Title: Long-term dynamics of adaptation to a warming environment is dependent on historical background

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Abstract

Climate change is leading to biodiversity decline at an unprecedented pace. Evolutionary responses may be crucial for organisms' ability to cope with prolonged effects of climate change. It is thus fundamental to understand the dynamics of adaptation to warming environments in order to predict and mitigate the impacts of climate change on biodiversity. In particular, addressing how reproductive success evolves in deteriorating environments is extremely relevant, as this trait is more likely constrained at lower temperatures than upper physiological thermal limits. Experimental evolution udner a warming environment can illucidate 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 adaptive response of Drosophila subobscura populations evolving under a warming environemnt, analysing the reproductive success after 39 and 52 generations of thermal evolution. We found that long-term adaptation to higher temperatures can occur but the pace of such response is slow and likely dependent on low rates of environmental change. Furthermore, we observed that the magnitude of the response is relatively mild. In addition, the evolutionary dynamics differs between populations of distinct geographical origin, with the higher latitude populations only showing an adaptive response to the warming environment in a more advanced generation. This study reinforces the need for long-term evolution experiments to fully reveal the potential for thermal response. It also highlight that the scrutiny of several populations in this context is needed for a measure of variation within a species. Accounting for these sources of variation - both temporal and spatial - will allow for more robust assessments of climate change responses.

Introduction

Climate change is promoting biodiversity decline at an unprecedented pace and is likely to continue to do so in the future (IPCC 2023). Evolutionary responses may play an important role in enhancing organisms' ability to cope with prolonged effects of climate change (Urban et al. 2016). It is crucial to understand the dynamics of adaptation to warming environments to predict impacts of climate change. In particular, addressing how reproductive success evolves in deteriorating environments is extremely relevant, as it has been shown that this trait is more likely constrained at lower temperatures than physiological impairment(Walsh et al. 2019a,b; Parratt et al. 2021; van Heerwaarden and Sgrò 2021). In fact, fertility patterns have been shown to track species' geographical distribution in nature, highlighting its role as a predictor of biodiversity changes due to climate change (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). When genetic variation for fitness-related traits is low and/or evolutionary constraints between traits occur, the speed of the adaptive response will be particularly limited. In that scenario, adaptive evolution may be only evident (if present at all) after prolonged imposition of the new environmental challenges and/or when the rate of environment change declines or ceases (Hoffmann and Sgró 2011; Lindsey et al. 2013; Bell 2017).

Experimental evolution is a powerful tool to deepen our understanding about the tempo and mode of thermal evolution, as it allows to follow the real-time evolution of populations under relevant thermal settings (Kawecki et al. 2012; Rogell et al. 2014; Kellermann and van Heerwaarden 2019; Santos et al. 2021b; van Heerwaarden and Sgrò

2021). Furthermore, it allows to directly test the role of specific environmental factors, e.g. such as temperature, while confounding environmental effects are minimized relative to field studies. Some studies in ectotherms (mostly Drosophila) have focused on evolutionary responses of reproductive traits to experimental warming but most have covered a limited temporal range – around 20 generations or less (Schou et al. 2014; Kinzner et al. 2019; van Heerwaarden and Sgrò 2021).

In general, these studies have found limited evolutionary response to warming in reproductive traits. In an exceptionally longer-term experimental study which spanned 41 generations, Rogell et al. (2014) found an increased reproductive performance in response to a fluctuating and increasingly warmer environment, with populations of seed beetle *Callosobruchus maculatus* evolving under higher temperatures presenting increased offspring production relative to their controls. Importantly, this effect on fecundity was only observed after the incremental setting (of 0.3ºC per generation) was halted upon 18 generations of selection (Rogell et al. 2014) and not immediately after the increment (Hallsson and Björklund 2012). These experiments suggest that the tempo of adaptive change to new thermal conditions is likely low – possibly due to limited genetic variation for reproductive traits - and that a rapid rate of environmental change poses serious limits to that response. In addition, reductions in genetic variation might result from prolonged evolution under stressful conditions due to the continuous effects of genetic drift when effective population size is rather low and/or there are population crashes causing bottlenecks. Nevertheless, it is possible - though not very likely - that a reshaping of the genetic architecture following such bottlenecks contributes to the release of genetic variation and increase of evolutionary potential (Bryant and Meffert 1993). Considering these different sources of genetic variation, long-term responses to incremental temperatures may be highly variable between populations and species. One caveat of the

thermal experimental evolution studies reported above is that they neglect effects of variation at the inter-population level - namely by studying different geographical populations of a species - and how it affects the adaptive response.

