Sex and population differences underlie variation in reproductive success in a warming environment

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Abstract

Current rising temperatures are threatening biodiversity. It is therefore crucial to understand how climate change impacts on male and female fertility and whether evolutionary responses can help in coping with heat stress. We use experimental evolution to study male and female fertility during real-time evolution of two historically differentiated populations of *Drosophila subobscura* under different thermal selection regimes for 23 generations. We aim to (1) tease apart sex-specific differences in fertility after exposure to warming conditions during development, (2) test whether thermal selection can enhance fertility under thermal stress, and (3) address the role of historically distinct genetic backgrounds. Contrary to expectations, heat stress during development had a higher negative impact on female fertility than on male fertility. We did not find clear evidence for enhanced fertility in male or females evolving under warming conditions. Population history had a clear impact on fertility response under thermal stress, particularly in males with those from lower latitude presenting better performance than their higher latitude counterparts. We show that the impact of thermal stress on fertility varies between traits, sexes and genetic backgrounds. Incorporating these several levels of variation is crucial for a deeper understanding of how fertility evolves under climate change.

Keywords: Thermal adaptation; Experimental evolution; Drosophila; Fertility; Climate change; Sex differentiation

1. Introduction

The rising temperatures, induced mostly by anthropogenic action, are one of the most alarming issues of the last decades. Recent climate models predict an increase in average surface temperature ranging from 0.2º C to 0.6º C per decade in this century as well as increased thermal amplitude on seasonal and daily timescales (IPCC 2022). These changes will affect various species across different taxa, given the fact that temperature has enormous repercussions on many traits of all organisms (Buckley and Huey 2016; Walsh et al. 2019). Ectotherms, in particular insects, could be amongst the organisms most affected by rising temperatures given their inability to regulate internal temperature (Kingsolver et al. 2013). The thermal impact on organism's performance may vary depending on the magnitude of temperature change (Vasudeva et al. 2014), the duration of exposure (Jørgensen et al. 2006) and the life stage where exposure occurs (Sales et al. 2021; Walsh et al. 2021).

To avoid negative repercussions or in more severe cases extinction, populations may respond locally to changes in temperature, either through adaptation or plasticity, or even resorting to both mechanisms (Kingsolver et al. 2013; Kellermann and van Heerwaarden 2019), despite evolutionary constraints to such heat stress responses (Araújo et al. 2013; MacLean et al. 2019). Developmental thermal plasticity can have an important role in shaping thermal response for key traits, namely by enabling increased adult performance after previous juvenile exposure to stressful conditions (Beaman et al. 2016; Sgrò et al. 2016; Rezende et al. 2020). However, several recent studies suggest that high temperatures during development can have negative carry-over effects on adult traits (Slotsbo et al. 2016; Klockmann et al. 2017; Sales et al. 2018; Santos et al. 2021a; Rodrigues et al. 2022). Considering the increasing occurrence of prolonged heat waves, further understanding of the impact of thermal stress during the developmental stage and the evolutionary response to this challenging selective pressure is needed.

To predict future population persistence, the impact of climate change should be addressed in a variety of key traits focusing not only on survival but also on fertility, i.e. the ability to generate offspring (Walsh et al. 2019). Particularly in ectotherms, negative impacts on fertility have been found following exposure to high developmental temperatures (Klockmann et al. 2017; Porcelli et al. 2017; Sales et al. 2018; Santos et al. 2021a). Furthermore, fertility loss has been observed when adult individuals are exposed to sub-lethal temperatures (Sales et al. 2018; Parratt et al. 2021; Vasudeva et al. 2021).

