Long-term evolution experiments fully reveal the potential for thermal adaptation

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ABSTRACT

Evolutionary responses may be crucial in allowing organisms to cope with prolonged effects of climate change. However, a clear understanding of the dynamics of adaptation to warming environments is still lacking. Addressing how reproductive success evolves in such deteriorating environments is extremely relevant, as this trait is constrained at temperatures below critical thermal limits. Experimental evolution under a warming environment can elucidate the potential of populations to respond to rapid environmental changes. The few studies following such framework lack analysis of long-term response. We here focus on the long-term thermal evolution of two *Drosophila subobscura* populations, from different European latitudes, under warming temperatures. We tested reproductive success of these populations in the ancestral (control) and warming environment after ~50 generations of thermal evolution. We found a general adaptive response to warming temperatures in the long term, since populations evolving in the warming environment showed increased performance in that environment relative to the respective control populations. On the other hand, no clear response was observed in the ancestral environment. Coupled with data from previous generations, we highlight a slow pace of adaptive response and differences in that response between populations of distinct histories. These findings demonstrate the need of long-term evolution experiments to fully reveal the potential for thermal adaptation. It also highlights that the scrutiny of different populations is needed as a measure of variation in evolutionary responses within a species. Accounting for these sources of variation - both temporal and spatial - will allow for more robust assessments of climate change evolutionary responses.

1. Introduction

Climate change is causing biodiversity to decline at an unprecedented pace (IPCC, 2023). Evolution may play an important role in enhancing organisms' ability to cope with the prolonged effects of climate change (Urban et al., 2016). It is thus crucial to understand the dynamics of adaptation to sustained warming temperatures to better predict the impacts of climate change (Edelsparre et al., 2024; Martin et al., 2023). In particular, addressing how reproductive success evolves in warmer environments is extremely relevant, since negative effects of heat stress on fertility occur quite often at lower temperatures compared to the critical thermal limits for the species (Bretman et al., 2024; Dougherty et al., 2024; Parratt et al., 2021; van Heerwaarden and Sgrò, 2021; Walsh et al., 2019). In fact, fertility patterns can track species' geographical distribution in nature, emerging as a predictor of species distribution changes due to global warming (Parratt et al., 2021; van Heerwaarden and Sgrò, 2021).

The possibility of an adaptive response to a deteriorating environment is shaped by two main factors: the standing genetic variation within populations and the rate of environmental change (Bell, 2017; Burger and Lynch, 1995). If genetic variation is present and provided that the environmental change is not too high, the average phenotype may evolve in parallel though lagging behind the changing optimum. In contrast, when genetic variation for fitness-related traits is low and/or evolutionary constraints between traits occur, the rates of evolution will be limited and populations may not be able to keep pace with environmental change (Burger and Lynch, 1995). Under such scenario, adaptation to the new conditions may only be possible if the rate of environmental change is very slow or ceases (Bell, 2017; Hoffmann and Sgró, 2011; Lindsey et al., 2013).

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Experimental evolution is a powerful tool to deepen our understanding of the potential for thermal evolution, as it allows to follow the real-time evolution of populations under relevant and controlled thermal settings (Kawecki et al., 2012; Kellermann and van Heerwaarden, 2019; Rogell et al., 2014; Santos et al., 2021a; van Heerwaarden and Sgrò, 2021). Some studies in ectotherms (mostly Drosophila) have focused on evolutionary responses of reproductive traits to experimental warming. In general, these studies have found limited evolutionary response to warming in reproductive traits (Schou et al., 2014; Kinzner et al., 2019; van Heerwaarden and Sgrò, 2021). On the other hand, Rogell et al. (2014) found that populations of the seed beetle *Callosobruchus maculatus* evolving under incrementally increasing temperature (from 30 to 36 °C, rate of 0.3 °C per generation) for 18 generations were more fecund (and lived longer) than the controls. Importantly, the effects on fecundity in these seed beetle populations were only observed after 23 generations of evolution at constant 36 °C, after the initial incremental setting mentioned above (Hallsson and Björklund, 2012; Rogell et al., 2014). These experiments suggest that the speed of adaptive change to new thermal conditions is likely slow – possibly due to limited genetic variation for reproductive traits - and that a rapid rate of environmental change poses serious problems to a timely adaptive response. One caveat of the thermal experimental evolution studies reported above is that they do not consider variation at the inter-population level - namely by studying different geographical populations of a species. Furthermore, most studies covered a limited temporal range – around 20 generations or less (Schou et al., 2014; Kinzner et al., 2019; van Heerwaarden and Sgrò, 2021), which is insufficient to address long-term evolutionary trends (Hoffmann et al., 2023).