Importantly, these real-time evolution studies of adaptation have also the potential to test for costs of adaptation to the warming conditions, that is whether adaptation to a specific thermal environment promotes a reduced performance in the ancestral (thermal) environment, for instance, due to the expression of genes with antagonistic pleiotropy (e.g. (Kawecki and Ebert 2004). However, detection of costs of adaptation in general has been evasive possibly because these are seldom strong (Hereford 2009).

Drosophila subobscura is a species that shows evolutionary responses to climatic factors with repeatable clines of body size (Huey et al. 2000; Gilchrist et al. 2004) and chromosomal inversions (Prevosti et al. 1988) that track global warming (Balanyá et al. 2006; Rezende et al. 2010). As expected, this species has also been found to respond plastically to thermal challenges (Fragata et al. 2016; Simões et al. 2020; Santos et al. 2021a). Our team has been studying how populations of this species evolve under an increasingly warmer environment (Santos et al. 2021c). We have been focusing on populations from northern (high latitude) and southern (low latitude) European locations to address the relevance of different historical backgrounds during thermal evolution (Santos et al. 2021c). The thermal setting in the warming regime was incremental (0.2ºC per generation) until generation 24 and then the thermal environment was kept constant across generations until the last assay, with a daily fluctuating regime spanning both high and low temperatures (with daily peaks of respectively 11ºC and 5ºC above and below 18ºC, the control conditions). We previously found that the evolutionary response of populations to warming conditions was slow and specific to the history of the populations. In fact, thermal adaptation was only observed in the populations of low latitude after 39

generations, but not after 22 generations in a warming environment (Santos et al. 2023b). We also found minor evidence supporting costs for this thermal adaptation (Santos et al. 2023b). These findings highlight the importance of a better understanding of the evolutionary dynamics of thermal responses in different populations. It is an open question whether longer-term evolution would allow for an adaptive response also in the higher latitude populations.

We here focus on the long-term adaptive response of our warming-evolved populations by analysing their reproductive success after more than 50 generations of thermal evolution. With this analysis we also aim to test the repeatability of patterns relative to those observed at generation 39 (Santos et al. 2023b). Coupling data from two long-term assays (generations 39 and 52) we expect to find a more robust adaptive response. Specifically, we expect that the reduction in the rate of environmental change - with the warming regime being kept constant for more than 25 generations by the time the last experiment was conducted - will have likely enabled a general adaptive response in both high and low latitude populations. Specifically, we expect to observe a previously undetected selective response in the high latitude populations, provided sufficient genetic variation is present. It is possible that evolutionary constraints such as trade-offs with other traits and costs of adaptation emerge during this longer-term response, contributing to a mild magnitude of evolutionary change.

Material and methods

Population maintenance and thermal selection regimes

The experimental populations used in this study were obtained from two collections of natural populations of *Drosophila subobscura,* done in August/September 2013 in

Adraga, Portugal (38º48′ N) and Groningen, The Netherlands (53º13′ N). 213 founder females were obtained in Adraga and 170 in Groningen (Simões et al. 2017). These collections gave rise to the PT and NL laboratorial populations. These populations were maintained in 30 cm^3 glass vials (in general 24 vials for both the adult and developmental stages) with discrete generations of 28 days, 12L:12D photoperiod with constant 18ºC temperature, controlled densities in eggs (70 eggs per vial) and adults (40 adults per vial), and reproduction close to peak fecundity (seven to ten days-old flies). By generation four, these populations were three-fold replicated, leading to PT1-3 and NL1-3 populations. After 70 generations, a warming regime was created from each population (Warming, WNL_{1-3} and WPT_{1-3} populations). This regime had a daily fluctuation cycle that ranged from 15ºC and 21ºC and increased its daily mean temperature (0.18ºC) and amplitude (0.54ºC, with a lower minimal and higher maximal daily temperature) every generation. All experimental populations were subjected to the maintenance protocol described above, only differing in the thermal regime imposed. Evolution in 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. PT and NL populations, representing the ancestral state of the WPT and WNL populations, were used as control populations in the experiment.