It is also critical to understand whether sex-specific variation shapes responses of fertility to thermal stress. Studies in this field support the hypothesis that males are more affected by the rising temperatures than females (David et al. 2005; Sales et al. 2018; Iossa 2019; Zwoinska et al. 2020). A lower energetic investment in sperm cells relative to oocytes as well as the fact that, in many insects, spermatogenesis starts earlier than oogenesis during development (Ashburner and Wright 1980) might in part explain these differences. At high temperatures during development, some males become sterile, while other males (sub-fertile) are likely to have lower sperm supply (Nguyen et al. 2013; Zwoinska et al. 2020). If females are less sensitive than males to heat-induced sterility, they could promote population persistence even at high temperatures. This is conditioned by the populations' mating system and by their level of dispersal (Iossa 2019). Thus, male sterility should be particularly costly for monandrous populations, or for populations with little ability to disperse. Recent studies in *Drosophila* indicate that male fertility limits are better predictors of species' distribution and persistence than the upper critical thermal limit (CT_{max}) of a species, which normally is relatively higher than the thermal fertility limit (Parratt et al. 2021; van Heerwaarden and Sgrò 2021). Despite the recent surge of studies, much is still unknown about the impact of evolution under increasingly warmer environments on fertility traits, possible differential effects on males and females (Walsh et al. 2019; Iossa 2019) and whether thermal selection responses are similar across sexes (Cally et al 2019; Gómez-Llano et al. 2021). In addition, a deeper understanding of the variation in fertility across populations within a species is needed as most studies of ectothermic thermal evolution do not consider such level of variation.

Drosophila subobscura, a native Palearctic fruit fly species (Rezende et al. 2010) is an extraordinary model organism to study the evolutionary potential for thermal response (Rezende et al. 2010; Castañeda et al. 2019; Mesas et al. 2021). *D. subobscura* exhibits evident plastic responses to temperature shifts, resulting in changes in reproductive performance (Simões et al. 2020; Santos et al. 2021a). Furthermore, this species displays chromosomal inversions that show pronounced variations in frequency that often correspond to temporal and spatial climatic trends. These latitudinal clines in chromosomal inversion frequencies seem to be responding to the impact of global warming (Rezende et al. 2010). This natural variation allows to study the impact of historically distinct backgrounds in the evolutionary responses (Fragata et al. 2014a,b; Matos et al. 2015). For these reasons, and considering its monandrous sexual system (Fisher et al. 2013), *D. subobscura* is a great species to study the impact of warmer environments on male and female fertility and address the effects of selection and history.

To assess how adaptation to a progressively warmer environment occurs, our team has been conducting a thermal experimental evolution study on *D. subobscura* populations from contrasting European latitudes - one from Portugal and one from The Netherlands (Santos et al. 2021b; 2023). After nine generations in a warming environment we did not observe relevant evolutionary changes in thermal reaction norms in any of the populations (Santos et al. 2021b). On the other hand, by generation 31, populations from higher latitude showed evolution of a plastic response to temperature (Santos et al. 2023). In these experiments, populations were not tested in the specific thermal environments in which populations evolved, which might impact on the conclusions about possible evolutionary responses to thermal selection. In addition, such studies did not analyze the differential impact of warming conditions on male and female fertility and the possible role of thermal selection in shaping potentially divergent response between sexes.

With this in mind, we here present an experimental evolution study on the evolution of male and female fertility in these two historically differentiated populations of *D. subobscura* after 23 generations under different thermal selection regimes - a warming environment and a benign, non-warming environment (see also Santos et al. 2021b; 2023). In this study we aim to: *i*) assess whether exposure to warming conditions during development affects the fertility of *D. subobscura* populations; *ii*) tease apart sex-specific differences in fertility and *iii)* determine whether temperature effects on fertility can be alleviated by long-term evolution under a warming environment. Finally, we aim to *iv*) understand if distinct historical genetic backgrounds have an impact on the thermal response. We here define fertility as the ability to generate offspring and characterize it estimating three traits: fecundity, juvenile viability and ultimately reproductive success (the composite of the two). With these goals in mind, we studied the adult performance under warming conditions of populations from different origins (lower *vs.* higher latitude) and selection regimes (warming *vs.* control), after males and /or females developed in either benign or warming conditions.

We expect that warming conditions experienced during development will negatively affect the fertility of both males and females, with previous evidence suggesting this detrimental impact should be higher in males (Iossa 2019, Zwoinska et al.

