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

Luis E. Castañeda

Programa de Genética Humana, Instituto de Ciencias Biomédicas (ICBM), Facultad de Medicina, Universidad de Chile, Santiago, Chile

Corresponding author: Luis E. Castañeda (luis.castaneda@uchile.cl)

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. Selection experiments for heat tolerance have been key to understanding the evolution of heat tolerance, but the effect of variable heat stress intensity on the correlated responses of resistance traits has not been investigated. Here, the effects of heat intensity selection (fast and slow ramping temperatures) for increasing knockdown temperature in *Drosophila subobscura* were evaluated on knockdown time at different stress temperatures (35, 36, 37 and 38 ºC), thermal death time (TDT) curves, and desiccation and starvation resistance. Correlated evolution was found for these four resistance traits in *D. subobscura*, indicating that the evolutionary response to tolerate higher temperatures also confers the ability to tolerate other stresses such as desiccation and starvation. However, these correlated responses were dependent on the intensity of thermal selection and on 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, but 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). Nevertheless, 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

51 et al. 2016; Castañeda et al. 2019). Thus, the intensity of heat stress may influence our
52 predictions regarding the evolutionary potential of heat tolerance, but how do
53 populations respond to variable selection driven by heat stress? Selection under
54 laboratory conditions has a long history of providing information on the adaptive
55 evolution of specific selective agents (Lenski and Bennett 1993; Garland Jr 2003; Fuller
56 et al. 2005; Gibbs and Gefen 2009). In particular, experimental evolution of heat
57 tolerance has been studied in several species, including fish, corals, and insects (Baer
58 and Travis 2000; Kelly et al. 2012; Geerts et al. 2015; Esperk et al. 2016). Experimental
59 evolution of heat tolerance has also been studied in several *Drosophila* species,
60 *including D. melanogaster* (Gilchrist and Huey 1999; Folk et al. 2006), *D. subobscura*
61 (Quintana and Prevosti 1990; Mesas et al. 2021; Mesas and Castañeda 2023), and *D.*
62 *buzzatti* (Krebs and Loeschcke 1996). Most of these studies reported the evolution of
63 heat tolerance using fast ramping protocols, ranging from 0.4 ºC/min in Folk et al. (Folk
64 et al. 2006) to 1 °C/min in Gilchrist and Huey (Gilchrist and Huey 1999), or static high
65 temperature assays (40 °C), as in Bubliy and Loeschcke (2005). Recently, Mesas et al.
66 (Mesas et al. 2021) reported that selected lines of *D. subobscura* evolved higher heat
67 tolerance, regardless of the heating rate used during the selection experiments (slow-
68 ramping rate: 0.08 °C/min and fast-ramping rate: 0.4 °C/min).
69 Interestingly, several of these selection experiments on heat tolerance in
70 *Drosophila* have found correlated responses in other traits such as starvation resistance,
71 desiccation resistance, and heat shock proteins (Hoffmann et al. 1997; Feder et al. 2002;
72 Bubliy and Loeschcke 2005). However, the intensity of thermal stress is expected to
73 have important effects on the correlated responses of other traits to heat tolerance
74 selection (Fragata and Simões 2022). For example, fast-ramping selected lines have
75 evolved thermal performance curves with higher optimum temperatures and narrower

76 thermal breadths than slow-ramping selected lines (Mesas et al. 2021). In addition,
77 Mesas and Castañeda (Mesas and Castañeda 2023) reported that the evolution of heat
78 tolerance was associated with reduced activity of the enzymes involved in the glucose-
79 6-phosphate branch point and increased performance of life-history traits in slow-
80 ramping selected lines. However, they did not observe any changes in the metabolic rate
81 of the selected lines, as predicted by Santos et al. (2012). In summary, there is evidence
82 that heat stress intensity determines the magnitude of the evolutionary responses of
83 performance, metabolic, and life-history traits to heat tolerance selection; however, the
84 correlated evolution of resistance traits has not yet been tested. This information
85 provides important clues as to how thermal stress intensity might determine the
86 evolution of cross-tolerance to stressful environmental conditions. Natural populations
87 are exposed to multiple environmental stressors, and it is known that increased tolerance
88 to one stressor can boost tolerance to another (Rodgers and Gomez Isaza 2023). Cross-
89 tolerance induced by thermal stress has been widely studied in several arthropod
90 species, increasing resistance to desiccation, insecticides, and pathogens (Kalra et al.
91 2017; Rodgers and Gomez Isaza 2021; Singh et al. 2022). However, the cross-tolerance
92 patterns at the evolutionary level can be constrained or facilitated by genetic
93 correlations between resistance traits depending on the environmental context (Lande
94 and Arnold 1983; Bubliy and Loeschcke 2005; Gerken et al. 2016).
95 The effects of heat stress intensity have previously been studied on the heat
96 knockdown temperature in *D. subobscura* and its correlated responses on the thermal
97 performance curves (Mesas et al. 2021), and on energy metabolism and fitness-related
98 traits (Mesas and Castañeda 2023). The evolutionary response of these traits was
99 evaluated using two thermal selection protocols that differed in the rate of temperature
100 increase (hereafter, ramping rate) used to measure the heat knockdown temperature:

101 slow-ramping selection (0.08ºC min^{-1}) and fast-ramping selection (0.4ºC min^{-1}). The
102 present study investigates the effects of heat intensity selection for increasing
103 knockdown temperature on the cross-tolerance evolution of four different resistance
104 traits in *D. subobscura*: knockdown time at different stress temperatures, thermal-death-
105 time curves (TDT), desiccation resistance, and starvation resistance. In particular, TDT
106 curves represent an integrative approach to assess how the probability of survival
107 depends on the intensity and duration of heat stress, as they allow the estimation of the
108 critical thermal maxima (CT_{max}) and thermal sensitivity using the thermal tolerance
109 measurements obtained at different stress temperatures (Rezende et al. 2014). Here, it is
110 expected that fast-ramping selected lines would exhibit higher knockdown time at
111 highly stressful temperatures and higher CT_{max} because fast-ramping protocols reduce
112 the confounding effects on heat tolerance associated with the assay length. In contrast,
113 slow-ramping selected lines should exhibit higher desiccation and starvation resistance,
114 because individuals with higher starvation and desiccation resistance exhibit higher
115 thermal tolerance during long assays.
116
117 **Materials and Methods**
118 ***Sampling and maintenance***
119 *D. subobscura* females were collected in the spring of 2014 at the Botanical Garden of
120 the Universidad Austral de Chile (Valdivia, Chile; 39º 48' S, 73º 14' W) using plastic
121 traps containing banana/yeast baits. A total of 200 females were collected and
122 individually placed in plastic vials containing David's killed-yeast *Drosophila*
123 medium to establish isofemale lines. In the next generation, 100 isofemale lines were
124 randomly selected, and 10 females and 10 males per line were placed in an acrylic
125 cage to establish a large outbred population. In the next generation, the flies contained

