Title: Biogeographical history shapes evolution of reproduction in a global warming scenario

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15 **Biogeographical history shapes evolution of reproduction in a global** 16 **warming scenario**

17

18 **Abstract**

19 Adaptive evolution might be critical for animal populations to thrive on the fast-
20 changing natural environments. Ectotherms are particularly vulnerable to global
21 warming and, although their limited coping ability has been suggested, few real-time
22 evolution experiments have directly accessed their evolutionary potential. Here, we
23 report a long-term experimental evolution study addressing the evolution of thermal
24 reaction norms, after ~30 generations under different thermal environments. We
25 analyzed the evolutionary dynamics of *Drosophila subobscura* populations as a
26 function of the thermally variable environments in which they evolved and their distinct
27 biogeographical background. Our results showed clear differences between the
28 historically differentiated populations: while the northern *D. subobscura* populations
29 showed a temporal increase in performance at higher temperatures, their southern
30 counterparts presented the opposite pattern. This suggests that the northern populations
31 might be better equipped to cope with the current rising temperatures. Remarkably, no
32 effect of thermal selection was found. The lack of a clear long-term adaptive response at
33 higher temperatures after evolution under a global warming scenario raises concerns
34 about the evolutionary potential of ectotherms. Our results highlight the complex nature
35 of thermal responses in face of environmental heterogeneity and emphasize the
36 importance of considering intra-specific variation in thermal evolution studies.

37 **Keywords:** *Drosophila*, experimental evolution, global warming, historical
38 background, thermal adaptation, thermal reaction norms

39 Introduction

40 Biodiversity is under great pressure due to rapid environmental changes
 41 associated with global warming. Adaptive evolution and plasticity may be important
 42 means for organisms to cope with the increasingly warmer temperatures (Chevin et al.
 43 2010; Merilä et al. 2016). Plasticity can also be adaptive if, for instance, environmental
 44 cues occurring during development allow for animals to better cope with environmental
 45 conditions as adults (see reviews in (Kelly 2019; Rodrigues and Beldade 2020; Buckley
 46 and Kingsolver 2021). Ectotherms are particularly vulnerable animals as their body
 47 temperature is highly constrained by the environment, which has a major impact on
 48 their biological processes and, thus, on their fitness (Angilletta 2009; Hoffmann and
 49 Sgrò 2018). To make matters worse, recent evidence showed a limited ability of
 50 ectotherms to evolutionarily respond to upper temperature shifts (Kellermann and van
 51 Heerwaarden 2019; MacLean et al. 2019), as well as limited scope for plasticity in that
 52 environmental context (Gunderson and Stillman 2015; Sørensen et al. 2016).

53 A thermal adaptive response will, expectedly, lead to an optimal performance
 54 within the range of temperatures to which the organisms have most exposure, and lower
 55 fitness at more extreme values (Angilletta 2009). In the context of global warming, one
 56 might expect a shift in the optimum performance towards higher temperatures, provided
 57 genetic variation is present and other evolutionary constraints do not play a major role
 58 (Kellermann and van Heerwaarden 2019; Kristensen et al. 2020). These shifts can be
 59 observed through changes in the thermal reaction norms, which describe the
 60 population's performance as a function of temperature range. According to Angilletta *et*
 61 *al.* (2003), different types of trade-offs may be involved in changes of elevation or
 62 shape of thermal reaction norms. On the one hand, the allocation of resources (*e.g.*,
 63 energy differently allocated to two traits) or acquisition trade-offs (*e.g.*, between

maximizing the acquisition of resources vs. minimizing the risk of mortality) will mainly lead to shifts in trait means, *i.e.*, to the elevation of the thermal reaction norm. On the other hand, the evolution under a specialist-generalist trade-off will expectedly lead to a change in shape, if an increase in performance at a given temperature causes a decrease at other temperatures. These different sources of variation of thermal reaction norms are not mutually exclusive. The genetic variation available for these different dimensions will govern the evolution of reaction norms (Huey and Kingsolver 1989; Angilletta et al. 2003; Berger et al. 2013). Generalist vs. specialist trade-offs can underlie patterns of local adaptation, even though adaptation costs are not always present (Hereford 2009). There is evidence of thermal plasticity suggestive of local adaptation in natural populations of ectotherms, with populations geographically distinct presenting different thermal reaction norms (Trotta et al. 2006; Berger et al. 2013; Austin and Moehring 2019; Klepsatel et al. 2019), but it is not universal (e.g., (Klepsatel et al. 2013; Clemson et al. 2016). It is, thus, critical to address how plasticity evolves in face of rapid thermal changes and whether this variation is consistent across populations, to better understand the consequences of the current global warming scenario.