Experimental evolution studies offer the possibility to assess adaptation to specific conditions as well as to detect associated costs of adaptation by testing control and experimental populations in both the ancestral and a novel environment, something that is not straightforward in field studies (Kawecki and Ebert, 2004). Adaptation to a specific thermal environment can lead to reduced performance in the ancestral environment due, for instance, to genes with antagonistic pleiotropy between traits that are differently expressed across environments (Griffiths et al., 2024; Kawecki and Ebert, 2004). However, detection of such costs of adaptation has been elusive, possibly because of their low magnitude (Hereford, 2009; Bono et al., 2017).

Drosophila subobscura is a species that shows evolutionary responses to climatic factors with repeatable clines of body size (Gilchrist et al., 2004; Huey et al., 2000) and chromosomal inversions (Prevosti et al., 1988). The inversion polymorphism shifts in this species are particularly relevant, with inversions more prevalent in warmer climates (i.e. warm-adapted inversions) increasing their frequency worldwide because of global warming (Balanyà et al., 2006; Rezende et al., 2010). This species also presents high levels of thermal plasticity for reproductive traits (Fragata et al., 2016; Santos et al., 2021b; Simões et al., 2020). Previous studies have used this species to address evolution under an increasingly warmer environment (Santos et al., 2021a, 2023a, 2023c). The studied *D. subobscura* populations were originally from northern (high latitude) and southern (low latitude) European locations and were maintained as separate populations for 70 generations in a benign laboratory environment to address the effects of different historical backgrounds during adaptation. Prior to the imposition of the thermal selection regime, in agreement with expectations, these populations showed fast adaptation to the lab environment during around 30 generations of laboratory evolution (Simões et al., 2017). Despite this clear phenotypic response, karyotypic and genomic differentiation resulting from the distinct historical origins was maintained (Barreira, 2023; Simões et al., 2017).

To study thermal adaptation in these populations a thermal regime was applied, with increases in both mean temperature and in daily thermal amplitude until generation 22 of evolution. With this warming thermal regime, the aim was to analyse the effect of these two key features of warming environments (IPCC, 2023). From generation 23 onwards, the thermal environment was kept the same until the end of the experiment, in a daily regime ranging from a low temperature peak of 13.5 °C to a high temperature peak of 29.4 °C, with constant 18 °C being the control conditions. The evolutionary response of these populations to warming conditions was found to be slow and specific to the history of the populations (Santos et al., 2023a, 2023c). In fact, thermal adaptation was only observed in the populations of low latitude, and only after 39 generations, while neither population showed signs of an evolutionary response after 22 generations (Santos et al., 2023c). Evidence supporting costs for thermal adaptation in these populations was weak (Santos et al., 2023c). It remained however to be tested whether longer-term evolution would allow for an adaptive response in the higher latitude populations.

To answer this, we here focus on the long-term adaptive response of such warming-evolved populations by performing an experimental assay to address the reproductive success of the warming populations relative to their controls under either warming or the ancestral (control) conditions, after more than 50 generations of thermal evolution. Given that the warming regime was kept unchanged for more than 25 generations by the time this study was conducted, we expect that this extended temporal period will have allowed for an adaptive response in both high and low latitude populations. Moreover, as a result of this expected long-term adaptation we anticipate the presence of adaptive costs, expressed by a decline in performance of warming populations in the control environment relative to their ancestral counterparts.