126 in this cage were divided into three population cages (R1, R2, and R3), attempting to
127 assign the same number of flies to each cage. After three generations, the flies of each
128 replicate cage were divided into four population cages, trying to assign the same
129 number of flies to each cage. This procedure allowed to establish 12 population cages
130 that were assigned to each artificial selection protocol in triplicate: fast-ramping
131 selection, fast-ramping control, slow-ramping selection, and slow-ramping control
132 lines (Fig. S1). During the selection experiments, population cages were maintained at
133 18 °C (12:12 light-dark cycle) in a discrete generation, controlled larval density
134 regime (Castañeda et al. 2015). Each population cage had a population size of 1000-
135 1500 breeding adults.
136
137 ***Heat tolerance selection***
138 For each replicate line, 120 four-day-old virgin females were randomly mated with
139 two males for two days, after which the females were individually placed in a capped
140 5-mL glass vial and the males were discarded. The vials were attached to a plastic rack
141 and immersed in a water tank with an initial temperature of 28 °C, controlled by a
142 heating unit (model ED, Julabo Labortechnik, Seelbach, Germany). After an
143 equilibration period of 10 min, the temperature was increased to a rate of 0.08 ºC min^{-1}
144 for the slow-ramping selection protocol or 0.4 ºC min^{-1} for the fast-ramping selection
145 protocol. Assays were stopped when all flies collapsed. Each assay was recorded using
146 a high-resolution camera (model D5100, Nikon, Tokyo, Japan) and then visualized to
147 score the knockdown temperature for each fly, which was defined as the temperature
148 at which each fly ceased to move. Flies were ranked by knockdown temperature, and
149 four virgin females were selected from the progeny of the 40 flies with the highest
150 knockdown temperature (top 30% of each assay) to establish the next generation. For

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

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

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

190

191 **Statistical analysis**

192 Normality and homoscedasticity were tested for all variables, and the knockdown times
193 were log_{10}-transformed to meet the parametric assumptions. All analyses were
194 performed with R software (R Development Core Team 2011).

195

196 ***Heat tolerance***

197 Analyses to evaluate the evolutionary response of the heat tolerance to ramping
198 selection was performed separately for the fast and slow ramping selection because it is
199 well known that the knockdown temperature is higher in fast ramping than in slow
200 ramping assays (Chown et al. 2009; Mesas et al. 2021). Mixed linear models with

ramping selection (fixed effect), sex (fixed effect), and replicate lines nested within thermal selection (random effect) were performed for knockdown temperature and knockdown time using the library *lme4* package for R (Bates et al. 2015). Fixed effects were tested by a type III ANOVA using the library *lmerTest* package for R (Kuznetsova et al. 2017), while the random effect was tested by a likelihood ratio test comparing the model with and without the replicate lines. If the selection effect was significant, *a posteriori* comparisons were performed using Tukey tests implemented in the *emmeans* package for R (Lenth et al. 2018).

Knockdown times were also used to plot the survival curves based on the Kaplan-Meier formula using the *survfit* function implemented in the *survival* package for R (Therneau 2023).

Thermal death time curves (TDT)

Average knockdown times were calculated for each sex, replicate lines, and selection treatment combination (Table S1). These values were regressed against the assayed temperatures according to equation 1 (Rezende et al. 2014):

$$log_{10}t = \frac{CT_{max}-T}{Z} \qquad \text{eqn. 1}$$

, where T is the assayed static temperature (ºC), CT_{max} is the upper thermal limit (ºC), t is the knockdown time (min), and z is the thermal sensitivity. These curves allowed the estimation of CT_{max} as the extrapolated temperature that would result in a knockdown time of $\log_{10} t = 0$ (i.e., knockdown time at 1 min) and the estimation of the thermal sensitivity ($z = -1/\text{slope}$), where the lower z values, the higher the thermal sensitivity.

225 Using equation 1, 24 TDT curves (2 sexes × 3 replicate lines × 4 selection
226 protocols) were fitted, from which CT_{max} and z values were estimated as described
227 above. A linear model with ramping selection treatment, sex, and their interaction was
228 performed to evaluate their effects on CT_{max} and z values.
229
230 ***Desiccation and starvation resistance***
231 To determine the lethal time at which 50% of flies of each vial were dead (LT_{50}), a
232 generalized linear model following a binomial distribution was fitted with the
233 proportion of flies alive as the dependent variable and time as the predictor variable.
234 The generalized linear model was run using the g*lm* function of the *lme4* package for R
235 (Bates et al. 2015). The LT_{50} of each vial was then estimated using the function *dose.p*
236 from the *MASS* package for R (Venables and Ripley 2002).
237 To estimate the median LT_{50} and the 95% confidence intervals for each selection
238 treatment and sex, each LT_{50} was transformed into a survival object using the *Surv* and
239 *survfit* functions of the *survival* package for R (Therneau 2023). This procedure also
240 allowed to estimate the survival curves in each vial. Finally, to test the effect of
241 selection treatment and sex on desiccation and starvation resistance, a Cox proportional
242 regression model was fitted with LT_{50} as the dependent variable, and selection protocol
243 and sex as predictor variables. The Cox model was run using the *coxph* function of the
244 *survival* package (Therneau 2023).
245
246 **RESULTS**
247 **Knockdown temperature evolution**
248 Knockdown temperature evolved in response to artificial selection for increased heat
249 tolerance, regardless of the ramping assay protocol: knockdown temperature was

250 significantly higher in fast-ramping selected lines than in to fast-ramping control lines
251 (mean fast-ramping selected lines [95% CI] = 37.71 ºC [37.63 – 37.78] and mean fast-
252 ramping control lines [95% CI] = 37.23 ºC [37.0 – 37.38]; $F_{1,4}$ = 32.0, P = 0.005); and
253 the knockdown temperature in slow-ramping selected lines was significantly higher
254 than in slow-ramping control lines (mean slow-ramping selected lines [95% CI] =
255 35.48ºC [35.41 – 35.55] and mean fast-ramping control lines [95% CI] = 34.97 ºC
256 [34.82 – 35.12]; $F_{1,4}$ = 41.7, P = 0.003). These results were previously reported by
257 Mesas et al. (2021) and are reported here to contextualize the following results.
258
259 ***Knockdown time evolution***
260 As expected, the knockdown time decreased significantly as the assay temperatures
261 increased ($F_{1,877}$ = 649.1, $P < 2\times10^{-16}$). The mean knockdown time and 95% CI for each
262 static assay are as follows: 35º C = 33.77 min [32.1 – 35.5]; 36º C = 16.98 min [16.1
263 – 17.9]; 37º C = 8.84 min [8.4 – 9.3]; and 38º C = 6.78 min [6.3 – 7.0].
264 Knockdown times differed significantly between selection treatments when flies
265 were assayed at 36 and 37ºC (Table 1; Fig. 1). At these temperatures, slow and fast
266 selected lines showed higher heat tolerance than slow and fast control lines (Table S1,
267 S2; Fig. 1C, E). Also, fast-selected lines showed a higher heat tolerance than slow-
268 selected lines in flies assayed at 37ºC but not at 36ºC (Table S1, S2; Fig. 1C, E),
269 whereas fast and slow control lines did not differ (Table S2; Fig 1). On the other hand,
270 replicate lines had no significant effect on knockdown time, indicating consistent
271 evolutionary responses within each selection and control treatment (variance among
272 replicate lines = 0, χ^2_1 = 0, and P =1 for all static assays). With respect to sex, females
273 showed a higher thermal tolerance than males but only when flies were assayed at 35

