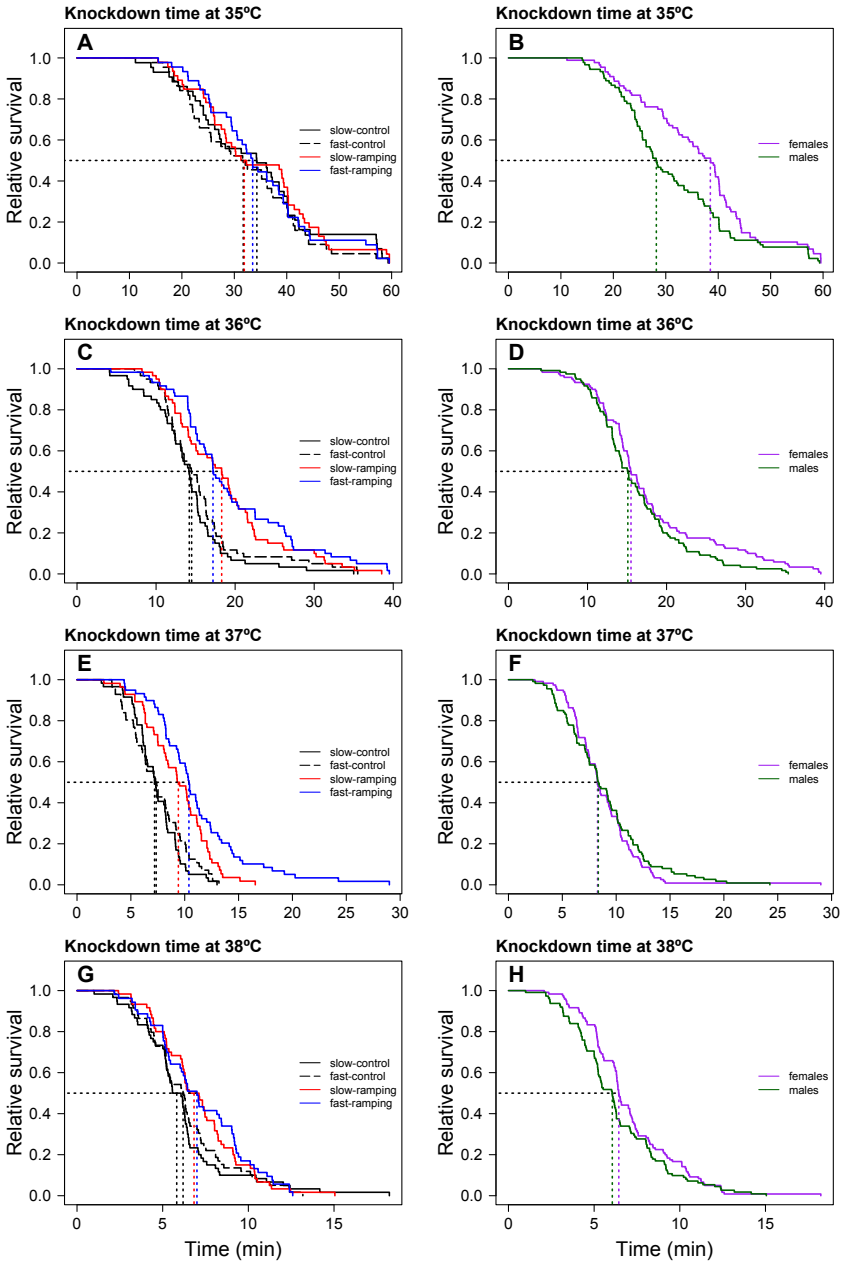
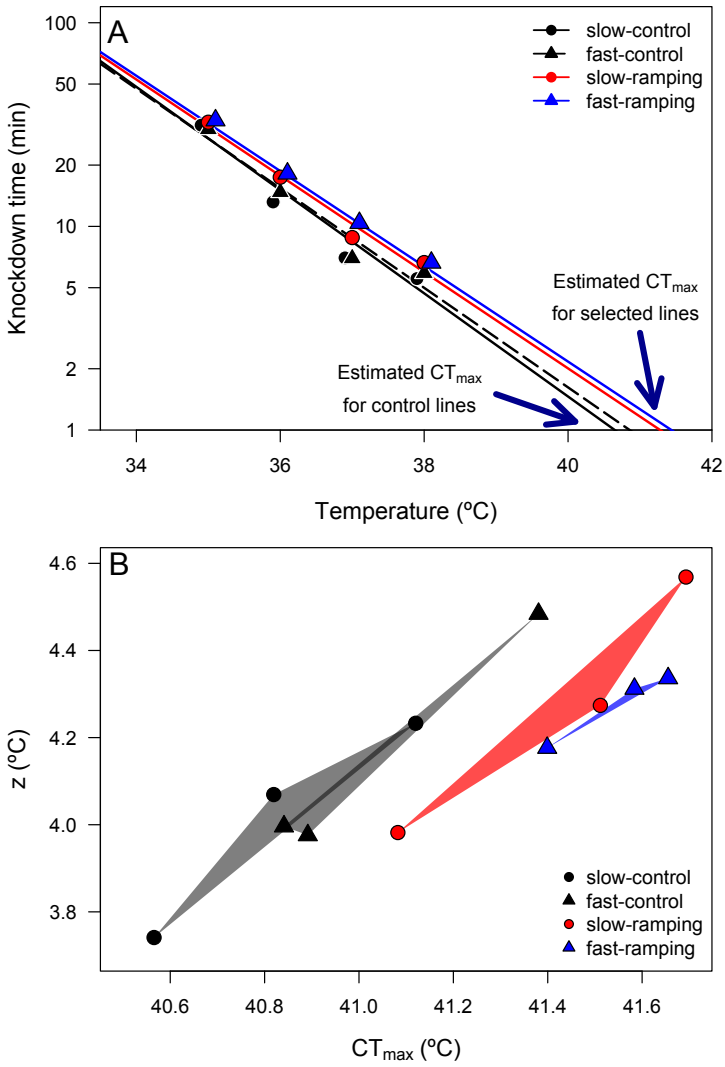
613