Experimental evolution, the real-time study of populations across several generations, in highly controlled and reproducible conditions (Kawecki et al. 2012), is a very powerful approach to address the adaptation to novel environmental conditions, including the evolution of plasticity. It has been frequently used to characterize the tempo and mode of thermal evolution of ectotherms, namely in ecological scenarios associated with global warming (Schou et al. 2014; Manenti et al. 2015; Kellermann and van Heerwaarden 2019; Kinzner et al. 2019; Liukkonen et al. 2021). The native Palearctic *Drosophila subobscura* is an emblematic case study of ectotherm thermal

89 adaptation. It shows local adaptation to distinct natural habitats and latitudinal clinal
 90 variation for chromosomal inversion frequencies in Europe, North America, and South
 91 America (Prevosti et al. 1988; Rezende et al. 2010). These karyotypic polymorphisms
 92 have been shown to change worldwide as a result of global warming (Balanyá et al.
 93 2006) and to respond to sudden heat waves (Rodríguez-Trelles et al. 2013). Local
 94 adaptation in the reproductive performance of *D. subobscura* during heat stress has also
 95 been reported (Porcelli et al. 2017). This species presents clear plastic responses to
 96 thermal variation (Fragata et al. 2016), with a key role of developmental temperature in
 97 shaping adult reproductive performance (Simões et al. 2020; Santos et al. 2021a). The
 98 developmental temperature range of *D. subobscura* is 6 – 26°C (Moreteau et al. 1997;
 99 David et al. 2005; Schou et al. 2017) with optimal viability and preferred body
 100 temperature between 16°C and 20°C (Rego et al. 2010; Castañeda et al. 2013; Schou et
 101 al. 2017). Shifts in this species' thermal reaction norms have been observed for
 102 locomotor behavior (Mesas et al. 2021) and thermal tolerance (Schou et al. 2016;
 103 MacLean et al. 2019), although in the latter case mostly for lower thermal limits.

104 For the last three years, we have been performing a thermal experimental
 105 evolution study on historically differentiated *D. subobscura* populations, derived from
 106 the extreme latitudes of the European cline (Simões et al. 2017). These populations have
 107 been subjected to thermal selective regimes that differ in mean temperature and/or
 108 thermal amplitude (Santos et al. 2021b). We have previously found no evidence for
 109 evolutionary changes in thermal reaction norms after nine generations in circadian
 110 fluctuating or global warming-like environments (Santos et al. 2021b). This finding may
 111 result from a small number of elapsed generations or a general lack of genetic variation
 112 for response to thermal selection.

113 Here, we present an extended report of the evolutionary response of these
 114 populations, spanning ~30 generations of thermal evolution, and thus testing a longer-
 115 term adaptive shift in thermal reaction norms. As in Santos et al. (2021), the thermal
 116 reaction norms were estimated through the population's reproductive performance at
 117 three different temperatures (14°C, 18°C, and 24°C). With this design, we aim to
 118 respond to three main questions: (i) Does longer-term evolution under thermally diverse
 119 environments change the populations' thermal reaction norm? (ii) Do thermal reaction
 120 norms change between short- and longer-term evolution? (iii) Can we find the signature
 121 of biogeographical history in the populations' thermal evolutionary response?

122 First and foremost, we expect that differential plasticity between thermal
 123 selection regimes may arise in a longer-term study, provided there is a correlated change
 124 in reaction norms due to adaptation to the dynamic thermal environments. Because of
 125 almost 30 generations experiencing a wide range of temperatures, we foresee a higher
 126 performance of the global warming-evolved populations in more extremal thermal
 127 environments. Second, we expect that fluctuating and warming-evolved populations to
 128 perform worse than the control lines in intermediate thermal conditions, assuming a
 129 trade-off between maximal performance and performance breadth of the reaction norms
 130 (Huey and Kingsolver 1989; Angilletta et al. 2003). Finally, we think that geographical
 131 variation for plasticity may become apparent, driven by an interaction of thermal
 132 selection with the distinct genetic backgrounds.

133

134 **Material and Methods**

135 *Population maintenance and thermal selection regimes*

136 The experimental populations derived from collections from natural populations
137 of *Drosophila subobscura* in two contrasting latitudes of the species' European cline:
138 Adraga, Portugal (38°48' N) and Groningen, The Netherlands (53°13' N). The
139 laboratory populations (PT, from Portugal, and NL, from The Netherlands) were
140 established in early September 2013 and three-fold replicated shortly after. All PT₁₋₃
141 and NL₁₋₃ populations were maintained in discrete generations with synchronous 28-day
142 cycle, 12L:12D photoperiod, and constant 18°C temperature (control conditions, C).
143 The flies were reared in ~30 mm³ glass vials with controlled densities in both eggs (70
144 eggs per vial) and adults (50 adults per vial). Egg collection for the following generation
145 was around peak fecundity (seven to ten days old flies) and the census sizes per
146 generation ranged between 500 and 1000 individuals (see (Simões et al. 2017), for more
147 details).