274 and 38ºC (Table 1; Fig. 1B, H). Finally, non-significant interactions between selection
275 and sex were found for all temperatures assayed (Table 1).
276
277 ***TDT curves evolution***
278 Linear regressions between $\log_{10}(LT_{50})$ and assayed temperatures allowed the
279 estimation of 24 TDT curves (4 selection treatments × 3 replicate lines × 2 sexes) with
280 high coefficients of determination (mean $R^2 = 0.946$, range: 0.820 – 0.989; Table S3),
281 confirming that heat knockdown time is linearly related to stressful sublethal
282 temperatures. From these TDT curves, the mean CT_{max} [95% CI] was 41.21ºC [41.02
283 – 41.41], and the mean *z* [95% CI] was 4.18ºC [4.03 – 4.32]. CT_{max} were significantly
284 different between selection treatments ($F_{3,20} = 4.46$, $P = 0.015$; Fig. 2A). A post hoc
285 analysis showed that fast-ramping selected and slow-ramping control lines were
286 significantly different in their CT_{max} values ($t_{20} = 3.195$, $P = 0.02$), whereas fast and
287 slow control lines had similar CT_{max} values ($t_{20} = 0.911$, $P = 0.80$). Thus, when control
288 lines are pooled, CT_{max} still differs between selection treatments ($F_{2,18} = 6.69$, $P =$
289 0.007), with fast-ramping (mean CT_{max} [95% CI] = 41.55 ºC [41.2 – 41.9]) and slow-
290 ramping selected lines (mean CT_{max} [95% CI] = 41.43 ºC [41.1 – 41.8]) had higher
291 CT_{max} than control lines (mean CT_{max} [95% CI] = 40.94 ºC [40.7 – 41.2]) ($t_{18} = 3.27$, P
292 $= 0.01$ and $t_{18} = 2.64$, $P = 0.04$, respectively). CT_{max} was not different between the
293 selected lines ($t_{18} = 0.54$, $P = 0.85$). On the other hand, sex and the interaction between
294 selection treatments and sex had no significant effect on CT_{max} ($F_{1,18} = 0.004$, $P = 0.95$
295 and $F_{3,18} = 2.11$, $P = 0.15$, respectively). Regarding *z* (i.e., thermal sensitivity), it shows
296 no significant effects of selection treatments ($F_{3,16} = 0.91$, $P = 0.46$; Fig. 2), sex ($F_{1,16} =$
297 1.30, $P = 0.27$), nor the interaction between selection treatments and sex ($F_{3,16} = 2.23$, P
298 $= 0.12$). In summary, the evolution of a higher CT_{max} is not associated with an

evolutionary change in thermal sensitivity (Fig. 2B). Indeed, the relationship between CT_{max} and z did not change with the evolution of increasing thermal tolerance ($r_{control\text{-}lines}$ = 0.979 and $r_{selected\text{-}lines}$ = 0.929; Z-test = 0.76, P = 0.45).

Desiccation resistance evolution

Survival analysis showed a significant interaction between selection treatments and sex on desiccation resistance (LTR: $\chi^2_5 = 83.55$, $P < 2\times10^{-16}$). Males showed a higher risk of desiccation than female flies (hazard ratio = 7.11, $P < 2\times10^{-7}$; Fig. 3). Females showed a significant difference between selected and control lines (LTR: $\chi^2_2 = 6.72$, P = 0.03; Fig. 3A). Specifically, females of the slow-ramping selection lines showed a higher desiccation resistance than females of the control lines (Hazard ratio = 0.42, P = 0.009), whereas females of the fast-ramping selection and control lines showed similar desiccation risk (hazard ratio = 0.56, P = 0.072). On the other hand, males showed no differences in desiccation resistance between selected and control lines (LTR: χ^2_2 = 1.88, P = 0.4; Fig. 3B). Results of the desiccation survival analysis testing the effect of selection protocol, sex, and their interaction are reported in the Table S4.

Starvation resistance evolution

Survival analysis showed a significant interaction between selection treatments and sex on desiccation resistance ($LTR_5 = 94.89$, $P < 2\times10^{-16}$). Males had a higher risk of starvation than female flies (hazard ratio = 22.75, $P < 1\times10^{-16}$; Fig. 4). In female flies (Fig. 4A), fast-ramping selection and slow-ramping selection lines showed a higher starvation risk than control lines (hazard ratio = 2.37, P = 0.009; and hazard ratio = 2.20, P = 0.014, respectively). In contrast, male flies had an opposite pattern (Fig. 4B): slow-ramping selection lines had a lower starvation risk than control lines (hazard ratio

324 = 0.50, $P = 0.03$), but nonsignificant differences were found between fast-ramping
325 selection and control lines (hazard ratio = 0.64, $P = 0.16$). Results of the starvation
326 survival analysis testing the effect of selection protocol, sex, and their interaction are
327 reported in Table S5.
328
329 **Discussion**
330 Studying the evolutionary responses of thermal limits is key to understanding the
331 adaptive responses and evolutionary constraints to global warming. Cross-tolerance
332 studies can then provide valuable information on the evolutionary response to multiple
333 environmental stressors. Cross-tolerance evolution has been reported among different
334 resistance traits (Hoffmann and Parsons 1993; Bubliy and Loeschcke 2005; Stazione et
335 al. 2020; Singh et al. 2022), but the magnitude of the evolutionary response could be
336 explained by the trait under direct selection or the stress intensity (Gerken et al. 2016).
337 Here, artificial selection for heat tolerance (i.e., knockdown temperature) resulted in
338 correlated responses in heat knockdown time, the thermal tolerance landscape (TDT
339 curves), desiccation resistance, and starvation resistance. However, these responses
340 depended on the intensity of thermal selection and sex, suggesting that the evolutionary
341 response to tolerate higher temperatures also confers partial tolerance to other stresses
342 such as desiccation and starvation.
343 Different approaches to measuring the upper thermal limit of ectotherms
344 produce different genetic and phenotypic estimates. In general, fast ramping assays
345 estimate higher upper thermal limits and higher heritabilities than slow ramping assays
346 (Chown et al. 2009; Rezende et al. 2011). In fact, the heritability of thermal tolerance of
347 was 0.13 for fast assays and 0.08 for slow assays (Castañeda et al. 2019). Because
348 heritability is commonly used as a predictor of the evolutionary response of a trait to