By generation 22 the peak temperature in the warming regime reached 30.2ºC. This originated a significant crash in adult census sizes due to high juvenile mortality for all warming populations in generations 22 and 24 (average sizes of 200 and 250 respectively). As a result of this, we reversed the warming cycle to the cycle of generation 20 (Santos et al. 2023b). This cycle, with a mean temperature of 21.4ºC and a fluctuation between 13.5ºC to 29.4ºC, was then maintained till the end of the study and was the warming environment applied in phenotypic assays (see details below). Apart from the

population crashes referred above that led to the halt in the thermal cycle (with census sizes below 250 individuals), very low population sensus sizes were also observed by generation 44 that motivated a two-generation transfer of the warming populations to control conditions to allow for population recovery. Apart from these previously referred generations, populations sizes were generally high throughout the study (between 600-1000 individuals) – see Table S1 with sample sizes across generations.

Experimental assays

This study includes data from the reproductive success of warming populations analysed at generations 39 and 52 of thermal adaptation. Data from the assay performed at generation 39 was already published elsewhere (Santos et al. 2023b). In both assays, an orthogonal design was applied with Warming and Control populations being tested in each (Warming and Control) environment.

The assay of generation 39 involved twenty pairs of flies per replicate population and environment. In total, 480 pairs of flies – placed in individual glass vials - were studied in this assay (20 pairs × 3 replicate populations × 2 selection regimes × 2 historically differentiated populations × 2 assay environments) – see also (Santos et al. 2023b). At generation 52, an additional assay involved sixteen pairs of females and males per replicate population and environment, in a total of 384 pairs of flies (16 pairs × 3 replicate populations × 2 selection regimes × 2 historically differentiated populations × 2 assay environments). In both assays, 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-hour laying period), that emerged during a 10-day period starting from the first day of emergence. The ninth day of adult life was chosen as it is within the interval of the age of

individuals contributing to the next generation during our population maintenance system (~ 6 to 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.

Statistical methods

Individual data from each mating pair was used as raw data in the analyses. These included Linear mixed effects models, defining a "sum to zero" contrast option for each factor. Generalized linear mixed-effects models (GLMM) were applied on the whole dataset and tested with different distributions - poisson, quasipoisson, and negative binomial. The best overall model, based on the lowest values of Akaike information criterion (AIC), was the one assuming a quasipoisson distribution 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 the data of assays from generation 39 and 52, that varied in the random factor defined (interactions with random factors are not presented but were included):

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

$$(2)\ Y = \mu + History + Selection + Env + Generation + Block + History \times Selection + History \times Env + History \times Generation + Selection \times Env + Selection \times$$

$$Generation + Env \times Generation + History \times Selection \times Env + History \times Selection \times Generation + History \times Env \times Generation + Selection \times Env \times Generation + History \times Selection \times Env \times Generation + \varepsilon$$

In model (1), *AP{History}* is a random factor, that represents the Ancestral population nested in the fixed factor *History* (PT low latitude vs NL high latitude) (e.g. NL1 is the ancestral population of NL1 and WNL1, nested in NL origin, etc.). *Block* is a random effect in model (2), being the set of same-numbered replicate populations assayed in the same pseudo-randomized experimental rack. Y is the trait under study, reproductive success. *Selection* is a fixed factor corresponding to the two selection regimes (Warming and Control), *Env* is the fixed factor Environment, with two categories (Warming and Control Environments), and *Generation* is the fixed factor corresponding to the two generations assayed (39 and 52). All other terms in the model represent the interactions between the fixed factors. Model (2) – block as random factor - was chosen based on lower AIC values. Additional models were applied to each latitudinal population separately and to each generation separately.

All analyses and figures were done in R v4.3.3, with glmmTMB, car and ggplot2 packages (Wickham 2016; Brooks et al. 2017; Fox and Weisberg 2019).