148 Two new thermal selection regimes were derived in January 2019, by the time
149 PT and NL populations had undergone 70 generations of lab evolution. The *circadian*
150 *fluctuation* regime (F populations, FPT₁₋₃ and FNL₁₋₃) and the *global warming* regime
151 (W populations, WPT₁₋₃ and WNL₁₋₃) – see (Santos et al. 2021b). The F populations are
152 under a daily temperature regime that fluctuates between 15°C and 21°C, with a mean
153 daily temperature of 18°C, a cycle that is constant across generations. The W
154 populations experience a daily fluctuation initially similar to the F lines but with a per
155 generation increase of 0.18°C in daily mean and 0.54°C in daily amplitude (see the
156 thermal profiles on Figure 1). This simultaneous increase in thermal mean and
157 amplitude per generation was, to our knowledge, not yet tested in other experimental
158 studies. Taking into account the global warming pace of 0.1-0.3°C per decade (IPCC
159 2018), a mean increase of 0.18°C per generation is equivalent to the thermal changes
160 experienced in nature by an organism with a ~10-year generation time. The

161 progressively divergent upper and lower temperature extremes (increases of 0.44°C in
162 upper and 0.08°C in lower limits, per generation) fit well with the projected 2-fold
163 increase in thermal extremes relative to mean temperature in mid-latitude location
164 (IPCC 2018).

165 The PT and NL populations have been kept at 18°C since their foundation and
166 were already adapted to lab conditions when the two new thermal regimes were created,
167 serving, thus, as controls. As they are expected to be at evolutionary equilibrium, they
168 reflect the ancestral state prior to the start of the F and W selection regimes. All
169 experimental populations were, otherwise, kept under the same conditions, as explained
170 above. Census sizes were, in general, high for all thermal selection regimes with the
171 exceptions referred below (see Figure S1).

172 The experimental populations were synchronously assayed twice: (1) after 9
173 generations of thermal evolution (F and W), when the control populations (C) had 79
174 generations of lab adaptation; and (2) after 31 generations of W selection and 29
175 generations in the F regime, together with the controls (evolved for 99 generations in
176 the standard lab conditions).

177 After 9 generations in the warming regime, the experimental populations had
178 been subjected to temperatures ranging from 14.4°C to 24.5°C, with a daily average of
179 19.4°C (Figure 1). By generation 22, the thermal mean and amplitude increases in the
180 warming regime (peak temperature of 30.2°C) had to be halted. Particularly low egg-to-
181 adult viability led to significant declines in population size mainly between generations
182 22 and 24 (see Figure S1). Since then, the warming populations have been kept in the
183 temperature cycle of generation 20, *i.e.*, with a mean temperature of 21.4°C, a lower
184 extreme of 13.5°C, and an upper extreme of 29.4°C (Figure 1). The temperature increase

185 caused a progressively shorter development time (*i.e.*, from egg to adult) and, by
186 generation 31, had led to a 96h reduction in the life-cycle length, that became 24 days.

187 *Thermal plasticity assays*

188 To study the evolution of thermal plasticity, we tested the effect of different
189 thermal environments on the reproduction of adult flies. We analyzed the reproductive
190 performance of eighteen experimental populations (three thermal selection regimes –
191 W, F and C, two histories – NL and PT, and three replicate populations) subjected to
192 three thermal treatments (14°C, 18°C, and 24°C) in two different time points – after nine
193 and 31 generations of thermal evolution; these assays are thereon referred as G9 and
194 G31, respectively. The results for the G9 assay were previously reported in (Santos et
195 al. 2021b). Experimental protocols for both G9 and G31 assays were identical. Briefly,
196 the temperature treatments involved a lifelong exposure to a constant temperature:
197 colder (14°C), intermediate (18°C), or warmer (24°C). These temperatures were chosen
198 considering the range of viable developmental temperatures for *D. subobscura* (6 –
199 26°C, (Moreteau et al. 1997; David et al. 2005; Schou et al. 2016)). Maternal effects
200 were minimized by rearing all populations in a common-garden environment, under
201 control conditions (18°C and 28-day life cycle), for one full generation, before each
202 assay.

203 In each generation, sixteen pairs of virgin males and females, per population and
204 treatment, were individually assayed with a total of 864 couples. The mating pairs were
205 transferred to fresh medium every other day, for eight days, and the presence of eggs
206 was checked daily. The eggs laid between days six and eight were counted under a
207 stereoscope. Four life history traits were assessed: (1) *age of first reproduction* (number
208 of days since emergence until the first laid egg), where lower values show faster
209 maturity; (2) *fecundity* (total number of eggs laid between days six and eight), which
210 addresses the females reproductive performance when the selective pressures are higher,
211 *i.e.*, near the age of egg collection for the following generation; (3) *productivity* (number

of offspring obtained from the eggs of day eight), which conveys the females' ability to produce viable progeny; and (4) *juvenile viability* (ratio between productivity and fecundity at day eight), which measures developmental success. To obtain more reliable estimates of viability, only vials with at least five eggs were considered for this trait, with ~9% of the total number of vials being excluded from this analysis. This was done to adjust for the increase in fungal infections observed in vials with a very low number of eggs.

