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

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ABSTRACT

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

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

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

INTRODUCTION

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

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

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

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

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

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

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

Materials and Methods

Sampling and maintenance

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

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

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

176 ***Desiccation and starvation resistance***

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

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

191

192 **Statistical analysis**

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

196

197 ***Heat tolerance***

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

see Mesas et al. 2021). For the knockdown time analysis, a mixed linear model with ramping selection (fixed effect with fast-control, slow-control, fast-selection, and slow-selection lines as levels), sex (fixed effect with females and males as levels), and replicate lines nested within the thermal selection (random effect with replicates 1, 2 and 3 as levels) was performed using the library *lme4* package for R (Bates et al. 2015). Fixed effects were tested by a type III ANOVA and the random effect was tested by a likelihood ratio test comparing the model with and without the replicate lines. Both tests were performed using the library *lmerTest* package for R (Kuznetsova et al. 2017). If the selection effect was significant, *a posteriori* comparisons were performed using false discovery rate adjustment 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 assay 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 assay 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.

Using equation 1, 24 TDT curves (2 sexes × 3 replicate lines × 4 selection protocols) were fitted, from which CT_{max} and z values were estimated as described above. A linear model with ramping selection treatment (levels: fast-control, slow-control, fast-selection, and slow-selection lines), sex (levels: females and males), and their interaction was performed to evaluate their effects on CT_{max} and z values. TDT curve analysis did not include replicate lines as a random effect because only one CT_{max} and z value was estimated by each replicate line. Additionally, a ~~mixed~~ linear mixed model with ramping selection (fixed effect with fast-control, slow-control, fast-selection, and slow-selection lines as levels), sex (fixed effect with females and males as levels), and replicate lines nested within the thermal selection (random effect with replicates 1, 2 and 3 as levels), and assay temperatures (as covariate) was fitted on the knockdown time using the *lmer* package for R.

Desiccation and starvation resistance

To determine the lethal time at which 50% of flies of each vial were dead (LT_{50}), a generalized linear model following a binomial distribution was fitted with the proportion of flies alive as the dependent variable and time as the predictor variable. The generalized linear model was run using the g*lm* function of the *lme4* package for R (Bates et al. 2015). The LT_{50} of each vial was then estimated using the function *dose.p* from the *MASS* package for R (Venables and Ripley 2002).

To estimate the median LT_{50} and the 95% confidence intervals for each selection treatment and sex, each LT_{50} was transformed into a survival object using the *Surv* and *survfit* functions of the *survival* package for R (Therneau 2023). This procedure also

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

RESULTS

Knockdown temperature evolution

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

Knockdown time evolution

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

static assay are as follows: 35º C = 33.77 min [32.1 – 35.5]; 36º C = 16.98 min [16.1 – 17.9]; 37º C = 8.84 min [8.4 – 9.3]; and 38º C = 6.78 min [6.3 – 7.0].

Knockdown times differed significantly between selection treatments when flies were assayed at 36 and 37ºC (Table 1; Table S2; Fig. 1). At these temperatures, slow and fast selected lines showed higher heat tolerance than slow and fast control lines (Table S1- S3; Fig. 1C, E). Also, fast-selected lines showed a higher heat tolerance than slow-selected lines in flies assayed at 37ºC but not at 36ºC (Table S1-S3; Fig. 1C, E), whereas fast and slow control lines did not differ (Table S3; Fig 1). On the other hand, replicate lines had no significant effect on knockdown time, indicating consistent evolutionary responses within each selection and control treatment (Table S2). Concerning sex, females showed a higher thermal tolerance than males but only when flies were assayed at 35 and 38ºC (Table 1; Fig. 1B, H). Finally, non-significant interactions between selection and sex were found for all assay temperatures (Table 1).