Results

To assess long-term adaptation in our warming populations we performed an overall analysis including both assays, done at generations 39 and 52. Significant effects were observed for the terms Environment and Selection (Table S2). Differences in selection corresponded to a general better performance of the warming populations relative to the controls (with the exception of the patterns for the low latitude populations in the control environment, see below). Differences observed between environments corresponded to a general decline when populations where assayed in the warming environment (see Figure 1). Importantly, a significant *Selection × Environment* interaction was observed (Table S2), indicating that differences between selection regimes changed across environments, with the warming populations showing a better performance than controls in that environment but less so in the control environment. This was particularly evident in the case of the low latitude populations where the sign of differences between populations reverses between environments (see Figure 1). Importantly, although we found general differences between generations (significant factor *Generation*), we did not find any significant interactions of other fixed factors with generation, indicating that the differences between populations of different selective regimes across environments that were mentioned above were maintained across generations (e.g. non significant *Selection × Environment × Generation* interaction see Table S2).

Analyses were performed to separately assess the performance of populations with different historical origin. In both cases, as expected there was a significant Environment effect (see Table 1). For the low latitude populations (but not for the high latitude ones) we also observed a significant effect of generation as well as a significant interaction between selection and environment (see Table 1 and Figure 1). As referred above, this

interaction resulted from a better performance of the warming populations of southern origin relative to their controls in the warming environment, while the reverse pattern occurred in the control environment, particularly at generation 39 (see Figure 1). As for the high latitude populations, we also found significant differences between generations (significant factor *Generation*) and a marginally significant effect of selection and of the interactions between selection and generation and between environment and generation factors (*Selection x Generation* and *Environment x Generation*, see Table 1). The interaction between selection and environment was not significant for these populations.

Given the marginal interaction between selection and generation in the high latitude populations we further analysed data from each generation separately to search for possible discrepant patterns between generations. Besides the expected effect of environment, we did not find any effect of selection or interaction between selection and the environment by generation 39 in the high latitude populations (see Table S3). On the other hand, by generation 52 a significant effect of selection and of the interaction between selection and environment was observed (see Table S3). This interaction corresponds to a higher difference in performance between warming and control populations in the warming environment, although performance was consistently higher in the warming populations in both environments (see Figure 1). Applying a similar analysis to the low latitude populations, selection was not significant in either generation, while the interaction between selection and environment was statistically significant at generation 39 but not at generation 52 (see Table S3).

Discussion

Here we provide evidence for long-term adaptation to the warming conditions in *Drosophila subobscura* populations evolving in a warming environment. In fact, we found that those populations showed an increased reproductive success than the controls when tested in their own (warming) environment while differences were reduced in the ancestral (control) environment. Importantly, the dynamics of the response was different between populations with distinct geographical origins, being faster in the southern – lowe latitude - ones (see below). Our findings point to the existence of sufficient standing genetic variation to allow for an adaptive response in our populations despite the occurrence of sporadic bottlenecks associated with thermal stress during development in particular generations. It might also be the case that some of these episodes of high stress have opened the opportunity for interactions between drift and selection (Cohan 1986; Cohan and Hoffmann 1989) and/or reshuffling of the genetic architecture with increased additive genetic variation following bottlenecks (Bryant and Meffert 1993) that may allow for further evolutionary potential and subsequent responses. In any case, the slow response suggests that levels of genetic variability are not high in our populations. Apart from the expected low heritability in traits closely related to fitness (Mousseau and Roff 1987), reduced genetic variation may also result from lower effective population sizes in some generations as a consequence of repeated episodes of high selection during development. In addition, high environmental effects on the phenotype also result in low heritabilities.

It is important to note that the adaptive responses we observe were only obtained after the thermal increase per generation in the warming cycle was halted by generation 24 (Santos et al. 2021c, 2023a,b). This finding is in agreement with those of another evolution experiment addressing adaptation to incremental warming in seed beetles

(Hallsson and Björklund 2012; Rogell et al. 2014). These results reflect the importance of the rate of environmental change in allowing for evolutionary rescue to occur (Bell 2017). This has been clearly demonstrated in experimental studies in bacteria, with more adaptive responses observed under slower environmental change (Liukkonen et al. 2021) and particular genotypes not being evolutionarily accessible under rapid environmental change (Lindsey et al. 2013). Experimental studies in ectotherms addressing short term evolution under continuously incremental temperatures have shown a lack of adaptive response (Schou et al. 2014; Kinzner et al. 2019; van Heerwaarden and Sgrò 2021). We here show that adaptive responses can arise after prolonged evolution in a thermally fluctuating environment but probably not when populations face a fast pace of environmental perturbation. Consequently, this raises concerns on the ability of populations to respond to sudden thermal shifts and in particular to sustained high rates of environmental change.