Statistical methods

Raw data for the analyses is the mean value for each replicate population and temperature combination (*e.g.*, three data values for FPT populations in each of the three temperature treatments). Data was analyzed by linear mixed effects models fitted with REML (restricted maximum likelihood). Three factor ANOVAs (Type III Wald F tests, Kenward-Roger degrees of freedom) were done to estimate significance levels of differences between thermal selection regimes, histories and temperature treatments, as well as their interactions. Homoscedasticity and normality were checked. Considering the robustness of the analysis of variance, minor violations were accepted (Knief and Forstmeier 2021). Arcsine transformation was applied to the juvenile viability data. All statistical analyses were performed in R v4.0.4, using the *lme4* (Bates et al. 2015), *car* (Fox and Weisberg 2019), *lawstat* (Hui et al. 2008), *emmeans* and *ggplot2* (Wickham 2016) packages. Two general models were used to analyze the evolution of thermal reaction norms:

$$(1) \quad Y = \mu + \text{History} + \text{Temp} + \text{Selection} + \text{Gen} + AP\{\text{History}\} + \text{History} \times \text{Temp} + \text{History} \times \text{Selection} + \text{Selection} \times \text{Temp} + \text{History} \times \text{Gen} + \text{Temp} \times \text{Gen} +$$

$$\begin{aligned} & \text{Selection} \times \text{Gen} + \text{Selection} \times \text{History} \times \text{Temp} + \text{History} \times \text{Temp} \times \text{Gen} + \text{History} \times \\ & \text{Selection} \times \text{Gen} + \text{Selection} \times \text{Temp} \times \text{Gen} + \text{Selection} \times \text{History} \times \text{Temp} \times \text{Gen} + \varepsilon \end{aligned}$$

238

$$\begin{aligned} (2) \quad Y = & \mu + \text{History} + \text{Temp} + \text{Selection} + \text{Gen} + \text{Block} + \text{History} \times \text{Temp} + \\ & \text{History} \times \text{Selection} + \text{Selection} \times \text{Temp} + \text{History} \times \text{Gen} + \text{Temp} \times \text{Gen} + \text{Selection} \times \\ & \text{Gen} + \text{Selection} \times \text{History} \times \text{Temp} + \text{History} \times \text{Temp} \times \text{Gen} + \text{History} \times \text{Selection} \times \\ & \text{Gen} + \text{Selection} \times \text{Temp} \times \text{Gen} + \text{Selection} \times \text{History} \times \text{Temp} \times \text{Gen} + \varepsilon \end{aligned}$$

243

244 with Y being the trait in study (age of first reproduction, fecundity, productivity, or
245 viability); *History*, as the fixed factor corresponding to geographical origin (with PT and
246 NL as categories); *Selection* as the fixed factor representing the thermal selection
247 regimes (with three categories: Control, Warming, and Fluctuating); *Temp* as the fixed
248 factor corresponding to the three temperature treatments; and *Gen* as the fixed factor
249 with two categories (generations 9 and 31). In these models, interactions between
250 Selection or History and Temperature test for differences in thermal plasticity due to
251 those factors, while interactions between Generation and Temperature indicate temporal
252 changes in plasticity between the two generations. In model (1), $AP\{History\}$ is the
253 random effect consisting of the ancestral replicate population (*i.e.*, PT₁₋₃; NL₁₋₃) nested
254 in *History*, from which the replicate populations of the three thermal selection regimes
255 were originated (*e.g.*, Ancestral PT₁ generated Control PT₁, Fluctuating FPT₁ and
256 Warming WPT₁). In model (2), *Block* was defined as the random effect, corresponding
257 to the set of same-numbered replicate populations from all thermal regimes, assayed in
258 the same pseudo-randomized experimental rack. For this analysis, we used as raw data
259 in each generation the difference between the mean of each replicate population and the
260 average of the three synchronously assayed control populations (*e.g.*, FPT₁-PT, with PT

261 as average of PT_{1-3}). As the control populations are long established in the laboratory
 262 and, thus, expectedly near evolutionary equilibrium, this procedure allows to remove
 263 environmental effects due to the between generation assay asynchrony (*e.g.*, see
 264 (Fragata et al. 2016). In fact, analysis for the control raw data did not show significant
 265 variation between generations. Thus, the temporal analyses included standardized data
 266 for Warming and Fluctuating populations. Models including or excluding interactions
 267 with the random factors (Block or Ancestral Population) were tested and AIC was used
 268 to define the best model for each trait. For age of first reproduction and productivity, the
 269 model with Block as random effect without its interactions was used; the best suited
 270 model for fecundity and viability was the one with Ancestral Population as random
 271 effect without its interactions. For viability data analyses, both models (1) and (2) were
 272 tested including fecundity of day eight (F8) as a covariate (with models including and
 273 excluding its interactions with fixed factors), to account for variation in fecundity across
 274 the several thermal treatments. This analysis was also done separately for each selection
 275 regime (W and F).