TDT curves evolution

Linear regressions between $\log_{10}(LT_{50})$ and assay temperatures enabled the estimation of 24 TDT curves (4 selection treatments × 3 replicate lines × 2 sexes) with high coefficients of determination (mean $R^2 = 0.946$, range: 0.820 – 0.989; Table S4), confirming that heat knockdown time is linearly related to stressful sublethal temperatures. From these TDT curves, the mean CT_{max} [95% CI] was 41.21ºC [41.02 – 41.41], and the mean z [95% CI] was 4.18ºC [4.03 – 4.32]. CT_{max} were significantly different between selection treatments ($F_{3,20} = 4.46$, $P = 0.015$; Fig. 2A). A post hoc analysis showed that fast-ramping selected and slow-ramping control lines were significantly different in their CT_{max} values ($t_{20} = 3.195$, $P = 0.02$). In contrast, fast and slow control lines had similar CT_{max} values ($t_{20} = 0.911$, $P = 0.80$). Thus, when control

lines are pooled, CT_{max} still differs between selection treatments ($F_{2,18} = 6.69$, $P = 0.007$), with fast-ramping (mean CT_{max} [95% CI] = 41.55 ºC [41.2 – 41.9]) and slow-ramping selected lines (mean CT_{max} [95% CI] = 41.43 ºC [41.1 – 41.8]) had higher CT_{max} than control lines (mean CT_{max} [95% CI] = 40.94 ºC [40.7 – 41.2]) ($t_{18} = 3.27$, $P = 0.01$ and $t_{18} = 2.64$, $P = 0.04$, respectively). CT_{max} was not different between the selected lines ($t_{18} = 0.54$, $P = 0.85$). On the other hand, sex and the interaction between selection treatments and sex had no significant effect on CT_{max} ($F_{1,18} = 0.004$, $P = 0.95$ and $F_{3,18} = 2.11$, $P = 0.15$, respectively). Regarding z (i.e., thermal sensitivity), it shows no significant effects of selection treatments ($F_{3,16} = 0.91$, $P = 0.46$; Fig. 2), sex ($F_{1,16} = 1.30$, $P = 0.27$), nor the interaction between selection treatments and sex ($F_{3,16} = 2.23$, $P = 0.12$). 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, $P = 0.45$). Additionally, using a linear mixed model with the assay temperature as a covariate, this~~This~~ result was corroborated by ~~a~~the non-significant interaction between selection treatment and assay temperature ($F_{3,865} = 0.30$, $P = 0.82$).

Desiccation resistance evolution

Survival analysis showed a significant effect of sex and selection treatment on desiccation resistance, but not for the interaction ~~between selection treatments and sex~~of the two effects ~~on desiccation resistance~~ (~~LTR: $\chi^2_5 = 83.55$, $P < 2\times10^{-16}$~~Table S5). 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 (hHazard 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: $\chi^2_2 = 1.88$, $P = 0.4$; Fig. 3B). The desiccation survival analysis results testing the effect of selection protocol, sex, and their interaction are reported in the Table S5.

Starvation resistance evolution

A significant effect of sex, selection treatment, and the interaction between the two effects on starvation resistance was found in the survival analysis Survival analysis showed a significant interaction between selection treatments and sex on desiccation resistance ($LTR_5 = 94.89$, $P < 2\times10^{-16}$Table S6). 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 = 0.50, $P = 0.03$), but nonsignificant differences were found between fast-ramping selection and control lines (hazard ratio = 0.64, $P = 0.16$). The starvation survival analysis results testing the effect of selection protocol, sex, and their interaction are reported in Table S6.