2. Material and methods

2.1. Population maintenance and thermal selection regimes

Experimental populations resulted from two collections of natural populations of *Drosophila subobscura*, done in August/September 2013 in Adraga, Portugal (38°48′ N) and Groningen, The Netherlands (53°13′ N). 213 founder females were collected in Adraga and 170 in Groningen (Simões et al., 2017). These collections gave rise to the PT and NL laboratory populations. In the two first generations in the lab, females were maintained as families to avoid losing genetic variability due to sampling effects in the initial generations of lab foundation (see Fragata et al., 2014, cf. Santos et al., 2013). Outbred populations were originated in generation 3 as described in Fragata et al. (2014). These populations were maintained in 30 cm³ glass vials with controlled densities in eggs (70 eggs per vial in a total of 24 vials) and adults (40 adults per vial, again 24 vials for a total of around 1000 individuals per replicate population), with discrete generations of 28 days, 12L:12D photoperiod and a constant 18 °C temperature (see details of the maintenance cycle in Supplementary Fig. 1 and below). The sex ratio in the adult vials was not directly measured during population maintenance but data from an assay directly focusing on sex ratio in these populations did not find a sex ratio bias at 18 °C (Santos et al., 2023b). 18 °C is a benign temperature for this species (e.g. see Fragata et al., 2014; Santos et al., 2005), so we do not expect that temperature *per se* to be a major factor in determining patterns of lab adaptation. At the start of each generation, eggs laid by adults during a 24h period in those vials were counted and allocated to developmental vials with fresh medium. Upon emergence, imagoes were collected from each development vial for four days and allocated to vials with fresh medium. At the fourth day of emergences, these individuals were mixed with CO_2 anaesthesia, redistributed in 24 vials per replicate population and maintained for 1 week as adults. After that, egg collection for the new generation took place. Adults were seven to ten days old at the timing of egg collection for the next generation. By generation four in the lab, three replicate populations were created of each founding population, leading to PT1-3 and NL1-3 populations.

PT and NL populations were kept under the maintenance protocol explained above for 70 generations, after which two thermal selection regimes were created to start the experiment. This was done by dividing the egg collection of each ancestral PT and NL replicate population into

equal parts, that were assigned to the two different temperature regimes: the control regime, with populations kept under the standard maintenance protocol at constant 18 °C and the warming regime, with populations under increased thermal mean and amplitude across generations (see Fig. 1 and below). For example, the NL1 replicate population generated the control NL1 and the warming WNL1 populations. PT and NL population, used as control populations in the experiment, and represent the ancestral of WPT and WNL populations respectively. These populations were maintained as much as possible in synchrony with the warming populations (see also Fig. 1).

The warming regime had a daily fluctuation cycle that oscillated between 15 °C and 21 °C in the first generation (see Supplementary Fig. 2) with an average temperature of 18 °C and increases in daily thermal mean and thermal amplitude every generation (respectively of 0.18 °C and 0.54 °C), reflecting the two main features of warming environments (IPCC, 2023). The imposed increase in mean temperature (0.18 per generation) agrees with the expected rate of temperature increase per decade (0.19–0.63°C, IPCC, 2023). In addition, the ratio of increase of thermal amplitude relative to the mean increase (0.54/0.18) is also aligned with the IPCC predictions - see Santos et al. (2021a). However, this variation in amplitude does not intend to mimic changes in diurnal:nocturnal amplitudes as expected in nature *per se*. The rate of environmental change we imposed was high, aiming to address the potential for thermal evolution in an ectotherm species. It is comparable to the warming rates used in other experimental evolution studies (e.g. Schou et al., 2014; Rogell et al., 2014).

Nevertheless, by generation 22, when the peak temperature in the warming regime reached 30.2 °C (with an average temperature of 21.78 °C), there was a significant drop in adult census sizes due to high juvenile mortality in both warming populations of distinct origin (estimated average population sizes of 200 flies across all populations – see Table S1). As a result of this, to prevent the loss of experimental populations and avoid continued low population sizes that could lead to a loss of genetic variation and inbreeding depression, we decided to reverse the warming cycle back to the cycle of generation 20 (Santos et al., 2023c). This allowed for population recovery, although not immediately (see Table S1). This cycle, with a mean temperature of 21.4 °C and a fluctuation between 13.5 °C to 29.4 °C, was maintained from generation 23 till the end of the study and was the warming environment applied in phenotypic assays (see Supplementary Fig. 2 and also details below). Apart from the population crashes referred

above, very low population census sizes were observed by generation 44 that motivated a two-generation transfer of the warming populations to control conditions to allow for population recovery. Otherwise, population sizes were generally high throughout the study (between 600 and 1000 individuals) – see Table S1.

The maintenance of the warming and control populations only differed in the thermal regime imposed. However, the warming regime led to a decoupling in generation time – from 28 days to 24 days by the time of the experiment - given the faster developmental time of populations evolving under this thermal regime. This is expected based on the association between developmental time and temperature. We do not expect high selective pressures for faster development as the vast majority of individuals emerged within the 4-day emergence collection period we applied and could therefore potentially contribute to the next generation.

