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Title: No evidence for short-term evolutionary response to a warming environment in *Drosophila*

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Abstract

Adaptive evolution is key in mediating responses to global warming and may sometimes be the only solution for species to survive. Such evolution will expectedly lead to changes in the populations' thermal reaction norm and improve their ability to cope with stressful conditions. Conversely, evolutionary constraints might limit the adaptive response. Here, we test these expectations by performing a real-time evolution experiment in historically differentiated *Drosophila subobscura* populations. We address the phenotypic change after nine generations of evolution in a daily fluctuating environment with average constant temperature, or in a warming environment with increasing average and amplitude temperature across generations. Our results showed that (1) evolution under a global warming scenario did not lead to a noticeable change in the thermal response; (2) historical background appears to be affecting responses under the warming environment, particularly at higher temperatures; (3) thermal reaction norms are trait-dependent: while lifelong exposure to low temperature decreases fecundity and productivity but not viability, high temperature causes negative transgenerational effects on productivity and viability, even with high fecundity. These findings in such an emblematic organism for thermal adaptation studies raise concerns about the short-term efficiency of adaptive responses to the current rising temperatures.

Keywords: global warming; thermal fluctuations; thermal adaptation; *Drosophila*; experimental evolution; temperature

1. Introduction

One catastrophic consequence of global warming is the worldwide decimation of biodiversity, which impacts on species abundance and distribution, and damages the ecosystem functioning (Somero 2012; Pecl et al. 2017; IPCC 2018). Ectotherms are a huge fraction of biodiversity, have an essential role in ecosystems, and are particularly vulnerable to global warming due to the profound effects of temperature on their biology, biochemistry, and physiology (*e.g.*, Dillon et al. 2010; Hoffmann and Sgrò 2018). In species with low dispersal ability or when migration to climatically appropriate habitats is not possible, adaptation to the changing environmental conditions is crucial for survival (Hoffmann and Sgró 2011; Huey et al. 2012). Recent evidence suggests that adaptation to higher temperatures might be highly limited by physiological constraints and/or possible lack of genetic variation (Kellermann et al. 2009, 2012; Araújo et al. 2013; Kristensen et al. 2015). It is, thus, crucial to understand the potential of species to adaptively respond to global warming, and the underlying molecular, physiological, and phenotypic changes (Franks and Hoffmann 2012; Bell 2017; Walsh et al. 2019). This will shed light on how evolution shapes populations' fitness and fate, increasing our ability to preserve biodiversity and forecast species distributions (Urban et al. 2016; Bay et al. 2018).

The elevated mean temperatures and extreme thermal events associated with global warming (IPCC 2018) have increased the thermal variation of natural environments, already experiencing both circadian and seasonal variation. During the last decade, there has been a growing interest on the impact of temperature variation on organism fitness in the last decade, with several studies addressing thermal adaptation (Bozinovic et al. 2011; Vasseur et al. 2014; Colinet et al. 2015; Cavieres et al. 2018) and the evolutionary potential to respond to global warming (Hallsson and Björklund 2012; Schou et al. 2014; Manenti et al. 2016; Sørensen et al. 2020). The amount and rate of evolutionary change in response to rising temperatures is dependent on several features, such as the levels of additive genetic variation,

trade-offs derived from different thermal physiology (within and across species), population size and structure, inbreeding, and the rate of environmental change (Chevin et al. 2010; Hoffmann and Sgró 2011; Bell 2017; Trubenová et al. 2019; Kristensen et al. 2020).

One important body of theory that allows envisioning how adaptation to thermally varying environments proceeds stems from the generalist *vs.* specialist trade-off. Specialists are predicted to evolve in the more stable thermal environments and generalists in the more dynamic, variable environments (Angilletta et al. 2003; Angilletta 2009). These predictions assume a trade-off between maximal performance and performance breadth across temperatures, resulting from antagonistic pleiotropy associated with constraints to the structure and function of enzymes at different temperatures (Huey and Hertz 1984; Huey and Kingsolver 1989; Angilletta et al. 2003). Important empirical work has been done on evolution under constant *vs.* variable thermal environments, in controlled laboratory experiments. Overall, the expectations of a specialist *vs.* generalist trade-off have not been supported (Ketola et al. 2013; Berger et al. 2014; Condon et al. 2014; Manenti et al. 2016; but see Le Vinh Thuy et al. 2016). However, there is some consistent evidence for the evolution of generalists under fluctuating environments (Ketola et al. 2013; Condon et al. 2014). The occurrence of indirect selection, the range of tested temperatures (Condon et al. 2015), and the estimation of performance based on constant rather than fluctuating temperatures (Ketola and Saarinen 2015) might explain, at least in part, why evidence for a specialist *vs.* generalist trade-off is lacking.

Evolutionary changes resulting from thermal adaptation can also be framed in the context of evolution of thermal reaction norms, that describe performance across a range of temperatures. These reaction norms can evolve through changes in elevation or shape (Angilletta et al. 2003). The former refer to overall (similar) changes in performance - *i.e.*

trait means - across environments, reflecting positive correlations between environments. The latter imply evolution of plasticity, possibly associated with specialist vs. generalist trade-offs as explained above. Some *Drosophila* studies have provided evidence for the evolution of plasticity of several life-history traits as a result of local adaptation, with populations from distinct geographical locations differing in their thermal reaction norms (*e.g.* (Trotta et al. 2006; Austin and Moehring 2019; Klepsatel et al. 2019), but see (Klepsatel et al. 2013; Clemson et al. 2016) for changes mostly in elevation).