Our findings reveal differences in the evolutionary dynamics of populations of different geographical origin. In fact, the high latitude populations showed a more tardy response to thermal selection, being only evident after more than 50 generations of thermal evolution, contrary to the response already obtained for the low latitude populations by generation 39 (Santos et al. 2023b). Apart from the tempo of the response, it is also important to note that the patterns observed in each geographical population were also somewhat different, with stronger evidence suggesting that the relative performance of thermal selection regimes varied between assay environments in the low latitude populations but less so in the high latitude ones. Specifically, part of this variation between geographical populations is associated with performance in the control (ancestral) environment where the low latitude populations show some indication of performing below the controls particularly at generation 39, which was not the case for

the high latitude populations. This pattern is suggestive of costs of adaptation in the low latitude populations (see (Santos et al. 2023b). Nevertheless, by generation 52 such differences in the control environment are no longer conspicuous, suggesting that such costs of adaptation were not particularly relevant. This is not a surprising finding considering other literature (see (Hereford 2009; Bono et al. 2017) for a review). It might be the case that evolution under fluctuating (heterogeneous) environments, as we use here, is less likely to allow for measurable costs of adaptation than homogeneous environments since in the latter case organisms are not exposed to different environmental conditions and consequently selection will not act to adjust/increase performance in a wider range of environments (Bono et al. 2017). Furthermore, our finding of an adaptive response with no clear costs involved suggests a possible advantage in future climatic scenarios where warmer seasons are projected to be warmer (IPCC 2023), while populations will need to keep their ability 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.

In summary, 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. Thus, this finding supports the evidence that ectotherms may have limited capability to respond evolutionarily to temperature shifts (Kellermann and van Heerwaarden 2019) and may struggle to show an adaptive response to a fast-paced global warming. In addition, we observed that such adaptive response is not particularly strong. The long-term evolutionary dynamics differs between populations of distinct geographical origin, with the higher latitude populations only showing an adaptive response to the warming environment in a more advanced generation (*cf.* Santos et al. 2023b). This study reinforces the need for long-term evolution experiments and the

scrutiny of several populations in the analysis to account for variation within a species. Both these components will allow for more robust assessments of species responses to sustained climate change.

Funding

This study is financed by Portuguese National Funds through 'Fundação para a Ciência e a Tecnologia' (FCT) within the projects PTDC/BIA-EVL/28298/2017 and cE3c Unit FCT funding project UIDB/00329/2020. PS is funded by national funds (OE), through FCT, in the scope of the framework contract foreseen in the numbers 4, 5 and 6 of the article 23rd, of the Decree-Law 57/2016, of August 29, changed by Law 57/2017, of July 19. MMA was funded through an FCT PhD fellowship (2020.09172.BD).

Data Availability statement

Raw data and scripts available at https://doi.org/10.6084/m9.figshare.25431880.v1

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Tables

Table 1 - Patterns of local adaptation for generations 39 and 52 in high latitude (a) and low latitude (b) populations

a) High latitude populations

Model parameters	$\chi 2$
Selection	3.468 m.s.
Environment	79.449 ***
Generation	5.214 *
Selection:Environment	2.289 n.s.
Selection:Generation	2.943 m.s.
Environment:Generation	3.362 m.s.
Selection:Environment:Generation	1.414 n.s.

b) Low latitude populations

Model parameters	$\chi 2$
Selection	4.218 *
Environment	172.83 ***
Generation	19.070 ***
Selection:Environment	10.650 **
Selection:Generation	0.230 n.s.
Environment:Generation	0.003 n.s.
Selection:Environment:Generation	0.822 n.s.