2.2. Experimental assay

This study involved an assay aimed at testing for adaptation to warming conditions by assessing the reproductive success of warming populations and their controls in both the warming environment and the control (ancestral) environment by generation 52 of thermal adaptation. As done in previous assays (see Santos et al., 2023c), an orthogonal design was applied with Warming and Control populations being tested in each (Warming and Control) environment. This assay involved sixteen pairs of flies – placed in individual glass vials - per replicate population and environment. We used an experimental design with blocks, with each block encompassing all same numbered replicate populations representing each Regime and History (e.g. block 1 included all the vials from NL1, WNL1, PT1 and WPT1 replicate populations). Vials were organized in several experimental racks in each block, making sure that the same number of vials per population was placed in each rack. To organize vials within experimental racks, a pseudo-randomized approach was implemented, with the relative position of the vials from each replicate population (distributed in rows) changing between racks. In total, 384 pairs of flies were studied in this assay (16 pairs × 3 replicate populations × 2 thermal selection regimes × 2 historically differentiated populations × 2 test environments, see also Santos et al., 2023c).

We estimated reproductive success as the total number of offspring derived from eggs laid by each mating pair at day 9 of adult age (a 24-h
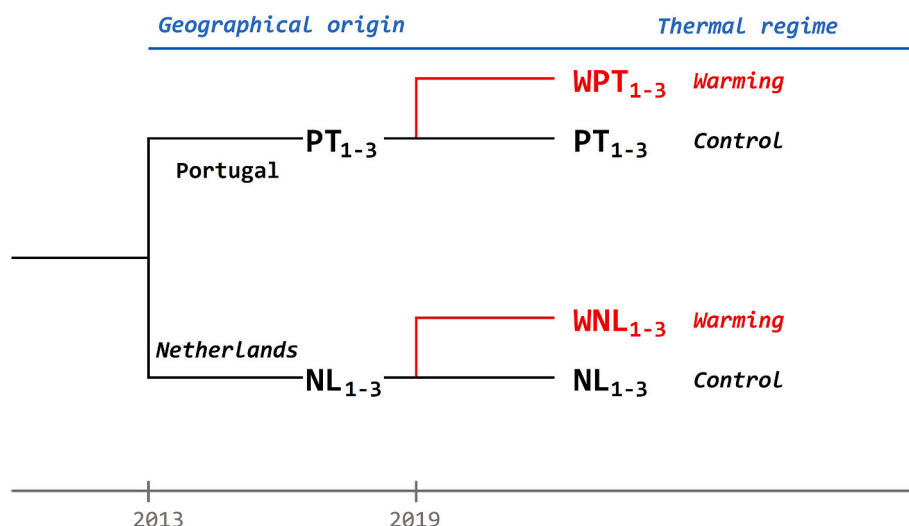

Fig. 1. Design of the thermal adaptation experiment. Populations were collected in Adraga, Portugal (PT) and Groningen, Netherlands (NL) in 2013. By generation 70 in the lab (in January 2019), from each NL and PT replicate population (NL1-3 and PT1-3, then on designated as Ancestral population - AP) a new replicate population was derived and moved to the warming environment originating the populations WNL1-3 and PT1-3 (e.g. WPT1 and PT1 derive from the ancestral population PT1 etc) that constitute the warming thermal selection regime.

laying period), that emerged during a 10-day period starting from the first day of emergence (maintained in the same test environment as the assayed parental generation). The ninth day of adult life was chosen as it is within the interval of the age of individuals that contribute to the next generation in our population maintenance system (~6–10 days of age). Maternal environmental effects were minimized by maintaining all assayed populations for one full generation in the control environment prior to the assay.

2.3. Statistical methods

Data from each mating pair was used as raw data in the analyses. These included Linear mixed effects models, with "sum to zero" contrasts for each factor. We applied Generalized linear mixed-effects models (GLMM) on the whole dataset and tested with different distributions - poisson, quasipoisson, and negative binomial. To account for zeros in our dataset, zero-inflated models were also tested. The best overall model, based on the lowest values of Akaike information criterion (AIC), was the one assuming a quasipoisson distribution with the inclusion of a parameter accounting for zero inflation. This distribution was used in all analyses. Significance levels were obtained by applying Type III Wald chisquare tests.

We applied two overall models to test for differences between selection regimes, test environments and the history of the populations. These varied in the random factor (for simplicity interactions with random factors are not presented here).