Discussion

Studying the evolutionary responses of thermal limits is key to understanding the adaptive responses and evolutionary constraints to global warming. Cross-tolerance studies can ~~then~~ provide valuable information on the evolutionary response to multiple environmental stressors. Cross-tolerance evolution has been reported among different resistance traits (Hoffmann and Parsons 1993; Bubliy and Loeschcke 2005; Stazione et al. 2020; Singh et al. 2022), but the magnitude of the evolutionary response could be explained by the trait under direct selection or the stress intensity (Gerken et al. 2016). Here, artificial selection for heat tolerance (i.e., knockdown temperature) resulted in correlated responses in heat knockdown time, the thermal tolerance landscape (TDT curves), desiccation resistance, and starvation resistance. However, these responses depended on the intensity of thermal selection and sex, suggesting that the evolutionary response to tolerate higher temperatures also confers partial tolerance to other stresses such as desiccation and starvation.

Different approaches to measuring the upper thermal limit of ectotherms produce different genetic and phenotypic estimates. Fast-ramping assays generally estimate higher upper thermal limits and higher heritabilities than slow-ramping~~slow ramping~~ assays (Chown et al. 2009; Rezende et al. 2011). For instance, the heritability of thermal tolerance was 0.13 for fast assays and 0.08 for slow assays in *D. subobscura* (Castañeda et al. 2019). Because heritability is commonly used as a predictor of the evolutionary response of a trait to natural or artificial selection, the evolutionary response of heat tolerance would be expected to depend on the ramping rate used during selection. However, previous work did not support this prediction for *D. subobscura*, finding that the evolution of heat tolerance was independent of the ramping rate (Mesas et al. 2021), but the correlated responses of the thermal performance curves or the energy metabolism depended on the intensity of the thermal selection (Mesas et al.

2021; Mesas and Castañeda 2023). In the present study, the evolution of knockdown temperature (e.g., heat tolerance measured in dynamic assays) induced a correlated response on the heat knockdown time (e.g., heat tolerance measured in static assays) when it was assayed at intermediate temperatures (36 and 37ºC), but not at less or more extreme assay temperatures (35 and 38ºC). These findings can be explained because stress tolerance at 35ºC should depend on the physiological state of the organism during prolonged thermal assays (e.g., availability of energy resources; see Rezende et al. 2011, but also see Overgaard et al. 2012) and not only on heat tolerance, whereas heat tolerance at 38ºC could be limited by physical properties of ectotherms (e.g., protein denaturation, membrane permeability). However, a previous study found a clinal pattern for heat tolerance in *D. subobscura* only for flies assayed in static assays (specifically at 38ºC), but this clinal pattern was not detected using ramping assays (Castañeda et al. 2015). Differences between these two studies could be explained by the number of generations under thermal selection, which could result in a different evolutionary response of heat tolerance. According to Begon (1976), *D. subobscura* can have between 4 and 6 generations per year, which makes it possible to estimate about 125 generations of selection from the introduction of *D. subobcura* in Chile until the study by Castañeda et al. (2015). On the other hand, the type of selection is completely different between the two studies (e.g., natural versus artificial selection), which could lead to various evolutionary outcomes. In any case, 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 (Rezende et al. 2014; Jørgensen et al. 2021).

TDT curves evolved in response to heat tolerance selection in *D. subobscura*. TDT curves showed that fast- and slow-ramping selected lines evolved higher CT_{max} than control lines (ΔCT_{max} = 0.49 ºC). This differential CT_{max} value is slightly lower than the population differences (0.9ºC) observed between the lowest and highest latitude populations (~8 latitudinal degrees) of *D. subobscura* studied by Castañeda et al. (2015) and even lower than the CT_{max} variation reported among *Drosophila* species (Jørgensen et al. 2019; Alruiz et al. 2022). On the other hand, although CT_{max} and z (i.e., thermal sensitivity) are phenotypically correlated (see Castañeda et al. 2015; Molina et al. 2023), the evolutionary increase in CT_{max} was not associated with a correlated response in thermal sensitivity (z). This result suggests that both thermal parameters are not genetically constrained, but further evidence from quantitative genetic studies is needed to assess the genetic association between CT_{max} and z. A caveat for this finding could be related to the fact that thermal selection for heat tolerance was carried out over 16 generations, followed by 7 generations of relaxed selection (i.e., no selection). However, previous evidence suggests that differences in heat tolerance between control and selected lines were consistent between generations 16 and 25 (Mesas et al. 2021). Indeed, Passananti et al. (2004) also reported that phenotypic values did not change after 35 generations of relaxed selection in desiccation-selected populations of *D. 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 than 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 due to heat thermal selection only being 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 than females). 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). Although energy content was not measured here, Mesas and Castañeda (2023) found that body mass and metabolic rate were similar between control and heat-tolerance selected lines of *D. subobscura*, suggesting that neither resource storage nor energy conservation explains the sex-dependent correlated response for stress resistance traits. However, the same study found that heat-tolerance selected lines had higher fecundity than control lines, whereas previous studies have found negative associations between fecundity and starvation