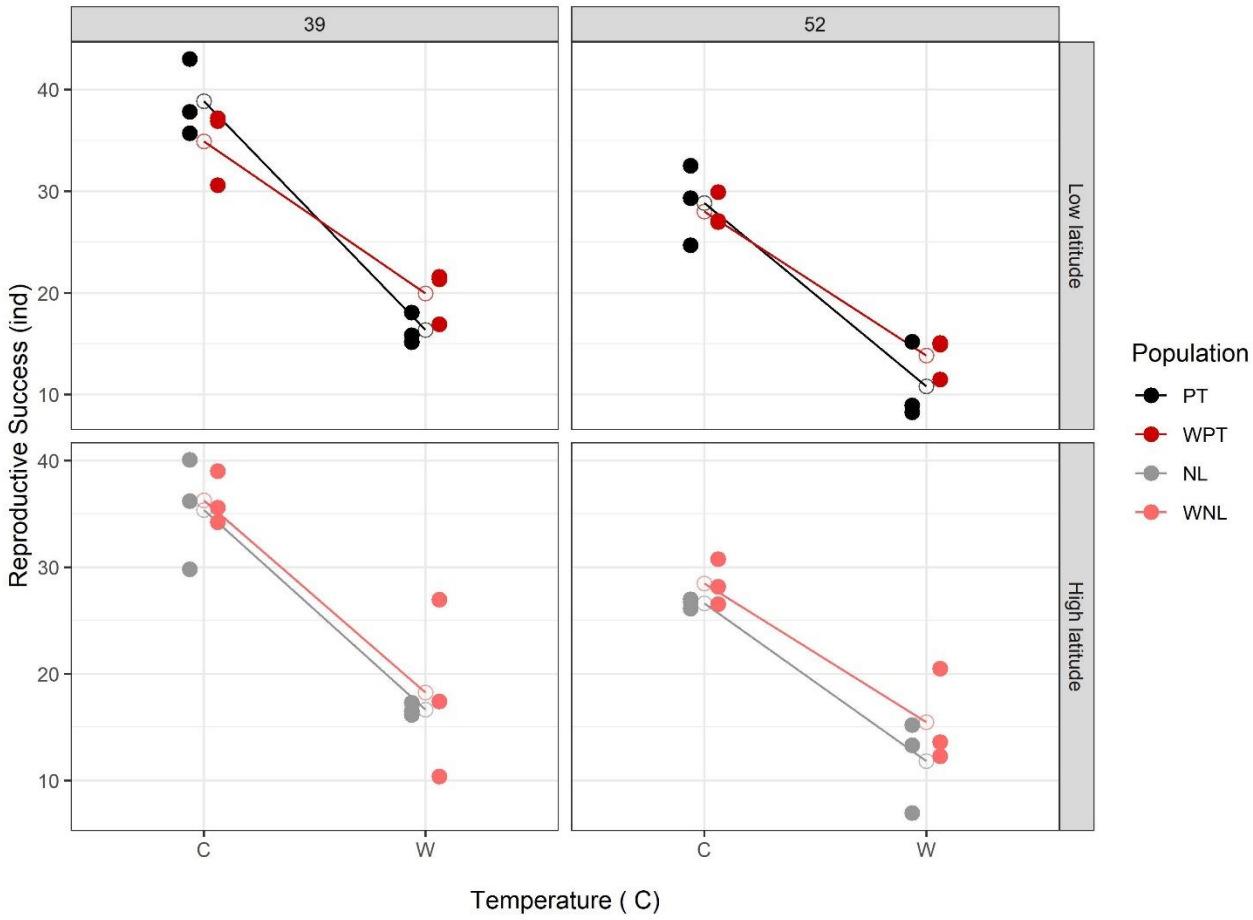
Note: significance levels: p> 0.05 n.s.; 0.05>p>0.01*; 0.01>p>0.001**; p<0.001 ***; d.f.=1 for all analyses

Figure Legends

Figure 1 - Patterns of local adaptation for low latitude and high latitude populations at generations 39 and 52.

Note: Low latitude populations are represented in the first line and high latitude in the second line; Generation 39 is represented in the first column and generation 52 in the second column.

Figure 1


39
52
Low latitude
High latitude
Reproductive Success (ind)
40
30
20
10
C
W
Temperature (C)
Population
PT
WPT
NL
WNL