349 natural or artificial selection, the evolutionary response of heat tolerance would be
350 expected to depend on the ramping rate used during selection. However, previous work
351 did not support this prediction for *D. subobscura*, finding that the evolution of heat
352 tolerance was independent of the ramping rate (Mesas et al. 2021), but the correlated
353 responses of the thermal performance curves or the energy metabolism depended on the
354 intensity of the thermal selection (Mesas et al. 2021; Mesas and Castañeda 2023). In the
355 present study, the evolution of knockdown temperature (e.g., heat tolerance measured in
356 dynamic assays) induced a correlated response on the heat knockdown time (e.g., heat
357 tolerance measured in static assays) when it was assayed at intermediate temperatures
358 (36 and 37ºC), but not at less or more extreme assayed temperatures (35 and 38ºC).
359 These findings can be explained because stress tolerance at 35ºC should depend on the
360 physiological state of the organism during prolonged thermal assays (e.g., availability of
361 energy resources; see Rezende et al. 2011, but also see Overgaard et al. 2012) and not
362 only on heat tolerance, whereas heat tolerance at 38ºC could be limited by physical
363 properties of ectotherms (e.g., protein denaturation, membrane permeability). However,
364 a previous study found a clinal pattern for heat tolerance in *D. subobscura* only for flies
365 assayed in static assays (specifically at 38ºC), but this clinal pattern was not detected
366 using ramping assays (Castañeda et al. 2015). Differences between these two studies
367 could be explained by the number of generations under thermal selection, which could
368 result in a different evolutionary response of heat tolerance. According to Begon (1976),
369 *D. subobscura* can have between 4 and 6 generations per year, which makes it possible
370 to estimate about 125 generations of selection from the introduction of *D. subobcura* in
371 Chile until the study by Castañeda et al. (2015). On the other hand, the type of selection
372 is completely different between the two studies (e.g., natural versus artificial selection),
373 which could lead to different evolutionary outcomes. In any case, beyond these results

374 from specific thermal assays, these findings support the idea that (1) the use of a single
375 static temperature would miss genetic or phenotypic effects on heat tolerance, and (2)
376 unifying several knockdown time estimates into a single approach (TDT curves) should
377 be necessary to elucidate genetic and phenotypic patterns of heat tolerance in
378 ectotherms (Rezende et al. 2014; Jørgensen et al. 2021).

379 TDT curves evolved in response to heat tolerance selection in *D. subobscura*.
380 TDT curves showed that fast- and slow-ramping selected lines evolved higher CT_{max}
381 than control lines (ΔCT_{max} = 0.49 ºC). This differential CT_{max} value is slightly lower
382 than the population differences (0.9ºC) observed between the lowest and highest latitude
383 populations (~8 latitudinal degrees) of *D. subobscura* studied by Castañeda et al. (2015)
384 and even lower than the CT_{max} variation reported among *Drosophila* species (Jørgensen
385 et al. 2019; Alruiz et al. 2022). On the other hand, although CT_{max} and z (i.e., thermal
386 sensitivity) are phenotypically correlated (see Castañeda et al. 2015; Molina et al.
387 2023), the evolutionary increase in CT_{max} was not associated with a correlated response
388 in thermal sensitivity (z). This result suggests that both thermal parameters are not
389 genetically constrained, but further evidence from quantitative genetic studies is needed
390 to assess the genetic association between CT_{max} and z. A caveat for this finding could be
391 related to the fact that thermal selection for heat tolerance was carried out over 16
392 generations, followed by 7 generations of relaxed selection (i.e., no selection).
393 However, previous evidence suggests that differences in heat tolerance between control
394 and selected lines were consistent between generations 16 and 25 (Mesas et al. 2021).
395 Indeed, Passananti et al. (2004) also reported that phenotypic values did not change
396 after 35 generations of relaxed selection in desiccation-selected populations of *D.*
397 *melanogaster*.

It was expected that flies selected for higher heat tolerance using slow-ramping rate protocols would exhibit greater desiccation and starvation resistance than flies selected using fast-ramping selection protocols. This is because flies assayed for heat tolerance in long assays are also exposed to desiccation and starvation stress (Santos et al. 2012) . This study provides partial support for this hypothesis. First, slow-ramping selected lines evolved a higher desiccation resistance compared to control and fast-ramping selected lines. However, this was only observed in female flies, while males of the different selection treatments did not show any difference in desiccation resistance. On the other hand, starvation resistance evolved in opposite directions depending on sex: females of the fast-ramping and slow-ramping selected lines showed lower starvation resistance than females of the control lines, whereas males of the slow-ramping selected lines showed higher starvation resistance than males of the control and fast-ramping selected lines. Differential evolutionary responses between the sexes could be explained because heat thermal selection was only applied to females, which could have exacerbated the evolutionary responses of female flies. However, previous studies that artificially selected exaggerated male traits also found fitness consequences in females (Harano et al. 2010). Differential evolutionary responses between females and males can then be explained by sexually antagonistic selection on genetically correlated traits (Eyer et al. 2019; Fanara et al. 2023). Kwan et al. (2008) reported that desiccation-selected females had higher desiccation resistance than desiccation-selected males (see also Chippindale et al. 2004), which can be explained by males using resources at a faster rate than females (e.g., males lose weight, water, and metabolites faster rate than females). In fact, sexual dimorphism in stress resistance traits has been mainly explained by differences in cuticular composition, resource storage, and energy conservation between the sexes (Schwasinger-Schmidt et al. 2012; Rusuwa et al. 2022).

423 Although energy content was not measured here, Mesas and Castañeda (2023) found
424 that body mass and metabolic rate were similar between control and heat-tolerance
425 selected lines of *D. subobscura*, suggesting that neither resource storage nor energy
426 conservation explains the sex-dependent correlated response for stress resistance traits.
427 However, the same study found that heat-tolerance selected lines had higher fecundity
428 than control lines, whereas previous studies have found negative associations between
429 fecundity and starvation resistance in *D. melanogaster* (Bubliy and Loeschcke 2005;
430 Kalra et al. 2017). Then, the decrease in starvation resistance in females of the heat-
431 selected lines could be related to increased fecundity, which is consistent with the
432 reported trade-off between stress resistance traits and life-history traits (van Noordwijk
433 and de Jong 1986; Rion and Kawecki 2007).

434 In conclusion, the present study shows that heat tolerance evolution is associated
435 with evolutionary responses in other stress resistance traits, which could be explained
436 by pleiotropic effects or linkage disequilibrium among the traits evaluated. However,
437 further evidence (e.g., quantitative genetic or genome-wide analysis studies) is needed
438 to elucidate the genetic basis of the cross-tolerance evolution in *D. subobscura*. In
439 addition, this study provides evidence for rapid evolutionary responses in ectotherms
440 mediated by thermal selection, but the evolutionary outcomes depend on the intensity of
441 the thermal stress (Mesas and Castañeda 2023) and sex (Rogell et al. 2014; Rusuwa et
442 al. 2022). This study also highlights the importance of *D. subobscura* as a suitable
443 model to study thermal adaptation mediated by natural selection (Huey 2000; Gilchrist
444 et al. 2008; Castañeda et al. 2013, 2015), and laboratory selection (Santos et al. 2005,
445 Santos et al. 2021; Simões et al. 2017; Mesas et al. 2021; Mesas and Castañeda 2023).
446 In addition, this study highlights the relevance of experimental evolutionary studies for
447 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.