449 resistance in *D. melanogaster* (Bubliy and Loeschcke 2005; Kalra et al. 2017). Then,
450 the decrease in starvation resistance in females of the heat-selected lines could be
451 related to increased fecundity, which is consistent with the reported trade-off between
452 stress resistance traits and life-history traits (van Noordwijk and de Jong 1986; Rion and
453 Kawecki 2007).

454 In conclusion, the present study shows that heat tolerance evolution is associated
455 with evolutionary responses in other stress resistance traits, which could be explained
456 by pleiotropic effects or linkage disequilibrium among the traits evaluated. However,
457 further evidence (e.g., quantitative genetic or genome-wide analysis studies) is needed
458 to elucidate the genetic basis of the cross-tolerance evolution in *D. subobscura*. In
459 addition, this study provides evidence for rapid evolutionary responses in ectotherms
460 mediated by thermal selection, but the evolutionary outcomes depend on the intensity of
461 the thermal stress (Mesas and Castañeda 2023) and sex (Rogell et al. 2014; Rusuwa et
462 al. 2022). This study also highlights the importance of *D. subobscura* as a suitable
463 model to study thermal adaptation mediated by natural selection (Huey 2000; Gilchrist
464 et al. 2008; Castañeda et al. 2013, 2015), and laboratory selection (Santos et al. 2005,
465 Santos et al. 2021; Simões et al. 2017; Mesas et al. 2021; Mesas and Castañeda 2023).
466 In addition, this study highlights the relevance of experimental evolutionary studies for
467 understanding the adaptive responses to climate change (Mitchell and Whitney 2018;
468 Brennan et al. 2022; Kelly 2022). Finally, these results suggest that ectotherms may
469 evolve in response to climate warming, but evolutionary responses may differ between
470 sexes and/or the warming rates experienced by natural populations, which may make it
471 difficult to propose general trends in the fate of ectotherms in a changing world where
472 temperature is not the only driver of climate change, but species are also expected to be
473 exposed to changes in precipitation patterns and food availability.

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Data availability

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

Conflict of interest disclosure

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

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709 **Table 1**. Mixed linear effect model for the knockdown time of *Drosophila subobscura*
710 assayed at four static temperature assays. For simplicity, results for the random effect
711 (replicate lines) are not shown because they were not statistically significant (see
712 Materials and Methods). Significant effects (P values < 0.05) are indicated in boldface
713 type.
714