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

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

In model (1), AP{History} is a random factor, that represents the Ancestral population (NL1-3; PT1-3) nested in the fixed factor History (PT low latitude vs NL high latitude). Thus, this term represents the level of replication of our experimental evolution study, accounting for the variation between replicate populations within each thermal regime and at the same time the pairing due to their shared ancestry (e.g. NL1 and its derived warming WNL1, nested in NL origin; etc.) – see also Fig. 1. Block is a random effect in model (2), corresponding to the set of same-numbered replicate populations assayed in the same experimental rack, with one replicate population from each regime (e.g. Block 1 involving the samples from NL1, WNL1, PT1 and WPT1, etc …). In both models the interaction between fixed factors and the random factor described above is the source of error in the significance testing of fixed factors. Y is the studied trait, reproductive success. Selection is the fixed factor corresponding to the two thermal selection regimes (Warming and Control regimes), while Environment is the fixed factor representing the test environments applied in the assays (Warming and Control test Environments). All other terms in the model represent the interactions between the fixed factors. Model (1) – ancestral population as random factor - was chosen based on lower AIC values. Considering the significant Selection $\times$ Environment interaction obtained (see results section), additional models were applied to analyse data from each test environment separately.

All analyses and figures were done in R v4.3.3, with glmmTMB, car and ggplot2 packages (Brooks et al., 2017; Fox and Weisberg, 2019; Wickham, 2016). Effect sizes for the differences between selection regimes in each environment were obtained by computing Cohen's d estimates (https://www.socscistatistics.com/effectsize/default3.aspx).

3. Results

To analyse the long-term adaptation in our warming populations we

assessed the reproductive success of our populations of distinct history in two test environments (warming and control) by generation 52 (see Table 1 and Fig. 2). We observed a significant Selection $\times$ Environment interaction (see Table 1), with the warming populations showing a significantly better performance than controls in the warming environment but less so in the control environment (see Fig. 2 and Supplementary Fig. S3). This interaction led us to test the performance of populations in each environment separately, an analyses that corroborated our previous finding: a significant differentiation between selection regimes was observed in the warming environment ($\chi 2 = 4.458$, d. f. $= 1$, p < 0.05) but not in the control one ($\chi 2 = 0.333$, d.f. $= 1$, p > 0.05) - see Table S2. In the case of the low latitude populations, there

Table 1

Analysis of adaptation to warming across populations of distinct history and environments.

Model parameters	Df	χ2	p-value
Intercept	**1**	**13703.4**	**<0.001**
History	1	1.696	0.193
Selection	**1**	**10.410**	**0.001**
Environment	**1**	**128.03**	**<0.001**
History x Selection	1	0.747	0.388
History x Environment	1	2.636	0.104
Selection x Environment	**1**	**7.249**	**0.007**
History x Selection x Environment	1	0.234	0.628

Note: A GLMM model (with the quasipoisson familiy) was applied with reproductive success as dependent variable and History, Selection and Environment as explanatory variables (see details in the Material and Methods). Total sample size: 468 observations. "Df": the degrees of freedom. Chisquare tests (X^2) are presented. Statistically significant terms are represented in bold (p < 0.05).
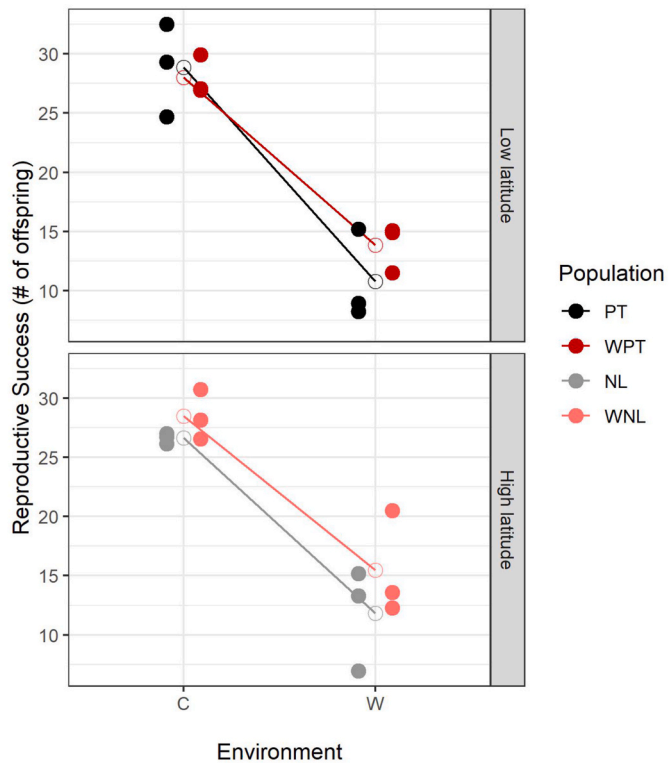