2020, van Heerwaarden and Sgrò 2021), and that populations evolving in the warming conditions for +20 generations will cope better under these stressful conditions. In addition, we predict to observe inter-population variation in fertility resulting from the distinct genetic backgrounds in populations with different history.

2. Methods

2.1 Experimental populations and thermal selection protocol

The laboratory populations (PT_{1-3} and NL_{1-3}) derived from collections from wild *Drosophila subobscura* in two locations: Adraga, Portugal (PT) and Groningen, The Netherlands (NL), as described in (Simões et al. 2017, 2020). Founder females were 213 and 170 for the Adraga and Groningen collections, respectively. Briefly, these populations were kept in discrete generations with synchronous 28-day cycle, 12L:12D photoperiod, and constant 18ºC. The flies were reared in glass vials (volume of ~30 cm^3) with controlled densities during development (70 eggs per vial) and adulthood (40 adults per vial). The egg collection for the following generation was done close to peak fecundity (seven to ten days-old flies) with per generation census sizes of 500 to 1000 individuals. Populations were three-fold replicated by generation four, giving rise to two sets of populations, NL1-3 and PT1-3. These are the Control populations in our study. Previously to the start of new thermal regimes the populations were already adapted to lab conditions (Matos et al. 2000; Simões et al. 2017). After 70 generations of lab adaptation (January 2019), the global warming selection regime was derived, originating the Warming populations, WPT_{1-3} and WNL_{1-3} (see Santos et al. 2021b), while the populations from which they derived serve as controls in the study, maintained at the standard lab conditions (e.g. constant 18ºC). The new thermal regime started under a daily temperature that fluctuated between 15ºC and 21ºC, with a mean daily temperature of 18ºC; every generation there was an increase of 0.18ºC in daily mean and 0.54ºC in daily amplitude (see Figure 1 for the thermal profiles). This thermal regime was kept unchanged from generation 20 on, *i.e.*, with a mean temperature of 21.4ºC, a lower extreme of 13.5ºC, and an upper extreme of 29.4ºC. This per generation mean thermal change is approximately equivalent to the one experienced in nature by animals with a ~10-year generation time and it fits well with the projected thermal extremes increase in mid-latitude locations (IPCC 2022). The average temperature increase led to a progressively shorter development time and reduction in the life-cycle length, that stabilized at 25 days. The adult age of reproduction in this regime was maintained as in the controls. All experimental populations were, otherwise, kept under the same conditions, as explained above.

2.2 Fertility assay

To address the impact of warming conditions during development on fertility and test for sex-specific differences we characterized in a fully orthogonal design the reproductive performance of pairs (1 male and 1 female) such that: (1) both male and female developed in the same thermal conditions (either warming or control environment), and (2) male and female differed in thermal conditions experienced during development (i.e. males developed under warming conditions and females under control conditions and vice-versa). Control and Warming populations were tested in this experiment. The warming populations were at their 23rd generation since implementation of thermal regimes.

After one full generation of common-garden rearing (18ºC) to minimize maternal effects, 18 vials (with 70 eggs in each) per experimental population were collected and placed in each of the two test environments: control (C) or global warming (W). Upon emergence, 24 males and females per population and environment were separated under CO_2 anesthesia and were paired in four types of mating crosses depending on their developmental environment: C×C (both male and female developed in C environment), W×W (both male and female developed in W), C×W (female developed in C, male developed in W), and W×C (female developed in W, male developed in C). A total of 12 pairs per replicate population, selection and environment combination were studied, with each pair being assigned to one experimental vial. From then on, the mating pairs were kept in the W environment and fed every other day during 9 days.