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

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

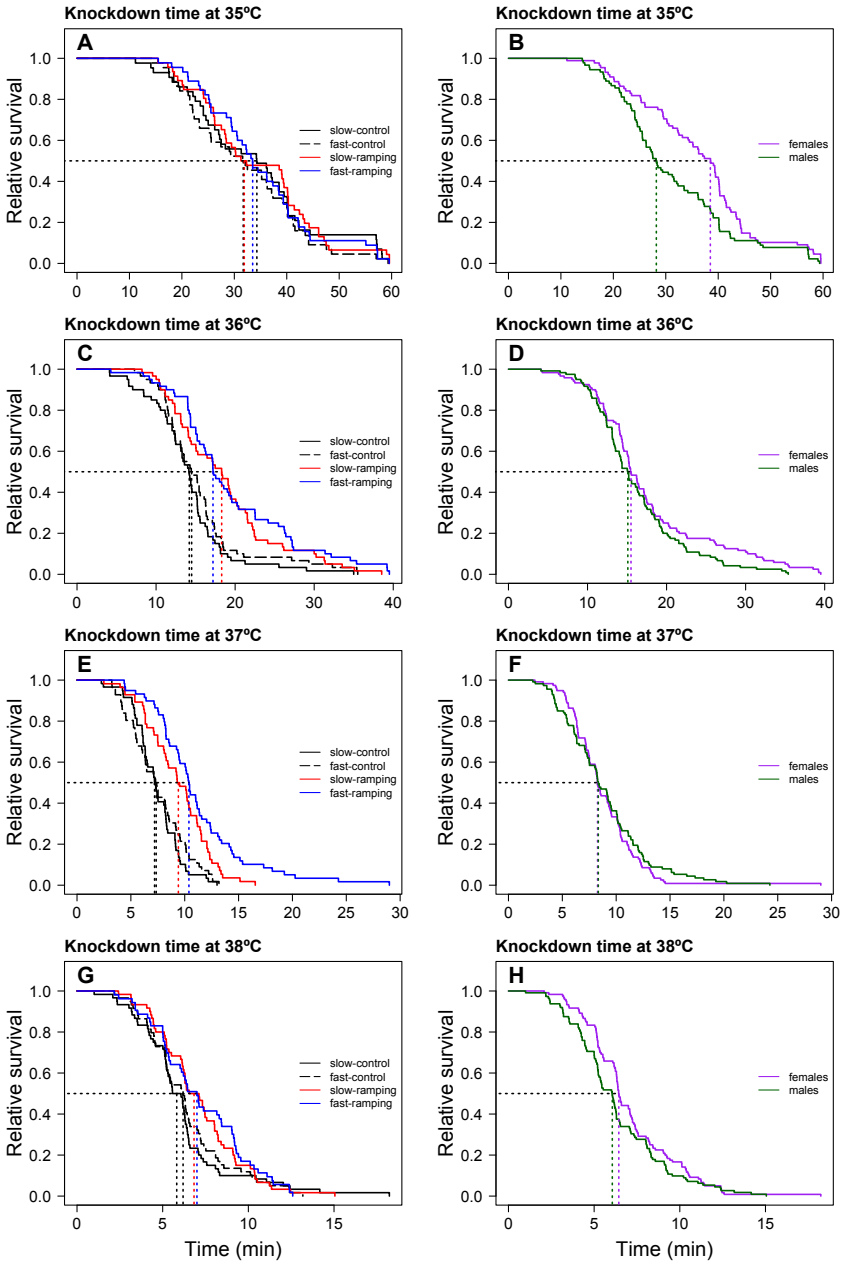
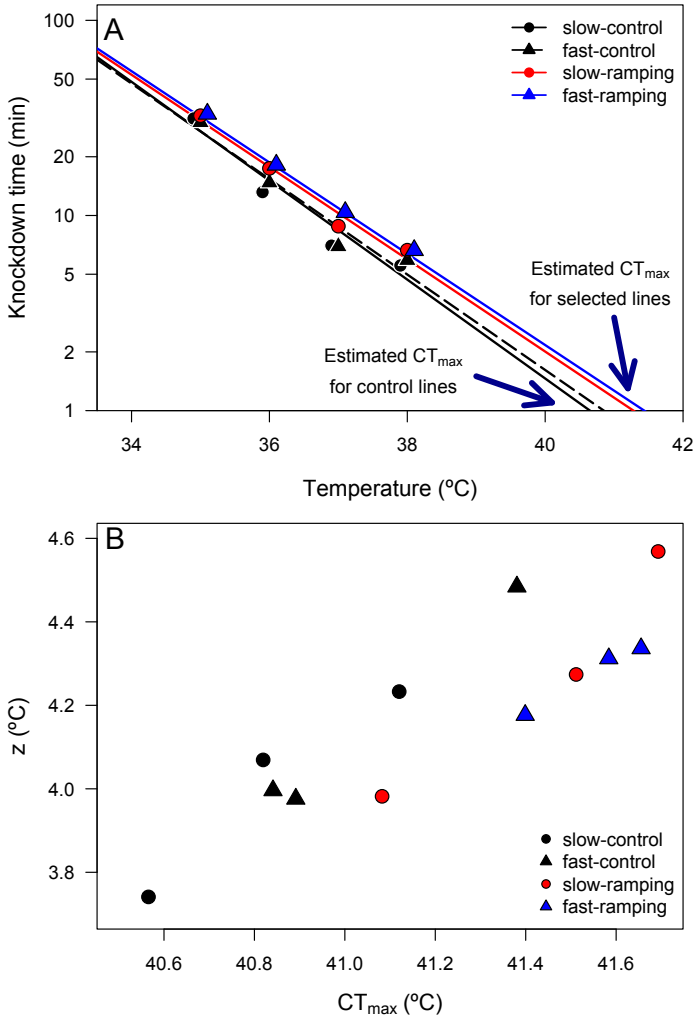