Fig. 2. Patterns of local adaptation for low latitude and high latitude populations at generation 52. Note: Full dots represent average replicate population values, while white dots represent the averages for each combination of thermal selection regime and history (i.e. NL, WNL, PT and WPT populations). Assayed individuals were subjected to Environment C - the "Control" test environment (constant 18 °C) or the Environment W - "Warming" test environment (a daily fluctuation regime with a mean temperature of 21.4 °C and a fluctuation between 13.5 °C to 29.4 °C).

was even a tendency for reversal in the sign of differences between selection regimes across the two test environments (see Fig. 2). On the other hand, we did not find evidence supporting differences between populations of distinct history (*History* factor, see Table 1) nor any indication of significant interactions of History with other factors.

We estimated effect sizes – through Cohen's d estimates – to assess the magnitude of the differences between selection regimes in each environment (see Table S3). These corroborated the statistical analyses presented above. Effect size estimates pointed to an increase in reproductive success of the warming populations relative to their controls in both test environments: a "large" differentiation in the warming environment (Cohen's d of 1.08, Warming > Control regime), whereas a "small" differentiation was obtained in the control environment (Cohen's d of 0.33, Warming > Control regime) - see Table S3.

4. Discussion

The study of the long-term response of populations allows for a deeper understanding of evolutionary processes and patterns (Stroud and Ratcliff, 2025). Here we provide evidence for long-term adaptation to increased temperatures in *Drosophila subobscura* populations evolving in a warming environment. We found that our warming populations showed a considerable improvement in reproductive success relative to the ancestral populations in the warming environment (around 30 %, see also below). Our findings show that there was sufficient standing genetic variation (SGV) for an adaptive response in our populations after more than 50 generations of thermal evolution. This slow evolutionary response occurred despite sporadic bottlenecks due to high mortality during development in particular generations. It is also interesting to note that our populations had been for 70 generations adapting to the ancestral environmental conditions previous to the imposition of the novel, warming regime. Both bottlenecks and the previous long-term lab maintenance may have reduced, but not suppressed, standing genetic variation (SGV) in our populations. Despite the well-known impact of new mutations on adaptation, we do not expect such an impact in our observed response, considering the timeframe of the study and the size of our populations (as generally occurs in Drosophila experimental evolution studies, e.g. Burke, 2012). Our finding agrees with the expectation of available genetic variability in our populations considering the genomic data from other populations studied in our lab, maintained under similar conditions and within a comparable time interval (Seabra et al., 2018).

We have previously shown that adaptation to warming conditions was detected after 39 generations of thermal evolution but only in the low latitude populations (see Santos et al., 2023c and below). The fact that an overall adaptive response across our latitudinal populations was only observed by generation 52 points to a slow adaptive response, suggesting that genetic variability is present in our populations – see discussion above - but is probably not high. Apart from the expected low additive genetic variance in traits closely related to fitness (Houle, 1992; Mousseau and Roff, 1987), the lower effective population sizes in some generations and the prior laboratory adaptation may have also contributed to some erosion of the initial standing genetic variation, as mentioned above.

The increased reproductive performance of the warming populations at higher temperatures can result from the evolution of improved physiological tolerance in pathways associated with oogenesis and/or spermatogenesis (e.g. Vasudeva et al., 2019; Wang and Gunderson, 2022). Direct studies on specific female and male reproductive traits would be needed to assess this hypothesis. In addition, it would be relevant to distinguish how males and females evolved in the warming environment, particularly considering that males may have stronger limitations due to sterility at high temperature (David et al., 2005; Iossa, 2019; Sales et al., 2021).