276 We addressed the evolutionary dynamics of thermal reaction norms by
 277 comparing longer (by generation 31) and shorter-term evolution data (by generation 9,
 278 published in (Santos et al. 2021b)). We analyzed differences in experimental
 279 populations associated with the thermal selective regime imposed (*Selection*, W vs. F),
 280 the geographical origin (*History*, PT vs. NL), the temperature treatments (*Temp*, 14°,
 281 18°C, and 24°C), and the two assayed generations (*Gen*, 9 and 31).

282 Thermal reaction norms were also analyzed after 31 generations of thermal
 283 evolution (*i.e.*, using only data from generation 31). The statistical models used were
 284 similar to (1) and (2), but without *Generation* as factor. Raw data for these analyses was
 285 the mean absolute value for each replicate population and temperature combination.

286 Models including and excluding interactions with random effects were assessed with
287 AIC. Considering the traits A1R, fecundity and productivity the best model was model
288 (1) for fecundity and productivity, excluding interactions with the random effect AP;
289 model (2) for age of first reproduction, excluding interactions with the random effect
290 block. For this trait, the model with the lowest AIC was the one defining the random
291 effect AP and F8 as covariate without interactions with fixed or random factors.

292

293 **Results**

294 *Evolutionary dynamics of thermal reaction norms*

295 To address the dynamics of temporal changes in thermal reaction norms between
296 generation 31 and 9, we compared the population's reaction norm after 31 generations
297 of selection considering the three lifelong temperature treatments (14°C, 18°C, and
298 24°C), with the data after 9 generations (see (Santos et al. 2021b) and Figures 2 and 3).

299 First, we analyzed the combined effects of thermal selection (W vs. F),
300 biogeographical history and their interaction on the reaction norms' evolution for the
301 three lifelong temperatures in the two assayed generations (Table 1). No overall effects
302 of selection (*Selection x Gen*) or its effects on the reaction norms (*Selection x Temp x*
303 *Gen*) across generations were observed. The only exception was a significant across-
304 generation difference in age of first reproduction between W and F lines, with an
305 increased performance at 18°C and 24°C in the warming populations (see Table 1, A1R,
306 *Selection x Generation*; and Figures 2 and 3). Overall temporal changes between
307 populations with contrasting history were observed for age of first reproduction and
308 viability (see Table 1, *History x Gen*). This effect resulted mainly from the temporal
309 pattern (better performance) of the northern populations in both selection regimes

(FNL₁₋₃ and WNL₁₋₃) compared to the southern populations (see Figures 2 and 3). Changes in reaction norms associated with biogeographical history were found for productivity and viability (see Table 1, *History x Temp*); however, the patterns of variation differed between generations, leading to a significant *History x Temp x Gen* interaction term (see below). Hence, the interpretation of this *History x Temp* interaction should be cautious. In terms of the thermal reaction norm's evolution, all traits but age of first reproduction showed significant temporal changes due to different geographical histories (see Table 1, *History x Temp x Gen*). This variation was mostly due to the relative performance of southern and northern populations at higher temperatures and across generations (see Figures 2 and 3). After 31 generations of thermal selection, the southern populations showed a general drop in performance at 24°C, when compared to generation 9, while the northern populations not only maintain their performance but also had an increase in productivity. Given the effect of geographical history on the evolution of thermal reaction norms, additional temporal analyses were performed on each set of populations from the same geographical origin (*e.g.*, WNL and FNL), see Figures S2 and S3. Temporal changes in thermal reaction norms were significant for productivity in both sets of geographical populations (*Temp x Gen*; northern: $F_{2,22}=5.64$, $p<0.011$; southern: $F_{2,22}=8.66$, $p<0.002$), and for fecundity in the southern populations (*Temp x Gen*; southern: $F_{2,22}=5.54$, $p<0.012$; northern: $F_{2,22}=1.27$, $p>0.29$). However, no significant temporal changes in reaction norms of populations from different thermal selective regimes were found for any trait (*Selection x Temp x Gen*; $p>0.05$ in all analyses). Finally, there were no temporal changes in the reaction norms for viability either for the northern or southern populations (*Temp x Gen*; $p>0.05$).