Acknowledgments

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

Funding

This work was funded by the Fondo Nacional de Investigación Científica y Tecnológica (FONDECYT) [grant number 1140066] – Chile. L.E. Castañeda is supported by the Agencia Nacional de Invertigación y Desarrollo (ANID) through the grants Anillo ATE230025, FOVI220194, and FOVI 230149.

Data availability

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

Conflict of interest disclosure

472 Author declares that he complies with the PCI rule of having no financial conflicts in
473 relation to the content of the article.
474
475 **References**

476 Alruiz, J. M., I. Peralta-Maraver, F. Bozinovic, M. Santos, and E. L. Rezende. 2022.
477 Thermal tolerance in Drosophila: Repercussions for distribution, community
478 coexistence and responses to climate change. J. Anim. Ecol. 91:655–667.
479 Angilletta, M. J. 2009. Thermal Adaptation: A Theoretical and Empirical Synthesis.
480 Oxford University Press.
481 Baer, C. F., and J. Travis. 2000. Direct and correlated responses to artificial selection on
482 acute thermal stress tolerance in a livebearing fish. Evolution (N. Y). 54:238–244.
483 Bates, D., M. Mächler, B. Bolker, and S. Walker. 2015. Fitting linear mixed-effects
484 models using lme4. J. Stat. Softw. 67:1–48.
485 Begon, M. 1976. Temporal variations in the reproductive condition of Drosophila
486 obscura Fallén and D. subobscura Collin. Oecologia 23:31–47.
487 Blackburn, S., B. Van Heerwaarden, V. Kellermann, and C. M. Sgrò. 2014.
488 Evolutionary capacity of upper thermal limits: beyond single trait assessments. J.
489 Exp. Biol. 217:1918–1924.
490 Brennan, R. S., J. A. deMayo, H. G. Dam, M. Finiguerra, H. Baumann, V. Buffalo, and
491 M. H. Pespeni. 2022. Experimental evolution reveals the synergistic genomic
492 mechanisms of adaptation to ocean warming and acidification in a marine copepod.
493 Proc. Natl. Acad. Sci. U. S. A. 119:1–10.
494 Bubliy, O. A., and V. Loeschcke. 2005. Correlated responses to selection for stress
495 resistance and longevity in a laboratory population of Drosophila melanogaster. J.
496 Evol. Biol. 18:789–803.

Castañeda, L. E., J. Balanya, E. L. Rezende, and M. Santos. 2013. Vanishing chromosomal inversion clines in Drosophila subobscura from Chile : is behavioral thermoregulation to blame ? 182:249–259.

Castañeda, L. E., E. L. Rezende, and M. Santos. 2015. Heat tolerance in Drosophila subobscura along a latitudinal gradient: Contrasting patterns between plastic and genetic responses. Evolution (N. Y). 69:2721–2734.

Castañeda, L. E., V. Romero-Soriano, A. Mesas, D. A. Roff, and M. Santos. 2019. Evolutionary potential of thermal preference and heat tolerance in Drosophila subobscura. J. Evol. Biol. 32:818–824.

Chippindale, A., M. R. Rose, A. G. Gibbs, A. K. Chippindale, and M. R. Rose. 2004. Methuselah Flies - A Case Study in the Evolution of Aging. , doi: 10.1142/9789812567222.

Chown, S. L., K. R. Jumbam, J. G. Sørensen, and J. S. Terblanche. 2009. Phenotypic variance, plasticity and heritability estimates of critical thermal limits depend on methodological context. Funct. Ecol. 23:133–140.

Cossins, A. R., and K. Bowler. 1987. Temperature Biology of Animals. Chapman and Hall.

Deutsch, C. A., J. J. Tewksbury, R. B. Huey, K. S. Sheldon, C. K. Ghalambor, D. C. Haak, and P. R. Martin. 2008. Impacts of climate warming on terrestrial ectotherms across latitude. Proceeding Natl. Acad. Sci. 105:6668–6672.

Esperk, T., A. Kjærsgaard, R. J. Walters, D. Berger, and W. U. Blanckenhorn. 2016. Plastic and evolutionary responses to heat stress in a temperate dung fly: Negative correlation between basal and induced heat tolerance? J. Evol. Biol. 29:900–915.

Eyer, P. A., A. J. Blumenfeld, and E. L. Vargo. 2019. Sexually antagonistic selection promotes genetic divergence between males and females in an ant. Proc. Natl.

522 Acad. Sci. U. S. A. 116:24157–24163.
523 Fanara, J. J., P. L. Sassi, J. Goenaga, and E. Hasson. 2023. Genetic basis and
524 repeatability for desiccation resistance in Drosophila melanogaster (Diptera:
525 Drosophilidae). Genetica, doi: 10.1007/s10709-023-00201-0. Springer
526 International Publishing.
527 Feder, M. E., T. B. C. Bedford, D. R. Albright, and M. Pawel. 2002. Evolvability of
528 Hsp70 expression under artificial selection for inducible thermotolerance in
529 independent populations of Drosophila melanogaster. Physiol. Biochem. Zool.
530 75:325–334.
531 Folk, D. G., P. Zwollo, D. M. Rand, and G. W. Gilchrist. 2006. Selection on
532 knockdown performance in Drosophila melanogaster impacts thermotolerance and
533 heat-shock response differently in females and males. J. Exp. Biol. 209:3964–
534 3973.
535 Fragata, I., and P. Simões. 2022. The other side of the evolution of heat tolerance:
536 correlated responses in metabolism and life-history traits. Peer Community Evol.
537 Biol. 100155.
538 Fuller, R. C., C. F. Baer, and J. Travis. 2005. How and when selection experiments
539 might actually be useful. Integr. Comp. Biol. 45:391–404.
540 Garland Jr, T. 2003. Selection experiments : an under-utilized tool in biomechanics and
541 organismal biology. Pp. 23–57 *in* V. L. Bels, J.-P. Gasc, and A. Casinos, eds.
542 Vertebrate Biomechanics and Evolution. BIOS Scientific Publisher Ltd, Oxford.
543 Geerts, A. N., J. Vanoverbeke, B. Vanschoenwinkel, W. Van Doorslaer, H. Feuchtmayr,
544 D. Atkinson, B. Moss, T. A. Davidson, C. D. Sayer, and L. De Meester. 2015.
545 Rapid evolution of thermal tolerance in the water flea Daphnia. Nat. Clim. Chang.
546 5:665–668.