It is important to note that this general adaptive response across populations was observed more than 25 generations after the thermal

increase per generation in the warming cycle was halted (by generation 24). This finding agrees with those of another evolution experiment addressing adaptation to incremental warming in seed beetles (Hallsson and Björklund, 2012; Rogell et al., 2014). These results are in accordance with the expectation that evolution lags behind environmental change, with such decoupling becoming larger with increasing rates of environmental change. Such high rates of environmental change are challenging for Evolutionary rescue (Gomulkiewicz and Holt, 1995), which is further hindered by expected reductions in population size and in levels of genetic variation (Bell, 2017; Bell and Gonzalez, 2011; Burger and Lynch, 1995). In fact, the rate of environmental change has been described as a critical factor for evolutionary rescue in experimental studies in bacteria (Liukkonen et al., 2021) with some genotypes not being evolutionarily accessible under rapid environmental change (Lindsey et al., 2013). Consistent with this, studies in ectotherms addressing short term experimental evolution under continuously, fast increasing temperatures have shown a lack of adaptive response (Kinzner et al., 2019; Schou et al., 2014; van Heerwaarden and Sgrò, 2021). We here show that adaptive responses can arise after prolonged evolution in a thermal environment with daily fluctuation and wide thermal extremes but likely not during periods of sustained, fast environmental perturbation. Consequently, this raises concerns about the ability of populations to respond to sudden thermal shifts and to high rates of environmental change.

We found that adaptation to the warming conditions produced a clear response in the warming environment, with no measurable differentiation between warming and control populations in the ancestral environment. This finding does not support the occurrence of costs of adaptation in the long term, which would involve a worse performance of the warming populations in the ancestral environment. We had previously reported slight evidence for costs of adaptation in a shorter period of evolution (see Santos et al., 2023c). In general, these findings are not surprising considering other studies in the literature (see Bono et al., 2017; Hereford, 2009 for a review). It might be the case that evolution under daily fluctuating environments, as we use here, is less likely to allow for measurable costs of adaptation than constant environments since in the former case organisms are exposed to different environmental conditions and consequently selection may act to adjust/increase performance in a wider range of environments (Bono et al., 2017). An absence of adaptation costs suggests a possible advantage in future climatic scenarios where warmer seasons are projected to be warmer (IPCC, 2023) but, at the same time, populations will need to cope with lower temperatures during colder seasons. However, this is most likely not enough to ensure population persistence, given the pace of the evolutionary response of the populations under study.

Even though populations of distinct history have a quite comparable adaptive response in the warming environment by generation 52 (higher latitude populations showed a 31 % increase relative to controls, quite similar to the 28 % increase of the lower latitude populations), differences in the speed of evolution are clear when considering our previous data. In fact, a response to thermal selection in the high latitude populations was only evident in this longer-term study (cf. Santos et al., 2023c). It is an open question whether the different evolutionary dynamics and patterns observed between populations (still) reflect the geographical differentiation of this species in nature, given they had been already evolving in the control environment for around 70 generations by the time the warming regime was imposed. Regardless, it is interesting to note that these populations still maintained clear genomic and karyotypic differentiation after the stronger phase of laboratory adaptation (~30 generations, see Barreira, 2023; Simões et al., 2017). More robust studies of the impact of historical backgrounds would need additional sampling of recently founded lab populations from varying latitudes.

In conclusion, we found that long-term adaptation to higher temperatures can occur *in D. subobscura* but the pace of such response is slow and likely dependent on low rates of environmental change. This finding

supports evidence that ectotherms may have limited capability to respond evolutionarily to temperature shifts (Kellermann and van Heerwaarden, 2019) and struggle to show an adaptive response to a fast-paced global warming. As expected, we observed more robust changes in the warming than in the ancestral environment, with no evidence for costs of adaptation. We conclude that the long-term evolutionary response differs between populations of distinct geographical origin, highlighting the need to scrutinize several populations to account for variation within a species. This study reinforces the relevance of long-term evolution experiments - assessing performance under both ancestral and novel environments - to allow for more robust assessments of species' responses to sustained climate change.

CRediT authorship contribution statement

Marta A. Antunes: Writing – review & editing, Methodology, Investigation. **Afonso Grandela:** Writing – review & editing, Investigation. **Margarida Matos:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Pedro Simões:** Writing – original draft, Methodology, Investigation, Funding acquisition, Conceptualization.

Data accessibility statement

Raw data and scripts are available online at https://doi.org/10.6084/m9.figshare.25431880.v4.

Ethical issues

Studies in Drosophila raise no ethical problems/no restrictions according to animal protection laws.

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Declaration of competing interest

The authors declare that they do not have any conflicts of interest in relation to the content of the article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtherbio.2025.104118.

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