A total of 576 pairs were assayed (12 pairs × 2 selection regimes × 2 historically differentiated populations × 3 replicate populations × 4 temperature combinations). Assays followed a block design, each block corresponding to a set of same-numbered replicate populations randomly distributed in the same racks and simultaneously manipulated (e.g. Block 1 including the samples from NL1, PT1, WPT1 and WNL1 populations). Three life-history traits were estimated: (1) *fecundity*, as the per female number of eggs laid on the ninth day since imago emergence, (2) *reproductive success*, as the number of emerged flies - during a 10-day period - from the eggs laid in the ninth day (used to estimate *fecundity*), a measure of the female's ability to produce viable progeny, and (3) *juvenile viability*, as the ratio between reproductive success and fecundity. The ninth day of adult life was chosen as it is within the age interval – near the median age - of individuals that contribute to the next generation in both thermal regimes. We are thus mimicking the maintenance protocol, focusing on a timing where selective pressures on fertility are likely higher. For males, fertility will be evaluated by the fertility of females with whom they mate. While these traits are the direct measure of females' reproductive performance, our orthogonal design can disentangle the effect of thermal conditions on the males and females' side, and thus measure the impact on sex-specific fertility. For reliability purposes, only vials with at least five eggs were considered for juvenile viability (this led to the exclusion of 1.5% of vials).

2.3 Statistical analysis

Raw data used in the analysis are the individual values (mated pairs) for each replicate population*male environment*female environment, *i.e.*, each replicate population has a total of twelve (mated pairs) values for each combination of male and female environment (C×C, C×W, W×C, W×W). Due to high deviations from normality, data for reproductive success and fecundity were analyzed through generalized linear mixed-effects models (GLMM) with different residual distributions (quasi-poisson with zero inflation parameter for reproductive success and a negative binomial distribution with zero inflation parameter for fecundity). The default estimation algorithm was chosen (Maximum Likelihood). For juvenile viability, linear mixed-effects models (LMM) fitted with REML (restricted maximum likelihood) were applied on the arcsine transformed values. P-values for differences between thermal regimes, historical differences between populations, male and female environment and their interactions were obtained through Type III Wald chi-square tests for the GLMM models and Type III Wald F tests (Kenward-Roger degrees of freedom) for the LMM models. Two general models were used (for simplicity interactions with random factors are not shown but were also included):

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

$$\times \mathit{Female\ Env} + \mathit{Selection} \times \mathit{Male\ Env} \times \mathit{Female\ Env} + \mathit{Selection} \times \mathit{History} \times \mathit{Male\ Env} + \mathit{Selection} \times \mathit{History} \times \mathit{Female\ Env} + \mathit{Selection} \times \mathit{History} \times \mathit{Male\ Env} \times \mathit{Female\ Env} + \varepsilon$$

$$(2)\ Y = \mu + \mathit{History} + \mathit{Block} + \mathit{Male\ Env} + \mathit{Female\ Env} + \mathit{Selection} + \mathit{Male\ Env} \times \mathit{Female\ Env} + \mathit{History} \times \mathit{Female\ Env} + \mathit{History} \times \mathit{Male\ Env} + \mathit{History} \times \mathit{Selection} + \mathit{Selection} \times \mathit{Female\ Env} + \mathit{Selection} \times \mathit{Male\ Env} + \mathit{History} \times \mathit{Male\ Env} \times \mathit{Female\ Env} + \mathit{Selection} \times \mathit{Male\ Env} \times \mathit{Female\ Env} + \mathit{Selection} \times \mathit{History} \times \mathit{Male\ Env} + \mathit{Selection} \times \mathit{History} \times \mathit{Female\ Env} + \mathit{Selection} \times \mathit{History} \times \mathit{Male\ Env} \times \mathit{Female\ Env} + \varepsilon$$

Y is the trait reproductive success, fecundity or juvenile viability. *Selection* is a fixed factor with the two selection regimes (Warming and Control), *History* is a fixed factor relative to population history (NL and PT), *Male Env* and *Female Env* are fixed factors that represent the male and female developmental environment respectively, each with two categories (W and C Environments). In model (1) *AP* represents the random factor that corresponds to the ancestral replicate populations nested in the fixed factor *History* (e.g. NL1 is the ancestral population of NL1 and WNL1, nested in NL origin, etc.). In model (2) *Block* is the random factor that represents the total of same-numbered replicate populations that were assayed and manipulated in synchrony (see above). All other terms correspond to the interactions between all factors (both between fixed factors and between random and fixed factors, also included in all analyses). Separate analyses were also performed for each historically differentiated population - lower latitude (derived from PT) and higher latitude (derived from NL) populations.