547 Gerken, A. R., T. F. C. Mackay, and T. J. Morgan. 2016. Artificial selection on chill-
548 coma recovery time in Drosophila melanogaster: Direct and correlated responses to
549 selection. J. Therm. Biol. 59:77–85. Elsevier.
550 Gibbs, A. G., and E. Gefen. 2009. Physiological Adaptations in Laboratory
551 Environments. Pp. 523–550 *in* T. Garland Jr and M. R. Rose, eds. Experimental
552 Evolution: Concepts, Methods, and Applications of Selection Experiments.
553 University of California Press, Berkeley.
554 Gilchrist, G. W., and R. B. Huey. 1999. The direct response of Drosophila melanogaster
555 to selection on knockdown temperature. Heredity (Edinb). 83 (Pt 1):15–29.
556 Gilchrist, G. W., L. M. Jeffers, B. West, D. G. Folk, J. Suess, and R. B. Huey. 2008.
557 Clinal patterns of desiccation and starvation resistance in ancestral and invading
558 populations of Drosophila subobscura. Evol. Appl. 1:513–523.
559 Harano, T., K. Okada, S. Nakayama, T. Miyatake, and D. J. Hosken. 2010. Intralocus
560 sexual conflict unresolved by sex-limited trait expression. Curr. Biol. 20:2036–
561 2039. Elsevier Ltd.
562 Heerwaarden, B. Van, M. Malmberg, and C. M. Sgrò. 2016. Increases in the
563 evolutionary potential of upper thermal limits under warmer temperatures in two
564 rainforest Drosophila species. Evolution (N. Y). 70:456–464.
565 Hoffmann, A. A., H. Dagher, M. Hercus, and D. Berrigan. 1997. Comparing different
566 measures of heat resistance in selected lines of Drosophila melanogaster. J. Insect
567 Physiol. 43:393–405.
568 Hoffmann, A. A., and P. A. Parsons. 1993. Direct and correlated responses to selection
569 for desiccation resistance: a comparison of Drosophila melanogaster and D.
570 simulans. J. Evol. Biol. 6:643–657.
571 Hoffmann, A. A., and C. M. Sgrò. 2011. Climate change and evolutionary adaptation.

Nature 470:479–85.

Huey, R. B. 2000. Rapid Evolution of a Geographic Cline in Size in an Introduced Fly. Science (80-.). 287:308–309.

Huey, R. B., C. A. Deutsch, J. J. Tewksbury, L. J. Vitt, P. E. Hertz, J. Héctor, Á. Pérez, T. Garland, P. R. S. B, R. B. Huey, C. A. Deutsch, J. J. Tewksbury, and L. J. Vitt. 2009. Why tropical forest lizards are vulnerable to climate warming. Proceeding R. Soc. B 2009:1939–1948.

Huey, R. B., and R. D. Stevenson. 1979. Integrating thermal physiology and ecology of ectotherms: a discussion of approaches. Am. Zool. 19:357–366.

Jørgensen, L. B., H. Malte, M. Ørsted, N. A. Klahn, and J. Overgaard. 2021. A unifying model to estimate thermal tolerance limits in ectotherms across static, dynamic and fluctuating exposures to thermal stress. Sci. Rep. 11:1–14. Nature Publishing Group UK.

Jørgensen, L. B., H. Malte, and J. Overgaard. 2019. How to assess Drosophila heat tolerance: Unifying static and dynamic tolerance assays to predict heat distribution limits. Funct. Ecol. 33:629–642.

Kalra, B., A. M. Tamang, and R. Parkash. 2017. Cross-tolerance effects due to adult heat hardening, desiccation and starvation acclimation of tropical drosophilid-Zaprionus indianus. Comp. Biochem. Physiol. -Part A Mol. Integr. Physiol. 209:65–73. Elsevier.

Kellermann, V., J. Overgaard, A. A. Hoffmann, C. Fljøgaard, J. C. Svenning, and V. Loeschcke. 2012. Upper thermal limits of Drosophila are linked to species distributions and strongly constrained phylogenetically. Proc. Natl. Acad. Sci. U. S. A. 109:16228–16233.

Kelly, M. W. 2022. Experimental evolution reveals complex responses to

environmental change. Proc. Natl. Acad. Sci. U. S. A. 119:1–2.

Kelly, M. W., E. Sanford, and R. K. Grosberg. 2012. Limited potential for adaptation to climate change in a broadly distributed marine crustacean. Proc. R. Soc. B Biol. Sci. 279:349–356.

Krebs, R. A., and V. Loeschcke. 1996. Acclimation and selection for increased resistance to thermal stress in Drosophila buzzatii. Genetics 142:471–479.

Kuznetsova, A., P. B. Brockhoff, and R. H. B. Christensen. 2017. lmerTest package: Tests in linear mixed effects models. J. Stat. Softw. 82:1–26.

Kwan, L., S. Bedhomme, N. G. Prasad, and A. K. Chippindale. 2008. Sexual conflict and environmental change: trade-offs within and between the sexes during the evolution of desiccation resistance. J. Genet. 87:383–394.

Lande, R., and S. J. Arnold. 1983. The measurement of selection on correlated characters. Evolution (N. Y). 37(6):1210–1226.

Lenski, R. E., and A. F. Bennett. 1993. Evolutionary response of Escherichia coli to thermal stress. Am. Nat. 142:47–64.

Lenth, R., H. Singmann, J. Love, P. Buerkner, and M. Herve. 2018. Package "Emmeans". R Package Version 4.0-3.

Mesas, A., and L. E. Castañeda. 2023. Evolutionary responses of energy metabolism, development, and reproduction to artificial selection for increasing heat tolerance in Drosophila subobscura. Evolution 77:509–518.

Mesas, A., A. Jaramillo, and L. E. Castañeda. 2021. Experimental evolution on heat tolerance and thermal performance curves under contrasting thermal selection in Drosophila subobscura. J. Evol. Biol. 34:767–778.

Mitchell, K. A., and A. A. Hoffmann. 2010. Thermal ramping rate influences evolutionary potential and species differences for upper thermal limits in

622 Drosophila. Funct. Ecol. 24:694–700.
623 Mitchell, N., and K. D. Whitney. 2018. Can plants evolve to meet a changing climate?
624 The potential of field experimental evolution studies. Am. J. Bot. 105:1613–1616.
625 Molina, A. N., J. M. Pulgar, E. L. Rezende, and M. J. Carter. 2023. Heat tolerance of
626 marine ectotherms in a warming Antarctica. Glob. Chang. Biol. 29:179–188.
627 Overgaard, J., T. N. Kristensen, and J. G. Sørensen. 2012. Validity of thermal ramping
628 assays used to assess thermal tolerance in arthropods. PLoS One 7:e32758.
629 Passananti, H. B., K. A. Beckman, and M. R. Rose. 2004. Relaxed stress selection in
630 Drosophila melanogaster. Pp. 323–352 *in* M. R. Rose, H. B. Passananti, and M.
631 Matos, eds. Methuselah Flies: A Case Study in the Evolution of Aging. World
632 Scientific, Singapore.
633 Quintana, A., and A. Prevosti. 1990. Genetic and environmental factors in the resistance
634 of Drosophila subobscura adults to high temperature shock. Theor. Appl. Genet.
635 80:847–851.
636 R Development Core Team, R. 2011. R: A Language and Environment for Statistical
637 Computing. R Foundation for Statistical Computing.
638 Rezende, E. L., L. E. Castañeda, and M. Santos. 2014. Tolerance landscapes in thermal
639 ecology. Funct. Ecol. 28:799–809.
640 Rezende, E. L., M. Tejedo, and M. Santos. 2011. Estimating the adaptive potential of
641 critical thermal limits: methodological problems and evolutionary implications.
642 Funct. Ecol. 25:111–121.
643 Rion, S., and T. J. Kawecki. 2007. Evolutionary biology of starvation resistance : what
644 we have learned from Drosophila. 20:1655–1664.
645 Rodgers, E. M., and D. F. Gomez Isaza. 2021. Harnessing the potential of cross-
646 protection stressor interactions for conservation: A review. Conserv. Physiol. 9.