Figure 2. (A) Thermal death curves for control (black solid and dashed lines) and selected (red and blue lines) lines for increasing heat tolerance in *Drosophila subobscura*. Symbols represent the average knockdown time at the different assay temperatures. Each symbol represents the average knockdown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



735 **Figure 3**. Desiccation survival curves of (A) females and (B) males from control (black
736 line), slow-ramping selection (red line), and fast-ramping selection lines (blue lines) of
737 *Drosophila subobscura*. Dashed lines indicate the median mortality time for each
738 selection protocol (pooled replicate cages).
739

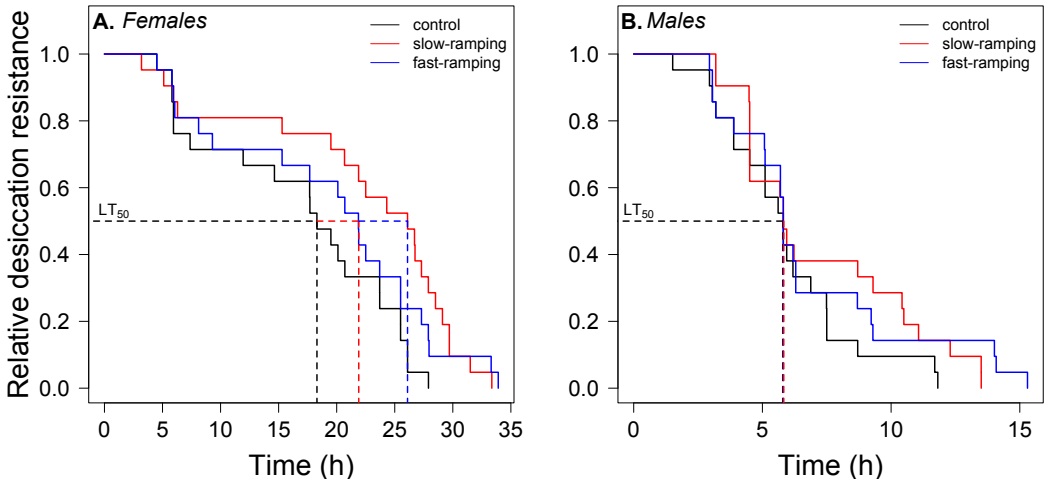


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Figure 4. Starvation survival curves of (A) females and (B) males from control (black line), slow-ramping selection (red line), and fast-ramping selection lines (blue lines) of *Drosophila subobscura*. Dashed lines indicate the median mortality time for each selection protocol (pooled replicate cages).