The study of real-time evolution of populations evolving under different realistic ecological scenarios can also provide invaluable insight on the adaptive potential to respond to temperature fluctuations and global warming (Kawecki et al. 2012; Karve et al. 2015, 2016; Manenti et al. 2015; Bailey and Bataillon 2016; Kellermann and van Heerwaarden 2019; Liukkonen et al. 2021). By means of experimental evolution, populations are studied across several generations in very well defined and reproducible conditions that are often achievable only in the laboratory (*e.g.*, Kawecki et al. 2012; Magalhães and Matos 2012). This powerful approach can provide direct evidence for adaptation to diverse thermal environments in different key adult and juvenile traits, allow the estimation of adaptive change rates, and clarify the link between phenotypic and genetic variation (Hoffmann and Sgró 2011; Porcelli et al. 2015). Experimental evolution has been a tool of choice to address several mechanisms and dynamics of thermal evolution in the last fifteen years, particularly in insects (*e.g.*, Santos et al. 2005, 2006; Hallsson and Björklund 2012; Rogell et al. 2014; Schou et al. 2014; Kellermann et al. 2015; Tobler et al. 2015; Manenti et al. 2016; Kinzner et al. 2019). Some of these studies have focused on evolution under increasing temperatures, as expected in a global warming scenario, although with varying rates of environmental change (Hallsson and Björklund 2012; Rogell et al. 2014; Schou et al. 2014; Kinzner et al. 2019). Most of them provided evidence of limited potential for evolutionary responses (Hallsson and

Björklund 2012; Schou et al. 2014; Kinzner et al. 2019). However, no empirical studies have yet addressed, to our knowledge, the impact on the evolutionary response of increasing both thermal mean and amplitude, two key aspects of global warming (IPCC 2018).

Drosophila is an excellent model organism to study thermal adaptation in ectotherms and has been widely used in experimental evolution studies. Disparities among studies have been obtained and highlight that the thermal adaptive responses are complex and caused by multiple factors, particularly, the specific thermal environments and populations under study. Different methodologies to estimate stress response also contribute to the different conclusions (Hoffmann and Sgró 2011; Kellermann and van Heerwaarden 2019; Kristensen et al. 2020). *Drosophila subobscura*, a native Palearctic species, is a well-known case study of thermal adaptation. It shows genetic variation associated with local adaptation, with evidence of latitudinal clinal variation for chromosomal inversion frequencies in Europe and, more recently, in both South and North America (Rezende et al. 2010). These polymorphisms have also been shifting worldwide, associated with global warming (Balanyá et al. 2006) and responding to heat waves (Rodríguez-Trelles et al. 2013). Previous selection experiments using different temperatures showed that evolutionary responses were not always as expected from clinal patterns (Santos et al. 2005). This species presents relevant thermal plasticity, with developmental temperature playing a decisive role in adult reproductive performance (Simões et al. 2020; Santos et al. 2021). Evolution of thermal performance curves for locomotor behavior in *D. subobscura* has been recently described as a result of selection for heat knockdown resistance (Mesas et al. 2021). The range of development temperatures suitable for this species is 6 – 26°C (Moreteau et al. 1997; David et al. 2005; Schou et al. 2017) with optimal viability between 16°C and 20°C (Schou et al. 2017), which is in agreement with their preferred body temperature (Rego et al. 2010; Castañeda et al. 2013). *D. subobscura* displays clear plastic responses to new thermal challenges (Fragata et al. 2016;

Simões et al. 2020; Santos et al. 2021), with some evidence for historical differences in plasticity between populations, particularly at colder temperatures (Simões et al. 2020).

Here, we use experimental evolution to analyze the evolutionary responses of historically differentiated *D. subobscura* populations, derived from extreme latitudes of the European cline (Simões et al. 2017, 2020), after nine generations under different thermal selective regimes that comprise differences in mean temperature and/or thermal amplitude. These regimes include (1) a constant thermal environment, corresponding to the thermal conditions of the long-established populations (controls), (2) a circadian thermal fluctuating environment (cooler nights, warmer days), and (3) a global warming-like environment, with increases in thermal mean and amplitude (progressively lower and higher thermal extremes) across generations. This experimental setup allowed us to address the effects of thermal fluctuations and warming conditions on the populations' evolutionary response. The adult reproductive performance of populations evolving under each of these environments was tested in different combinations of juvenile and adult temperatures. Thermal reaction norms for the different populations were estimated by focusing on the performance at different developmental and adult temperatures. We have three general (non-exclusive) expectations for the evolutionary response: (1) populations evolving under more heterogeneous (fluctuating or warming) thermal conditions are expected to evolve a better performance in more extreme thermal environments (Huey and Kingsolver 1993); (2) populations evolving in warming conditions will, most likely, perform better than the others in more stressful thermal environments, as these populations experience a wider range of temperatures during their life cycle; and (3) assuming there are costs of adaptation to more extreme temperatures (Huey and Kingsolver 1989; Angilletta et al. 2003), populations under fluctuating and (even more so) warming conditions will perform worse in intermediate thermal conditions than the control populations. All these expectations imply evolution of plasticity, through changes in

the shape of the thermal reaction norms. Alternatively, trade-offs between traits or lack of genetic variation might prevent the thermal evolutionary response. We, therefore, aimed to address the following questions: (i) Does evolution under warming or fluctuating environments change the population's thermal reaction norm? (ii) Does lifelong (or adulthood only) exposure to more extreme temperatures lead to a decline in performance? If so, does evolution in a warming environment change performance under these stressful conditions? (iii) Can we find evidence of geographical differences in the populations' thermal evolutionary response?

2. Material and Methods

2.1. Laboratory Populations and Thermal Selection Regimes

In late August/early September 2013, two natural populations of *Drosophila subobscura* were sampled. The collections were done in Adraga, Portugal (lat. 38°48' N) and Groningen, The Netherlands (lat. 53°13' N), two contrasting latitudes of the European cline (ranging from Scandinavia, ~60°N, to Northern Africa, ~30°N; Prevosti et al. 1988) that experience distinct environmental temperatures. A total of 213 founding females from Adraga and 170 from Groningen were collected. From these samples two sets of laboratory populations were established: PT, from Adraga, and NL, from Groningen – see details in (Simões et al. 2017). The founder females started separate families, that were kept during the first two generations, and inbreeding was prevented by outcrossing with males from other families. The outbred populations were formed at generation three, with an equal contribution of offspring from each family. One generation later, each population was three-fold replicated in the lab, originating PT₁₋₃ and NL₁₋₃ populations. The replication was done by dividing the egg collection of each outbred population in three equal parts (one for each replicate).