Second, we tested the evolutionary change of each thermal regime's reaction norm between generations 9 and 31. We did not detect a significant effect of the

335 intercept or significant temporal changes in the overall performance of any thermal
 336 regime (Table S1, *intercept*, and *Gen*). A significant effect of temperature was observed
 337 for age of first reproduction and productivity in the W populations (Table S1, *Temp*) but
 338 no temporal changes in the reaction norms were observed for any trait (Table S1, *Gen x*
 339 *Temp*). Also, historical variation did not present a significant effect either on overall
 340 performance (Table S1, *History*) or across generations (Table S1, *History x Gen*).
 341 However, history had a significant effect on the overall shape of the reaction norms for
 342 productivity, in both warming and fluctuating populations, but not for the other traits
 343 (Table S1, *History x Temp*). In addition, a significant *History x Temp x Gen* interaction
 344 was observed for fecundity in the F populations and productivity in both F and W
 345 populations, indicating historical differences in the evolution of thermal reaction norms
 346 (see Table S1 and Figures 2 and 3, see also below). This temporal analysis was also
 347 done separately for each combination of Selection and History populations (*i.e.*, WPT,
 348 WNL, FPT, and FNL) – see Table S2. Temporal changes in thermal reaction norms
 349 (Table S2, *Temp x Gen*) were observed for fecundity and productivity in the FPT
 350 populations, and productivity for the WNL populations. Trait changes in FPT
 351 populations resulted from the temporal decline in performance at higher temperatures,
 352 while a temporal increase in productivity was observed for the WNL populations. This
 353 productivity increase occurred at both lower (approaching control values) and higher
 354 temperatures (diverging from control values) – see Figures 2 and 3.

355

356 *Longer-term changes of thermal reaction norms after 31 generations under*
 357 *varying thermal environments*

358 We first analyzed the differences in thermal reaction norms between each
359 selective regime, fluctuating or warming, and the controls (Figures 2 and 3,
360 respectively). Differences between temperature treatments were observed for all traits in
361 both F *vs.* C and W *vs.* C comparisons (significant *Temp* factor, Tables S3 and S4).
362 Different traits showed different thermal responses: both colder (14°C) and warmer
363 (24°C) temperatures caused a decline in fecundity and productivity, when compared to
364 control conditions (18°C); age of first reproduction showed reduced performance at
365 lower but not at warmer temperatures, while viability showed the reverse pattern (lower
366 performance at the warmest temperature) – see Figures 2 and 3; Tables S3 and S4. No
367 overall effects of biogeographical history or thermal selection were observed for most
368 traits. The exception was for age of first reproduction, where W populations showed
369 significantly lower values (see Table S3 and Figure 3).

370 The reaction norms of populations from different selection regimes (F *vs.* C or
371 W *vs.* C) or from different biogeographical histories (Portugal *vs.* The Netherlands)
372 were also not significantly different (*Selection x Temp* and *History x Temp* interactions,
373 respectively, Tables S3 and S4). A significant triple interaction *History x Selection x*
374 *Temp* was observed for productivity in the F *vs.* C comparison (Table S4, Figure 2),
375 indicating that populations from distinct geographical origin respond differently to
376 thermal selection. This effect was mainly due to a lower performance at 24°C of the
377 southern populations subjected to the fluctuating regime (FPT).

378 Finally, we compared the performance of populations evolving under the two
379 varying thermal environments (fluctuating *vs.* warming). Like what was found above,
380 differences between temperature treatments were observed for all traits (Table S5,
381 *Temp*). Overall differences in performance between selective regimes were only found
382 for age of first reproduction (Table S5, *Selection*) while no differences were found in

383 the reaction norms between F and W regimes for any trait (Table S5, *Selection x Temp*).
384 Interestingly, differences in the reaction norms of populations with diverse
385 biogeographical histories were found for productivity, mostly due to a better
386 performance of northern populations at higher temperatures (Table S5, *History x Temp*).

387 Discussion

388 The temperature increase and fast-changing environmental conditions associated
389 with global warming impose great stress on ectothermic animals and adaptation can be
390 key for their survival. Here, we present a 31 generation-long experimental evolution
391 study addressing the evolutionary dynamics of thermal plasticity in populations of *D.*
392 *subobscura*. Two populations with distinct biogeographical histories (northern and
393 southern Europe) were subjected to different thermal selection environments (daily
394 fluctuating and global warming scenarios).

395

396 *Historical differences lead to contrasting evolutionary dynamics of thermal* 397 *reaction norms*

398 We found that the evolutionary dynamics of thermal reaction norms across 22
399 generations of evolution (generation 9 vs. 31) for several life history traits varied
400 between populations of contrasting geographical origin. This temporal variation in
401 plasticity was mostly driven by a differential performance at higher temperatures, with
402 the southern populations decreasing their reproductive performance across generations,
403 while their northern counterparts presented the opposite pattern; this effect was more
404 evident in their progeny productivity (Figures 2 and 3, Table 1). Geographical variation
405 in the shape of thermal reaction norms has been found in some studies of thermal
406 response in ectotherms (*e.g.*, (Trotta et al. 2006; Berger et al. 2013; Austin and
407 Moehring 2019; Klepsatel et al. 2019) but not in others (Klepsatel et al. 2013; Clemson
408 et al. 2016). Particularly in *D. subobscura*, variation in thermal reaction norms has been
409 observed in populations from Sweden and Spain, with northern populations performing
410 worse at higher temperatures (Porcelli et al. 2017). One could, thus, expect northern *D.*