Akaike information criterion (AIC) was used to choose the best model (between 1 and 2) for each trait. Models with *AP* as random factor were chosen for all traits, as they presented consistently lower AIC values.

To have a comparative measure of the magnitude of differentiation between effects of (male and female) environment across populations and traits we estimated the effect sizes –using Cohen's *d* (Cohen 1988) - for the effect of warming *vs.* control environment in both males and females for the different populations and traits. Effect size values were calculated using the standard deviation between replicate population means in each environment to account for heterogeneity at the replicate population level.

Statistical analyses were performed in R v4.0.4. The *glmmTMB* package (Brooks et al. 2017) was used to compute GLMM models, the *lme4* package (Bates et al. 2015) for the LMM models and the *car* package (Fox and Weisberg 2019) for computing Type III Wald F tests for the LMM models. The *ggplot2* package (Wickham 2016) was used for the graphical representations.

3. Results

We studied the effect of the developmental environment (under control *vs.* warming conditions) experienced by the males and females on their fertility. We measured three traits expressed by females (but affected also by males): reproductive success, fecundity and juvenile viability. An overall model applied including factors *History*, *Male* and *Female Environment* and *Selection* was initially applied. A significant effect of the female environment was observed for all traits (significant *Female Env*, see Table S1), with a decreased performance in females that developed in warming conditions (Figures 2 and

3, see also Supplementary Figures S1 and S2 including individual error bars). Significant interactions between the male environment and history were observed for reproductive success and viability with a better performance of low latitude males relative to their higher latitude counterparts following exposure to warming conditions (significant *History x Male Env*, see Table S1 and Figures 2 and 3). Given these significant interactions further analyses were carried out separately for populations of different historical origin.

When analyzing the high latitude populations, we observed a negative effect of both male and female warming environment in reproductive success (*Male Env* and *Female Env*, Table 1 and Figure 2). Fecundity and juvenile viability showed contrasting patterns across sexes: for fecundity there was a significant negative effect when females were exposed to the warming environment, while not for males (that even tended to increase in the controls, Figure 2 and below); for juvenile viability, a significant negative effect was observed when males where exposed to such stressful conditions, though not for females (see Table 1 and Figure 2). Interestingly, a significant interaction between selection and the effects of both male and female environments was found for fecundity (*Selection x Male Env x Female Env*, see Table 1), with the warming environment having a negative effect on female fecundity and a positive effect on male fecundity in the controls but clearly less pronounced effects in both male and female fecundity in the warming populations (see Figure 2).

For the low latitude populations, the effects of the warming development environment were only observed for fecundity, with a positive effect on males and a negative effect on females (*Male Env*, *Female Env* and *Male Env x Female Env*, Table 1 and Figure 3). No significant differences in any trait were detected between populations of different selective regimes nor variation in these thermal regimes associated with male or female environments (*Selection* and interaction terms with *Selection*, see Table 1).

Cohen's *d* values were computed for all traits to address the magnitude of the effect of the warming *vs.* control environment for different sexes and populations of distinct selective regimes or history (see Table S2 and Figures 2 and 3). For reproductive success and fecundity, there was a consistently larger (negative) effect of the warming environment in females than in males. For viability, sex differentiation patterns varied between historically differentiated populations: while for the high latitude populations the negative effect of warming environment was clearly higher on males (a "huge" effect in both high latitude control and warming populations, $d>7$ and $d>2$ respectively), for the low latitude populations the detrimental effect of warming conditions was bigger in females although with a lower magnitude in size ($d>1.10$ and $d>1.94$ for low latitude control and warming populations respectively) – see Table S2. Importantly, the negative effects observed for reproductive success and viability were consistently of a very high magnitude in the high latitude controls in both males and females (a "huge" effect, with $d>2$ in all four comparisons), with warming populations clearly less affected. The same did not occur for low latitude populations, with much less pronounced differences between warming and control populations for these traits (see Table S2).