Maintenance involved discrete generations with a synchronous 28-day cycle, 12L:12D photoperiod, constant temperature of 18°C, controlled densities in both adults (50 adults per vial) and eggs (70 eggs per vial) in ~30 mm³ glass vials, and reproduction for the following generation around peak fecundity (seven to ten days old imagoes). Census size ranged between 500 and 1000 individuals (see Simões et al. 2017). In January 2019, after the PT and NL populations had undergone 70 generations of lab evolution, two new thermal selection regimes were derived: *circadian fluctuation* (F, originating FNL₁₋₃ and FPT₁₋₃) and *global warming* (W, originating WNL₁₋₃ and WPT₁₋₃). The experimental regimes are summarized in Figures 1a and 1b. The F regime is under a daily temperature fluctuation between 15°C and 21°C, with a mean daily temperature of 18°C, constant across generations. The W regime has a daily fluctuation similar to the F regime, but has a per generation increase of 0.18°C in daily mean and 0.54°C in daily amplitude (as the difference between the highest and lowest temperature). A 0.2°C mean increase per generation corresponds to that expected to be faced in nature by organisms with a ~10-year generation time, considering the current pace of global warming: 0.1-0.3°C per decade – see IPCC 2018. It is also comparable to the rate of temperature increase in other experimental evolution studies in ectotherms (Hallsson & Bjorklund 2012; Schou et al 2014). Additionally, and in contrast with those studies, we imposed a simultaneous increase in thermal amplitude, with progressively upper and lower thermal extremes across generations (increases of 0.44°C in upper and 0.08°C in lower extremes, per generation). The projected increase in thermal mean and the forecasted occurrence of thermal extremes associated with global warming accounted for our choice of per generation amplitude increase. The upper thermal increase of 0.44°C corresponds to roughly twice the mean increase (0.18°C), which is comparable to the projected increase in thermal extremes related to the mean temperature in mid-latitudes (IPCC 2018).

After nine generations of thermal selection, when this experiment was carried out, the warming populations have been subjected to temperatures ranging from 14.4°C to 24.5°C, with a daily average of 19.4°C; the population's thermal profiles are presented in Figure 1b. By generation seven, the temperature increase had led to a 24h reduction in the life-cycle length which became 27 days due to a shorter development time (*i.e.*, from egg to adult). The PT and NL populations are kept at constant 18°C and were already lab-adapted when the two new thermal regimes were started – they are the experimental controls. Since these controls are expected to be at evolutionary equilibrium, they reflect the genetic background (ancestral state) prior to the start of the F and W regimes. All experimental populations were, otherwise, maintained under the experimental protocol referred to above for the PT and NL populations. Census sizes were generally high for all thermal selection regimes (see Table S1; average census for W= 935.6, F= 976.5, and C= 1006.1).

2.2. *Experimental assays*

To study the effect of different thermal environments on the reproduction of the experimentally evolved populations, we analyzed the performance of our eighteen experimental populations subjected to five thermal treatments (Figure 2). In three of these treatments, flies were exposed to constant developmental and adult temperatures, either a colder temperature (14°C), intermediate (18°C), or warmer temperature (24°C) – 14-14, 18-18, and 24-24 treatments, respectively. Because reduced performance under lower adult temperatures and higher developmental temperatures was previously shown (Simões et al. 2020; Santos et al. 2021), two additional and potentially stressful thermal treatments were tested: 18-14 (lower temperatures in the adult stage) and 24-18 (higher temperatures during the developmental stage). To minimize maternal effects, the experiment was preceded by one generation of common garden rearing under control conditions (18°C).

The assayed flies were obtained from the egg collection of 18 vials (~70 eggs each) per population for flies developed at 18°C and 24°C, and 12 vials per population for flies developed at 14°C. Sixteen recently emerged mating pairs (virgin males and females) were individually assayed, per population and treatment, with a total of 1440 pairs (16 pairs × 18 populations × 5 temperature treatments) analyzed. For each temperature treatment, all populations were synchronously assayed, as common garden rearing allowed for a similar developmental time between all experimental populations. The treatments with different developmental temperatures (*e.g.*, 18-18 and 24-18) were not assayed in synchrony due to varying developmental times. During the experiment, the mating pairs were transferred to fresh medium every other day, the vials were daily checked for the presence of eggs, and the eggs laid by each female were counted between days six and eight since emergence. Four life-history traits were studied: (1) *age of first reproduction* (number of days since emergence until the first egg laying), which addresses the rate of sexual maturity; (2) *fecundity* (total number of eggs laid between days six and eight), which refers to a period that is close to the age of egg collection for the following generation (seven to ten-day-old imagoes), where selective pressures are likely higher; (3) *productivity* (number of emerged flies from the eggs laid on day eight and that developed at the same temperature of the assayed adults, *e.g.*, 18°C for the 24-18 treatment), which shows the ability of a female to produce viable progeny, and (4) *juvenile viability* (ratio between productivity and fecundity at day eight), which conveys to the efficiency of reproduction. For reliability purposes, only vials with at least five eggs were considered for juvenile viability and ~2% of the total number of vials were excluded. This decision was based on our observation of increased fungal infections in vials with very low number of eggs, leading to an almost absence of emerged flies. Including such vials would artificially bias viability estimates towards lower values.