411 *subobscura* natural populations to exhibit a steeper evolutionary response to rising
 412 temperatures, as these populations are likely further away from the new optimum in a
 413 warming scenario – and assuming that all populations evolve towards the same adaptive
 414 peak (Matos et al. 2002; Losos 2011). Prior to the establishment of the thermal regimes,
 415 our northern populations did not show a worse performance at higher temperatures
 416 when compared to their southern counterparts (Simões et al. 2020; Santos et al. 2021a).
 417 However, after 9 generations of thermal selection, though there was no robust evidence
 418 for historical effects, we detected a tendency for lower performance of the northern
 419 populations at higher temperatures across traits (Santos et al. 2021b). In this sense, the
 420 contrasting temporal dynamics observed between geographical populations at higher
 421 temperatures agrees with these expectations. Nevertheless, and assuming convergence,
 422 this variation in dynamics would lead to lower historical differences after 31 generations
 423 of thermal evolution. On the contrary, we found that populations are diverging through
 424 time, with the northern populations increasing their performance at higher temperatures
 425 and southern ones decreasing theirs. This pattern is particularly evident for productivity.
 426 This is an interesting finding as it suggests that, given enough time, populations
 427 historically from cooler climates might better overcome the current thermal challenges.
 428 The increased performance in the northern populations could result from a better
 429 developmental acclimation to higher temperatures, as both developmental and adult
 430 stages were subjected to the same temperature, which is worthy of further investigation.
 431 The presence of high genomic differentiation between the southern and northern
 432 populations at the start of our experiment might also play a role in the observed
 433 patterns. This is corroborated by previous findings in other *D. subobscura* populations
 434 that were founded from the same natural locations and still exhibit a high degree of
 435 genomic differentiation, even after 50 generations of lab evolution (Seabra et al. 2018).

436 Further studies are needed to address the relevant genomic variation between the
437 evolving populations, so that we understand the driving force behind the geographical
438 variation and the temporal increase in performance in the northern populations.

439 Our results suggest non-linear and complex evolutionary trajectories in which an
440 interplay between geographical backgrounds and the varying thermal environments has
441 an important role. The absence of clear geographical variation in our short term study
442 (Santos et al. 2021b) and the later emergence of such differences reinforces the need for
443 caution when interpreting and extrapolating short term evolution patterns to longer term
444 evolution.

445

446 *Evolution under different thermal selection regimes does not lead to*
447 *different thermal reaction norms*

448 Considering the higher thermal variation in the warming vs. the fluctuating
449 environment with much more pronounced thermal extremes, one could expect a higher
450 performance of the warming-evolved populations at higher and lower temperatures, as
451 well as a lower performance at intermediate ones. This expectation is based on a
452 possible trade-off between thermal breadth and maximal performance (Huey and
453 Kingsolver 1993). However, we did not find differences in the evolution of thermal
454 reactions norms between the warming and fluctuating populations, despite their
455 geographical origin. It might be the case that enzymatic constraints limit the evolution
456 of improved performance at both extremes (Angilletta et al. 2003), which, in turn, might
457 hinder the evolution of a more generalist performance of the warming populations. In
458 fact, other experimental evolution studies have seldom verified the evolution of such
459 predicted phenotypes as a response to thermal environmental heterogeneity, including

the evolution of thermal specialists *vs.* generalists (*e.g.*, (Berger et al. 2014; Condon et al. 2014; Manenti et al. 2015) but see (Le Vinh Thuy et al. 2016).

Can populations adapt to a global warming environment?

We did not find a clear long-term adaptive response to higher temperatures after 31 generations of thermal selection under a warming scenario, despite the trend of improved productivity (*i.e.*, number of offspring) of the northern populations (see Figure 3 and Tables S2 and S3). Other experimental evolution studies addressing adaptation to cumulative higher temperatures have also failed to find a clear adaptive response to these more stressful environments (*e.g.*, (Schou et al. 2014; Kinzner et al. 2019). Constrained plastic response of physiological traits at high temperatures was also observed in comparative studies across species (Gunderson and Stillman 2015; Schou et al. 2016; Sørensen et al. 2016; MacLean et al. 2019). Several causes might contribute to the absence of a clear adaptive response, which was particularly evident in our southern populations. Evolutionary constraints, such as trade-offs or an overall low genetic variation for the measured traits, might play an important role (Hoffmann et al. 2017; Kristensen et al. 2020). Methodological issues, such as the decoupling between the assay environments and the selection regimes (*e.g.*, constant *vs.* fluctuating temperatures, low *vs.* high density reproduction) might also contribute. Another possible explanation is that a part of the population's response to the thermal conditions might be environmentally-based, resulting, for instance, from epigenetic and/or maternal environmental effects (*e.g.*, (Hoffmann and Bridle 2021). That being true, such effects would not be detected in our experimental setup as they could have been mitigated or removed by the one-generation common garden protocol at 18°C imposed prior to the plasticity assays. The impact of common garden procedures and the environment in

485 which they occur is an issue worthy of further consideration and analysis, particularly in
486 the context of experimental evolution.