614 **Figure 1**. Thermal-induced mortality in *Drosophila subobscura* flies assayed at four
615 static temperatures. Left panels show the heat knockdown time of slow-ramping control
616 (solid black line), fast-ramping control (dashed black line), slow-ramping selection (red
617 line), and fast-ramping selection lines (blue lines). Right panels show the heat
618 knockdown time of female (purple line) and male flies (green line). The dotted lines
619 indicate the median knockdown time for each selection protocol (left panels) and sex
620 (right panels).



621

622 **Figure 2**. (A) Thermal death curves for control (solid and dash black lines) and selected
623 (red and blue lines) lines for increasing heat tolerance in *Drosophila subobscura*.
624 Symbols represent the average knockdown time at the different assayed temperatures.
625 (B) Relationship between CT_{max} and z for slow-ramping control (solid black line), fast-
626 ramping control (dashed black line), slow-ramping selection (red line), and fast-ramping
627 selection lines (blue lines). Each symbol represents the average value for each replicated
628 population and the polygons represent the space that occupies each selection protocol in
629 the thermal tolerance landscape.



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631

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

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

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

635 selection protocol (pooled replicated cages).

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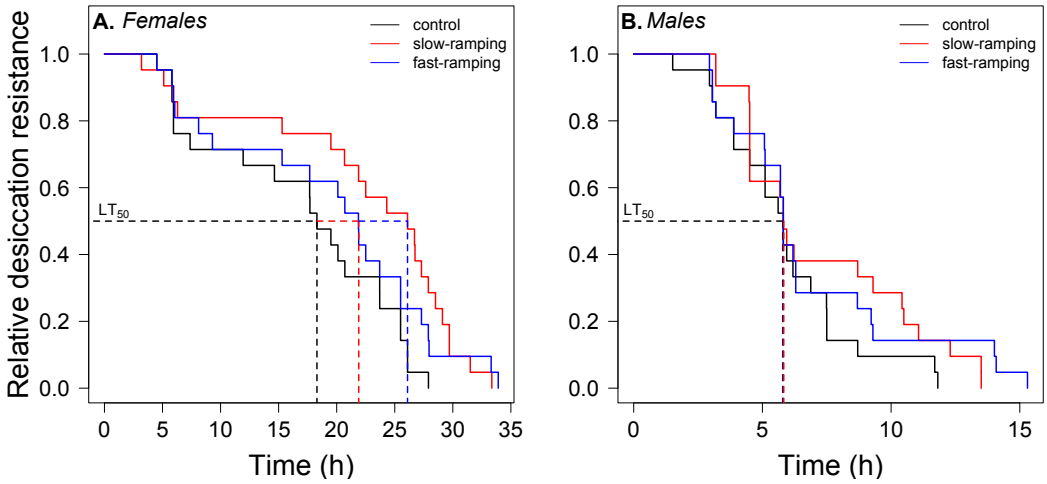

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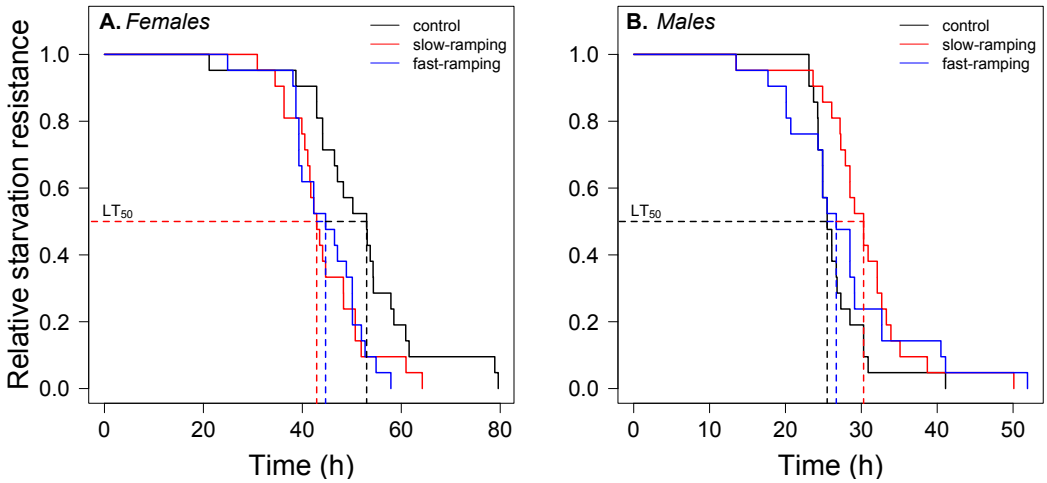
639 **Figure 4**. Starvation survival curves of (A) females and (B) males of control (black

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

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

642 selection protocol (pooled replicated cages).



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