2.3. Statistical Methods

Raw data used in the analyses is the mean value for each replicate population and temperature treatment (*e.g.*, the mean of PT_1 for the 14-14 treatment is one of the three data values for PT in this treatment). Data was analyzed by linear mixed effects models fitted with REML (restricted maximum likelihood). *p-values* for differences between temperature treatments, thermal regimes, populations as well as their interactions were obtained through analyses of variance (Type III Wald F tests, Kenward-Roger degrees of freedom). Two general models were applied (for simplicity we do not present interactions with random factors):

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

$$(2) Y = \mu + History + Temp + Selection + Block + History \times Temp + History \times Selection + Selection \times Temp + Selection \times History \times Temp + \varepsilon$$

where Y is the studied trait (age of first reproduction, fecundity, productivity, or viability), $History$ is the fixed factor corresponding to distinct geographical origin (with categories PT and NL), $Selection$ is the fixed factor representing the Thermal Selection Regimes (with three levels: Control, Warming, and Fluctuating), and $Temp$ is the fixed factor corresponding to the five different temperature treatments. In model (1), $AP\{History\}$ is the random effect consisting of the ancestral replicate population (*i.e.*, PT1-3; NL1-3) nested in History, from which each of the replicate populations of the three thermal selection regimes were generated (*e.g.*, Ancestral PT1 originating Control PT1, Fluctuating PT1 and Warming PT1). In model (2) Block was defined as random effect, corresponding to the set of same-numbered replicate populations from all different thermal regimes that were assayed synchronously in the same

experimental rack in a randomized manner. Models with and without interactions with random factors were assessed using AIC and the best model for each trait was chosen: model (1) for age of first reproduction and fecundity including interactions with AP; model (2) for productivity, without defining interactions with the random block. For the viability data, models (1) and (2) were tested including fecundity of day eight (F8) as covariate to account for the variation in fecundity across thermal treatments. The model without interactions with AP (random factor) and defining F8 as covariate presented the lowest AIC and was, therefore, chosen.

This body of data allowed to address how differences in the experimental populations associated with thermal regime (*Selection*) and geographical origin (*History*) impacted on their thermal performance. First, comparisons between thermal regimes were performed for the lifelong thermal treatments (14-14 vs. 18-18 vs. 24-24), allowing to analyze the populations' thermal reaction norms. Specific paired combinations of thermal regimes were used to test for (1) the effect of evolution under thermal fluctuations (F vs. C); and (2) the effect of evolution under global warming (W vs. C).

Second, specific effects of different temperature combinations were assessed to analyze how thermal evolution impacted on the flies' performance under stressful environments. Two models were then applied to compare the Warming and the Control regimes: (1) under colder conditions (treatments 14-14, 18-14, and 18-18); and (2) under warmer conditions (treatments 24-24, 24-18, and 18-18). The factor History and its interaction with temperature were also included in these models to test for differences between populations with distinct biogeographical origin (PT vs. NL). Tukey post-hoc tests were used to compare performance in the three different thermal treatments, whenever the temperature treatment factor was significant, allowing to identify which thermal treatments showed reduced performance. We further tested for differences in performance due to

selection (W vs. C) in the traits that presented a significant temperature effect, either for colder or warmer temperatures. In the latter analyses False Discovery Rate (FDR, Benjamini and Yekutieli 2001)) was applied to correct for multiple testing.

The homoscedasticity and normality assumptions for analysis of variance were checked and met in our dataset. Arcsine transformation was applied to the viability data to meet normality assumptions. All statistical analyses were performed in R v4.0.0, with *lme4* (Bates et al. 2015), *car* (Fox and Weisberg 2019), *lawstat* (Hui et al. 2008), *emmeans* and *ggplot2* (Wickham 2016) packages.

3. Results

3.1. Evolution of thermal reaction norms under dynamic thermal environments

We tested the evolutionary response of populations evolving under the following dynamic thermal environments: (1) circadian fluctuating and (2) global warming, by comparing the performance of each thermal regime with the controls (Figures 3 and 4, respectively). These analyses included the life-long temperature test environments (14-14, 18-18, and 24-24). Both fluctuating vs. control and warming vs. control analyses showed differences between temperature treatments for all traits (significant temperature factor; $0.001 < p < 0.01$ for fecundity and $p < 0.001$ for all other traits in both comparisons, see Tables S2 and S3). However, these patterns differed between traits. For the fecundity characters (*i.e.*, age of first reproduction and fecundity), a general reduction of performance relative to control (18-18) conditions was observed at lower (14-14 treatment) but not at warmer (24-24) temperatures. As for productivity, both colder and warmer temperatures showed a lower performance relative to control conditions. Viability showed yet a different pattern, with lower performance occurring only at the warmer temperature (see Figures 3 and 4).

The reaction norms of populations from the fluctuating or warming regimes were not significantly different from those of the controls (selection*temperature interaction, see Tables S2 and S3; Figures 3 and 4). Also, the reaction norms did not differ between populations with distinct histories (Portugal vs. Netherlands) – history*temperature interaction. Finally, the overall effects of history or selection were also not significant for any trait (see Tables S2 and S3).

3.2. Evolutionary response under stressful conditions

We aimed to test whether populations reared in an increasingly warmer environment for several generations have evolved increased performance when tested under stressful thermal environments. With this in mind, we assessed whether different juvenile-adult temperature treatments showed indications of cold stress (14-14, 18-14, and 18-18) or heat stress (24-18, 24-24, and 18-18), by comparing data from warming and control thermal selection regimes. Significant differences in performance between temperature treatments were obtained in both models – testing for either cold or heat stress – although this response varied across traits (see Table 1, Figures 4, S1 and text below). The exception was viability that did not present significant effect of lower temperatures.

When testing for heat stress, fecundity characters (*i.e.*, age of first reproduction and fecundity) showed a reduction of performance when populations developed at 24°C were exposed to 18°C as adults (24-18 treatment) relative to those under the 18-18 and 24-24 treatments (see Table 1, Figure S1). However, no differences were found in performance between the 24-24 treatment and the control (18-18) conditions (Table 1). As for productivity and viability, both 24-24 and 24-18 show lower performance relative to control conditions. Interestingly, no significant differences in productivity were found between these warmer

treatments, while viability was significantly lower in the 24-24 treatment relative to the 24-18 conditions – see Table 1 and Figure S1.