647 Rodgers, E. M., and D. F. Gomez Isaza. 2023. The mechanistic basis and adaptive
648 significance of cross-tolerance: a 'pre-adaptation' to a changing world? J. Exp.
649 Biol. 226:jeb245644.
650 Rogell, B., W. Widegren, L. R. Hallsson, D. Berger, M. Björklund, and A. A.
651 Maklakov. 2014. Sex-dependent evolution of life-history traits following
652 adaptation to climate warming. Funct. Ecol. 28:469–478.
653 Rusuwa, B. B., H. Chung, S. L. Allen, F. D. Frentiu, and S. F. Chenoweth. 2022.
654 Natural variation at a single gene generates sexual antagonism across fitness
655 components in Drosophila. Curr. Biol. 32:3161-3169.e7. Elsevier Ltd.
656 Santos, M. A., A. Carromeu-Santos, A. S. Quina, M. Santos, M. Matos, and P. Simões.
657 2021. No evidence for short-term evolutionary response to a warming environment
658 in Drosophila. Evolution (N. Y). 75:2816–2829.
659 Santos, M., W. C,spedes, J. Balany…, V. Trotta, F. C. F. Calboli, A. Fontdevila, and L.
660 Serra. 2005. Temperature-related genetic changes in laboratory populations of
661 Drosophila subobscura : Evidence against simple climatic-based explanations for
662 latitudinal clines. Am. Nat. 165:258–273.
663 Santos, M., L. E. Castañeda, and E. L. Rezende. 2012. Keeping pace with climate
664 change: What is wrong with the evolutionary potential of upper thermal limits?
665 Ecol. Evol. 2:2866–2880.
666 Schwasinger-Schmidt, T. E., S. D. Kachman, and L. G. Harshman. 2012. Evolution of
667 starvation resistance in Drosophila melanogaster: Measurement of direct and
668 correlated responses to artificial selection. J. Evol. Biol. 25:378–387.
669 Simões, P., I. Fragata, S. G. Seabra, G. S. Faria, M. A. Santos, M. R. Rose, M. Santos,
670 and M. Matos. 2017. Predictable phenotypic, but not karyotypic, evolution of
671 populations with contrasting initial history. Sci. Rep. 7:1–12.

Singh, K., M. Arun Samant, and N. G. Prasad. 2022. Evolution of cross-tolerance in Drosophila melanogaster as a result of increased resistance to cold stress. Sci. Rep. 12:19536. Nature Publishing Group UK.

Stazione, L., F. M. Norry, F. H. Gomez, and P. Sambucetti. 2020. Heat knockdown resistance and chill-coma recovery as correlated responses to selection on mating success at high temperature in Drosophila buzzatii. Ecol. Evol. 10:1998–2006.

Therneau, T. M. 2023. A package for survival analysis in R. R package version 3.2-7.

van Noordwijk, A. J., and G. de Jong. 1986. Acquisition and Allocation of Resources: Their Influence on Variation in Life History Tactics. Am. Nat. 128:137–142.

Venables, W. N., and B. D. Ripley. 2002. Modern Applied Statistics with S. Fourth edi. Springer US, New York.

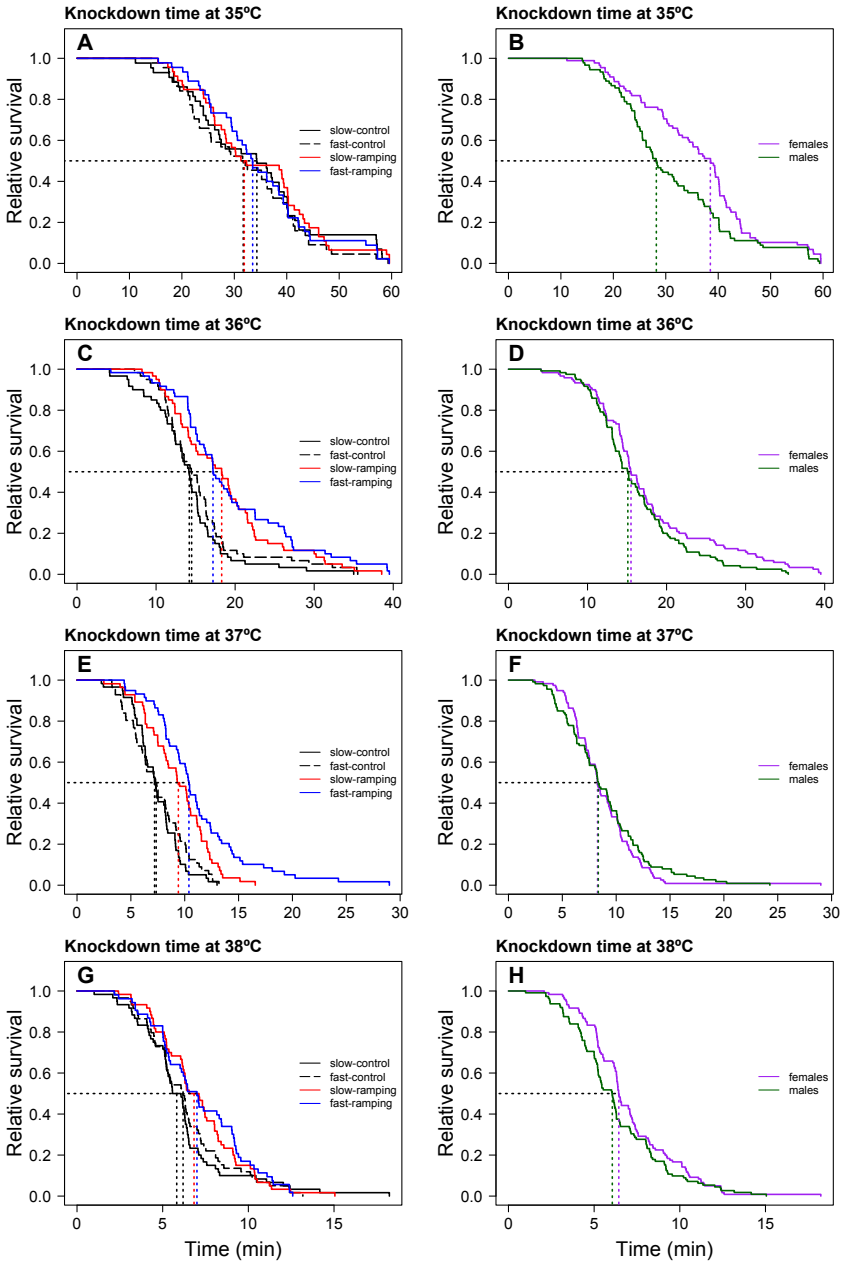
Visser, M. E. 2008. Keeping up with a warming world; assessing the rate of adaptation to climate change. Proc. R. Soc. B Biol. Sci. 275:649–659.