487 The selective pressure experienced by populations in the new thermal
488 environment is also an important point to consider. There were times during thermal
489 evolution when the selective pressures in the warming environment were most likely
490 very high, as there were severe drops in the W population sizes' due to extremely low
491 juvenile viability. Nevertheless, in more recent generations, the populations showed an
492 obvious improvement in their ability to endure the maintenance conditions, with
493 consistently higher census sizes (Figure S1). This makes us wonder why such an
494 apparent adaptive response is not clearly detected in our life-history assays. We believe
495 that this might stem from a decoupling between the test environment and the imposed
496 selection environment. However, even if adaptation to the different thermal conditions
497 occurred, it did not produce conspicuous changes in the thermal reaction norms. In
498 future experiments, it will be important to test the exact environment in which the
499 populations evolve (*i.e.*, local adaptation assays), alongside with changes in the thermal
500 reaction norms measured at constant temperatures.

501

502 *Final remarks*

503 We, here, report differential plasticity during evolution under thermally varying
504 environments, driven by the contrasting biogeographical history of the evolving
505 populations. The northern European populations appear to be coping better with these
506 dynamic thermal environments, as they showed a temporal increase in performance at
507 higher temperatures, particularly in terms of productivity. Contrary to our expectations,
508 the evolutionary changes in thermal reaction norms did not lead to an across-trait

509 increased performance at lower and higher temperatures after long-term thermal
 510 selection. Remarkably, our study shows a high impact of the previous biogeographical
 511 history in the population's thermal evolutionary response, even after tens of generations
 512 evolving in the same environment. These results highlight the importance of extending
 513 the evolutionary studies to populations from different geographical sources, which have
 514 adapted to local environmental conditions, to make more accurately intraspecific
 515 generalizations. Our findings reinforce the likely complex nature of thermal responses
 516 and illustrate the hardships of predicting evolutionary responses to global warming.

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684 Tables

685 **Table 1.** Evolutionary dynamics of W and F populations. Data refers to average
686 population values standardized to the controls. The F statistic shows the degrees of
687 freedom of effect and error. Significance levels for F values: $p > 0.05$ n.s.;
688 $0.05 > p > 0.01$ *; $0.01 > p > 0.001$ **; $p < 0.001$ ***. Only the model parameters with a
689 significant effect in at least one trait are presented.

690

Model parameters	Age of First Reproduction (A1R)	Fecundity	Productivity	Viability
History*Temp	$F_{2,4} = 2.300$ n.s.	$F_{2,44} = 1.111$ n.s.	$F_{2,46} = 7.250$ **	$F_{2,43.6} = 3.594$ *
History*Gen	$F_{1,28} = 11.08$ **	$F_{1,44} = 0.007$ n.s.	$F_{1,46} = 2.236$ n.s.	$F_{1,43.1} = 4.613$ *
Selection*Gen	$F_{1,28} = 7.185$ *	$F_{1,44} = 0.571$ n.s.	$F_{1,46} = 0.108$ n.s.	$F_{1,43.0} = 0.001$ n.s.
History*Temp*Gen	$F_{2,28} = 2.911$ n.s.	$F_{2,44} = 6.054$ **	$F_{2,46} = 14.811$ ***	$F_{2,43.8} = 5.459$ **

691

692

693 Figure Legends

694

695 **Figure 1.** Daily temperature profiles of the experimental regimes after nine (G9) and
696 thirty-one (G31) generations of thermal selection. The warming profile was kept
697 unchanged from generation 20 on.

698

699 **Figure 2.** Evolution of the thermal reaction norms of the Fluctuating (F) and Control
700 (C) thermal selection regimes after 9 and 31 generations of selection. Data show the
701 average and 95% confidence intervals for each thermal regime at each time point, with
702 average values of each replicate population as raw data. Viability data is arcsine
703 transformed. A1R – Age of first reproduction.

704

705 **Figure 3.** Evolution of the thermal reaction norms of the Warming (W) and Control (C)
706 thermal selection regimes after 9 and 31 generations of selection. Data show the average
707 and 95% confidence intervals for each thermal regime at each time point, with average
708 values of each replicate population as raw data. Viability data is arcsine transformed.
709 A1R – Age of first reproduction.

710

Figures

712

713

714 **Figure 1**

715

716 **Figure 2**

Thermal Performance Curves in W vs. C populations

717

718 **Figure 3**