As for the cold stress analysis, significant reduction in performance was found in both colder treatments (14-14 and 18-14) relative to control conditions for age of first reproduction, fecundity, and productivity – see Table 1. For these same traits, performance of flies in the 14-14 conditions was always significantly higher than those of the 18-14 treatment. Viability showed no significant differences between these thermal treatments.

The linear mixed effects models applied to the pairwise combinations of temperature treatments showed no significant effects of selection or selection*temperature, in either cold or warm scenarios (see Table S4), which suggests that both warming and control populations do not differ in their response under the tested conditions. Interestingly, for the warming regime, we observed that southern populations (WPT₁₋₃) presented consistently higher average values than their northern counterparts (WNL₁₋₃) for all analyzed traits when tested in the warmer conditions (24-18 and 24-24; see Figures 4 and S1). However, no significant effects of biogeographical history or its interactions with temperature and selection were found for either heat or cold stress tolerance for any trait (Table S4).

4. Discussion

Recent empirical data casts doubt on the ability of ectotherms, including *Drosophila*, to respond to temperature increases, with stress resistance traits being evolutionarily constrained (reviewed in (Kellermann and van Heerwaarden 2019; Kristensen et al. 2020). This is problematic, given the fast-adaptive demand caused by the rapid and globally changing environment. In contrast, some thermal selection experiments have demonstrated

that heat tolerance can evolve, when directly selected upon (Bubliy and Loeschcke 2005; Hangartner and Hoffmann 2016), although the ecological relevance of such selection protocols might be questioned. Using the terminology of Diamond and Martin (2020), the question stands: can thermal adaptation be regarded as the *silver bullet* to fight global warming? According to our experimental data, the quick answer is no, not in the short run at least. It is important to mention that our experiment focuses on the two major features of global warming – the increased thermal mean and variance – and it does not cover the effects of all other climatic variables that necessarily change in a global warming scenario. The increases in thermal mean and variance that we imposed are relevant as they are (1) within the interval of past and projected thermal changes occurring worldwide and (2) within the forecasted association of increases in mean and extreme temperatures for mid-latitude regions (IPCC 2018).

Evolution in dynamic thermal environments did not substantially change life-history reaction norms.

Short-term thermal evolution (nine generations) did not alter the thermal reaction norms of our experimental populations, nor did adaptation to a warming environment improve their ability to cope with more stressful conditions. This lack of a clear adaptive response has been found in other studies addressing short-term evolution (<20 generations) of ectotherms in increasingly warmer fluctuating environments, although with non-increasing thermal amplitudes (Hallsson and Björklund 2012; Schou et al. 2014; Kinzner et al. 2019). In addition, evolution of *Drosophila simulans* populations in fluctuating environments with a constant average led to changes in the elevation of thermal reaction norms (increased mean performance across environments) for several life history and stress traits, but not in their shape (Manenti et al. 2015).

Several reasons could account for the deviation from our initial expectations. First, the number of elapsed generations might have been too small for evolutionary response to be detected. Because life-history traits and resistances to environmental stress are, in general, polygenic traits with low-to-intermediate heritabilities (Mousseau and Roff 1987; Diamond 2017; Castañeda et al. 2019), the evolutionary rates of adaptation are expected to be low (Hoffmann et al. 2017). This may explain our results, even with a high rate of environmental change (Chevin et al. 2010; Hoffmann and Sgró 2011; Kristensen et al. 2020) as occurs in the warming regime (an increase of 0.2°C per generation, with a final daily peak temperature ~7°C above control conditions). Additionally, the colder extreme temperatures to which the warming regime is exposed, even if slight, might contribute to an overall reduction in selection intensity. Analyzing thermal reaction norms after more generations of thermal selection will allow to test whether more time is required to achieve a significant evolutionary response.

Second, environmental stress can lead to population bottlenecks, which may dramatically decrease effective population size with the consequent loss of genetic variation and evolutionary response impairment (Hoffmann et al. 2017; Ørsted et al. 2019). However, demographic data from the experimental lines did not show any relevant census sizes drops (Table S1). Furthermore, the control populations (in which this experiment was based) were found to have abundant genomic variation at least during the first 26 generations after lab establishment (data not shown). This is corroborated by a previous study from our team, using lab populations collected from the same geographical locations, where only a very modest decline in genome diversity after 50 generations of lab evolution was found (Seabra et al. 2017). In addition, inversion polymorphism is known to have an adaptive response to thermal changes in this species (*e.g.*, Rezende et al. 2010). A total of thirteen different inversions were still segregating in these populations after 68 generations in the laboratory

(unpublished data). This level of polymorphism was already found by Santos et al. (2016) in other laboratory populations. Other *Drosophila* experiments involving increasing temperatures did not find an overall decline in genetic diversity (Schou et al. 2014; Kinzner et al. 2019), although we cannot exclude that low additive genetic variation for the analyzed phenotypic traits might be a factor in both those studies and ours.

Third, the idiosyncrasies of the dynamic thermal regimes, such as the simultaneous selection for lower and higher thermal extremes, may have slowed down the evolutionary response due to the existence of unfavorable genetic correlations and trade-offs. Though Manenti et al. (2016), in a study of evolution of *D. simulans* in fluctuating environments, found significant negative correlations between life-history and stress resistance traits, such trade-off between measures of cold and heat tolerance was not observed. Nevertheless, in a fluctuating environment, the time spent in each environment is necessarily shorter than in constant conditions, which reduces the strength of selection for each given environment (Kristensen et al. 2020).