687 **Table 1**. Mixed linear effect model for the knockdown time of *Drosophila subobscura*
688 assayed in four static temperature assays. For simplicity, results for the random effect
689 (replicate lines) are not shown because they were not statistically significant (see
690 Materials and Methods). Significant P-values (< 0.05) are indicated in boldface type.
691

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

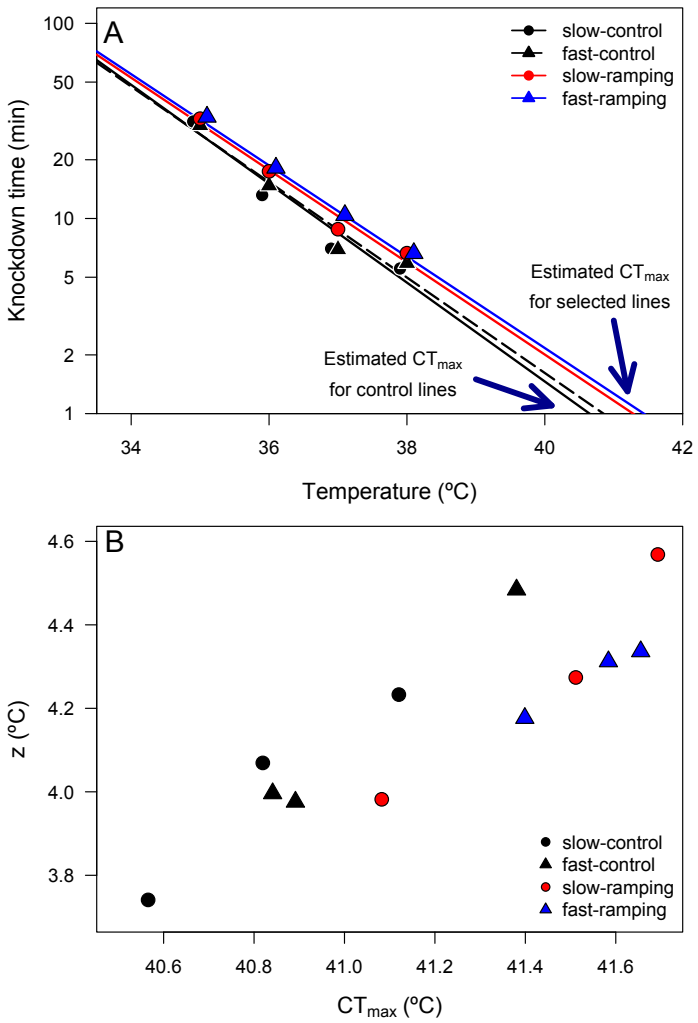
692
693

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



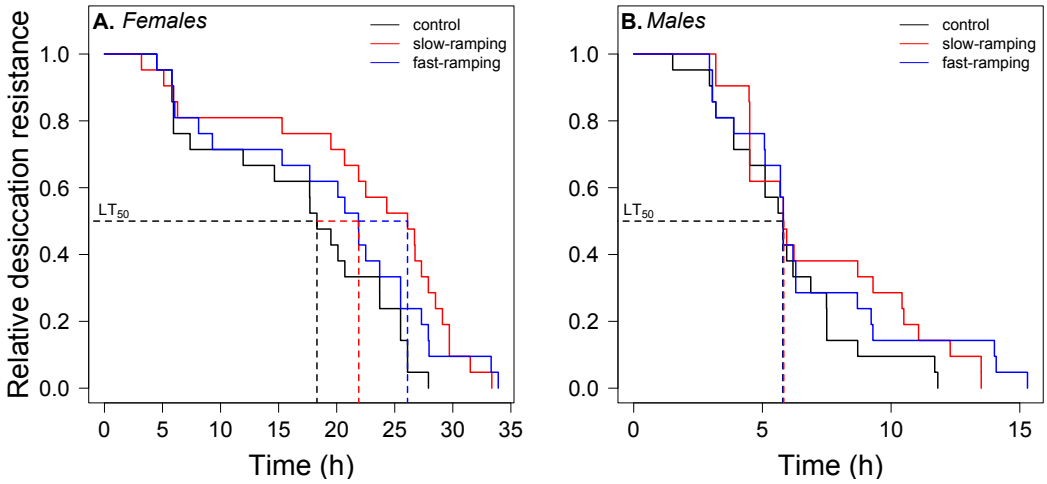
700

701 **Figure 2**. (A) Thermal death curves for control (black solid and dashed lines) and
702 selected (red and blue lines) lines for increasing heat tolerance in *Drosophila*
703 *subobscura*. Symbols represent the average knockdown time at the different
704 temperatures assayed. Each symbol represents the average knockdown time for each
705 replicate line for each thermal regime: slow-control (black circle), fast-control (black
706 triangle), slow-ramping (red circle), and fast-ramping (blue triangle). (B) Relationship
707 between CT_{max} and z for slow-ramping control (solid black line), fast-ramping control
708 (dashed black line), slow-ramping selection (red line), and fast-ramping selection lines
709 (blue lines). Each symbol represents the CT_{max} and z estimated for each replicate line.
710



711

712 **Figure 3**. Desiccation survival curves of (A) females and (B) males from control (black
713 line), slow-ramping selection (red line), and fast-ramping selection lines (blue lines) of
714 *Drosophila subobscura*. Dashed lines indicate the median mortality time for each
715 selection protocol (pooled replicate cages).
716



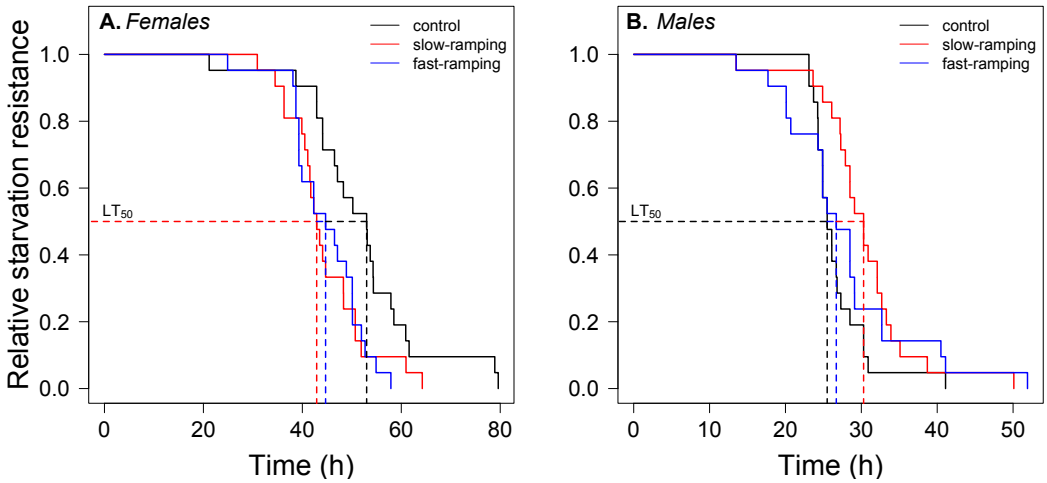
717
718

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

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

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

722 selection protocol (pooled replicate cages).



723