Finally, genotype by environment (GxE) interactions might arise out of differences between the dynamic selection environments and the constant thermal conditions at which the assays were carried out (Ketola and Saarinen 2015; Kellermann and van Heerwaarden 2019). These GxE interactions could potentially lead to a lower performance of populations evolving in the dynamic thermal environments (fluctuating and warming). The pervasiveness of such interactions and, specifically, their effect on the shape of thermal reaction norms is still unknown (Kellermann and van Heerwaarden 2019). Interestingly, a large evolution experiment in *Drosophila simulans* showed that the shape of the reaction norm for fecundity was not affected by the evolution under constant vs. fluctuating environments, when tested in each environment (Manenti et al. 2015). Further and already planned experiments will

include, alongside with the thermal reaction norm approach, testing the populations in all the environmental conditions under which they evolved (local adaptation, *sensu* Kawecki and Ebert 2004).

In general, our data does not provide evidence for the impact of the geographical source of our populations on the thermal reaction norms nor their evolution. Nevertheless, we found that the warming populations of southern origin (WPT) performed consistently better at warmer temperatures in all measured life-history traits (Figure 3), suggesting that history may affect the evolutionary response to global warming. Higher thermal performance in southern *D. subobscura* populations has been shown by Porcelli et al. (2017). Considering the larger projected impacts of global warming at higher latitudes, due to mean and cold-season warming rates above the global average (IPCC 2018), a higher potential for evolutionary response would be needed in high-latitude populations. On the one hand, we cannot exclude the possibility that the low geographical differentiation in our study results, to some extent, from the evolutionary convergence of populations adapting to similar laboratorial conditions prior to the onset of this study (see Simões et al. 2017). On the other hand, it is quite likely that these populations still show a high degree of historical genomic differentiation, based on evidence from a genomic survey of populations founded from similar locations after 50 generations of lab adaptation (Seabra et al. 2018). It is, thus, possible that evolution in stressful conditions uncovers such underlying genomic differences. Longer-term experiments will evaluate if the incipient geographical differentiation we found in the thermal response expands further as populations evolve in the warming conditions.

Contrasting effects of thermal stress on fecundity and juvenile viability

In general, we found clear evidence for a plastic response to both cold and heat stress, despite general similarity of patterns between the different experimental populations.

Exposure to colder temperatures led to an overall decline in fecundity (and productivity) of all populations, but not in viability. This reduction in reproductive performance was due, most likely, to lower metabolic rates, which may reduce oogenesis and lead to lower reproduction output, as previously observed (Simões et al. 2020). Unsurprisingly, life-long cold-exposed flies had better performance than flies that were only exposed to cold as adults, probably due to increased ovariole number during cold development (Moreteau et al. 1997) and showing evidence for beneficial cold acclimation in fecundity patterns (Huey et al. 1999; Simões et al. 2020).

Heat stress is known to significantly affect *D. subobscura* reproduction and, most importantly, the stage at which high temperature stress is experienced strongly influences reproductive performance (Porcelli et al. 2017; Simões et al. 2020; Santos et al. 2021). High developmental temperatures have shown to cause within-generation, negative carry-over effects in ectotherms (Klockmann et al. 2017; Porcelli et al. 2017; Cao et al. 2018; Iossa et al. 2019; Klepsatel et al. 2019), maybe due to the irreversible physiological and metabolic damage brought upon them (*e.g.*, during gametogenesis). Conversely, adult exposure to warmer temperatures has, previously, shown to have little to no effect on fecundity and to increase the sexual maturity rate, leading to earlier reproduction (Santos et al. 2021). Our experimental data corroborates this dual effect in control and warming-selected flies: high lifelong temperature led to negative effects in productivity and, also, in progeny viability, despite the high levels of fecundity. Furthermore, the longer the flies were exposed to the heat stress during their life cycle, the higher the damage in viability. In any case, the reduced viability of the eggs laid by flies exposed to high temperature in the developmental stage only (24-18) shows possible negative transgenerational (or carry-over) effects for this trait. Interestingly, fecundity showed a quite different pattern: flies with lifelong exposure to higher temperatures showed higher egg production than those experiencing thermal stress

only in the development stage – this suggests some rescue in performance because of higher adult metabolic rates. The decoupling between fecundity and productivity/viability patterns further suggests that heat stress is (1) leading to the occurrence of male sterility; (2) affecting to a higher degree egg quality rather than egg production; (3) increasing juvenile mortality. In sum, our results confirm the previously noted vulnerability of the insects' development stage to global warming and its associated heat waves (Klockmann et al. 2017).

Conclusions

It is becoming apparent that the recovery of populations, *i.e.*, *evolutionary rescue* (Bell 2017), may not occur quickly enough to keep up with the fast pace of current environmental change (Diamond and Martin 2020). In line with those expectations, here we report no evidence for short-term evolution in the elevation or shape of the reaction norms of *D. subobscura* populations under dynamic thermal environments. In particular, our results indicate that evolution in a warming environment does not lead to an increased performance in key life-history traits at higher temperatures. This casts doubts on the possibility of a timely thermal adaptation to counteract the detrimental effects of global warming. Combining data from our populations on thermal reaction norms with additional generations of thermal selection and further research focusing on the patterns of local adaptation will allow a broader understanding of the pace of evolution in face of rapid environmental change.

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Tables

Table 1 – Differences in thermal performance between warming and control populations for colder (a) or warmer (b) temperature treatments.

a)

Trait	Model parameters	$F_{(df1, df2)}$
	Temp	$F_{2,8} = 347.90$ ***
Age of First Reproduction (A1R)	18-14 vs 18-18	t.ratio = 25.457 ***
	14-14 vs 18-18	t.ratio = 18.712 ***
	14-14 vs 18-14	t.ratio = - 6.745 ***
	Temp	$F_{2,8} = 123.71$ ***
Fecundity (F6-8)	18-14 vs 18-18	t.ratio = - 15.665 ***
	14-14 vs 18-18	t.ratio = - 9.060 ***
	14-14 vs 18-14	t.ratio = 6.606 ***
	Temp	$F_{2,22} = 22.050$ ***
Productivity	18-14 vs 18-18	t.ratio = - 6.588 ***

	14-14 vs 18-18	t.ratio = - 4.071 **
	14-14 vs 18-14	t.ratio = 2.571 *
	Temp	$F_{2,19,3} = 0.575$ n.s.
Viability	18-14 vs 18-18	-
	14-14 vs 18-18	-
	14-14 vs 18-14	-

Note: significance levels: $p > 0.05$ n.s.; $0.05 > p > 0.01$ *; $0.01 > p > 0.001$ **; $p < 0.001$ ***

First line refers to the statistics of the temperature factor (temp) in the overall linear mixed effects model. Below are presented the Tukey tests between thermal treatments using similar degrees of freedom of the error term as those used in the mixed effect model (for temperature).

b)

Trait	Model parameters	$F_{(df1, df2)}$
	Temp	$F_{2,8} = 7.463$ *
Age of First Reproduction (A1R)	24-18 vs 18-18	t.ratio = - 3.356 *
	24-24 vs 18-18	t.ratio = - 0.021 n.s.
	24-24 vs 24-18	t.ratio = 3.335 *
	Temp	$F_{2,8} = 7.317$ *
Fecundity (F6-8)	24-18 vs 18-18	t.ratio = 3.563 *
	24-24 vs 18-18	t.ratio = 0.575 n.s.
	24-24 vs 24-18	t.ratio = - 2.988 *
	Temp	$F_{2,22} = 15.447$ ***
Productivity	24-18 vs 18-18	t.ratio = 3.434 **
	24-24 vs 18-18	t.ratio = 5.502 ***
	24-24 vs 24-18	t.ratio = 2.068 n.s.
	Temp	$F_{2,19,2} = 35.747$ ***
Viability	24-18 vs 18-18	t.ratio = 3.110 *

24-24 vs 18-18 t.ratio = 8.410 ***

24-24 vs 24-18 t.ratio = 5.104 ***

Note: significance levels: $p > 0.05$ n.s.; $0.05 > p > 0.01$ *; $0.01 > p > 0.001$ **; $p < 0.001$ ***

First line refers to the statistics of the temperature factor (temp) in the overall linear mixed effects model. Below are presented the Tukey tests between thermal treatments using similar degrees of freedom of the error term as those used in the mixed effect model (for temperature).

Figure Legends

Figure 1a. Derivation of the experimental lines from natural *Drosophila subobscura* populations.

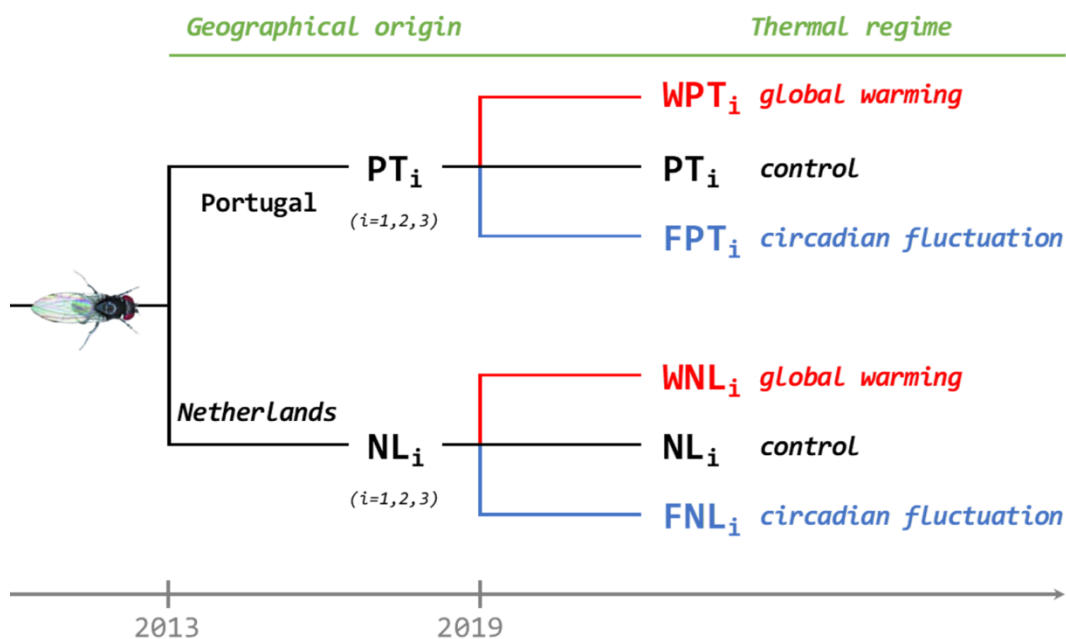


Figure 1b. Daily temperature profiles of the experimental regimes after nine generations of thermal selection.

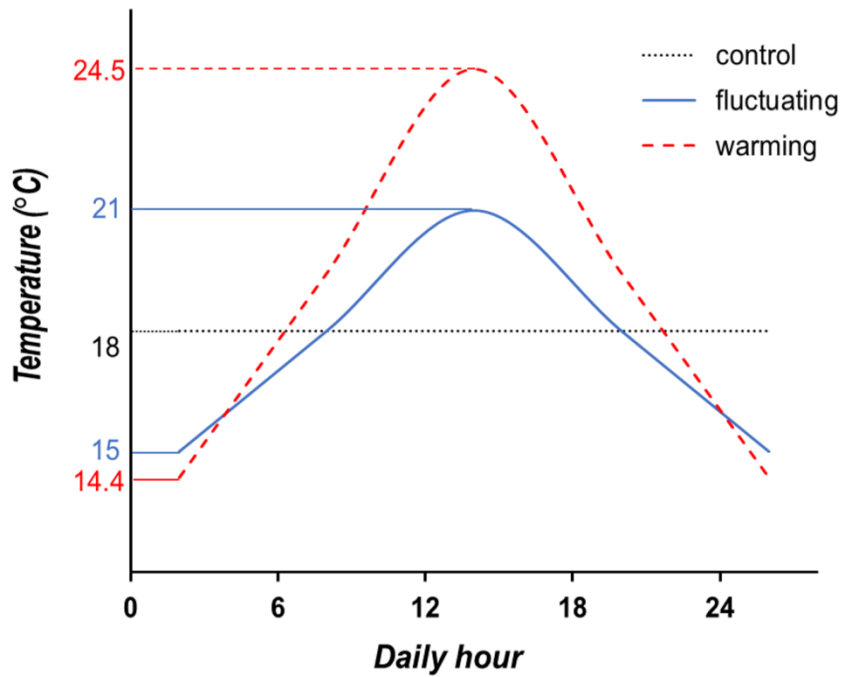


Figure 2. Experimental design: combinations of three developmental and three adulthood test temperatures.

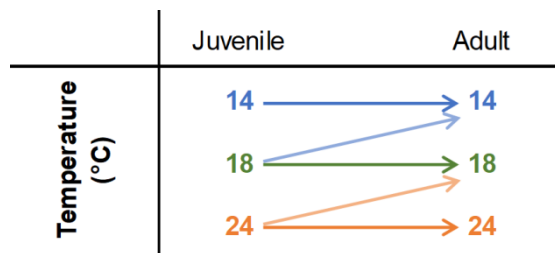


Figure 3. Thermal reaction norms of the Fluctuating (F) and Control (C) thermal selection regimes. A1R – Age of first reproduction. Data shows the average and 95% confidence intervals for each thermal regime (with average values of each replicate population as raw data).

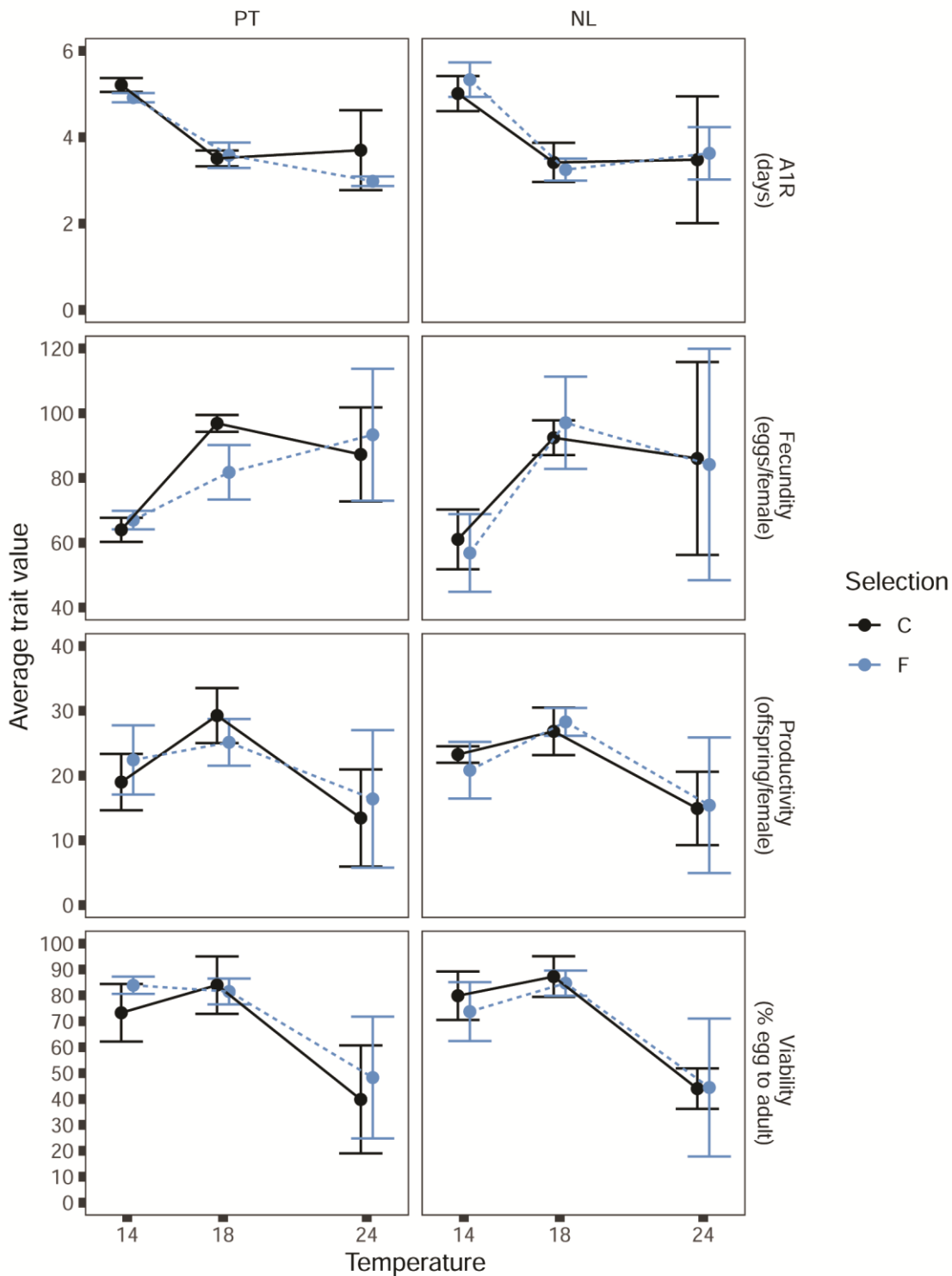


Figure 4. Thermal reaction norms of the Warming (W) and Control (C) thermal selection regimes. A1R – Age of first reproduction. Data shows the average and 95% confidence intervals for each thermal regime (with average values of each replicate population as raw data).