4. Discussion

In this experiment we analyze the effect of development under both mild and warming environment on the subsequent fertility of males and females under warming conditions in several *Drosophila subobscura* populations. These populations differed in their thermal selection and historical genetic background.

Fertility loss following development in a warming environment

We found that development under warming environmental conditions reduced reproductive success in the high-latitude females and males. This drop in performance was mostly driven by a decline in juvenile viability of eggs laid by mated pairs in which males developed under stress, and to a reduction of fecundity (egg number) in females following heat stress. Heat stress during development has been previously shown to cause lowered reproductive performance (e.g. (Klockmann et al. 2017; Porcelli et al. 2017; Santos et al. 2021a; Simões et al. 2020). Fertility of flies in the warming environment was not enhanced by development in such conditions - it was in fact reduced - indicating no positive effects of developmental plasticity (i.e. acclimation). This contrasts with recent evidence of adaptive developmental plasticity for fertility (Sgrò et al. 2016; Vasudeva et al. 2019; Wang and Gunderson 2022). Our findings are likely due to a detrimental impact of long exposure to stressful conditions during development (~ 16 days, with daily peaks of high temperature ~30ºC) on male spermatogenesis and female oogenesis. Lower acclimation periods and thermal peaks might have yielded a different outcome, despite recent work in *Drosophila virilis* suggesting that acclimation for fertility may be difficult to attain (Walsh et al. 2021).

Males and females show contrasting responses to a warming environment

Based on previous evidence that thermal stress has a higher impact on male fertility than on female fertility (Sales et al. 2018; Zwoinska et al. 2020), we would expect a general lower reproductive success in males than in females, due to a drop in juvenile viability of offspring, from males exposed to the warming environment than in females. However, this was not the case in our study. While both males and females from populations of higher latitude - but not lower latitude - showed decreased reproductive success, the magnitude of such decline was clearly higher in females than in males (see Figure 2 and Table S2). Fecundity patterns can help in explaining these unexpected differences between male and female reproductive success, as stressed females showed a decline in fecundity in both populations while males show indications of increased fecundity. These positive effects in males might mitigate the negative impact of the decline in viability that was observed (particularly in the higher latitude males), most probably due to the expected increase in adult male sterility upon heat stress.

In a largely monandrous species such as *Drosophila subobscura*, this increase in male sterility under thermal stress can be particularly troublesome and potentially lead to population decline at higher latitudes. A meta-analysis of experimental evolution studies also points to increased variation between sexes associated with sexual selection under stressful conditions, with greater benefits for females than males (Cally et al. 2019; but see Gómez-Llano et al. 2021 for evidence of decreased sexual dimorphism). Adding to this, in an experimental evolution study under warming conditions in the seed beetle *Callosobruchus maculatus,* evolution of sex-specific variation in longevity was observed, possibly because of a different reproductive investment between sexes (Rogell et al. 2014). These findings point to an interplay between sexual selection and thermal evolution. Although we find here evidence for sex-specific variation in reproductive success under a warming environment, such differences did not arise from thermal selection as they were observed in both warming and control populations.

As expected, the effect of exposure to a warming environment in males and females was trait specific. In contrast with the general negative (or no) effect described above for reproductive success and viability, we found that males and females exposed to warming conditions showed opposing responses for fecundity particularly in the populations of lower latitude. While males developed under heat stress increased the fecundity of the females with whom they mated, a general decline was observed for females under the same conditions. This positive effect on fecundity from the males is counter intuitive, though a similar effect has been described in butterflies (Janowitz and Fischer 2011). It might be the case that the warming environment overstimulates metabolism leading to an increase in production of sperm (Snook et al. 2000) and/or sex peptides, known to increase female fecundity (Chen et al. 1988), a hypothesis that needs additional testing.

In all, our results show that, increased fecundity in pairs where males were exposed to heat stress might help mitigate detrimental effects of heat stress observed on females thus reducing the drop in overall levels of reproductive success in the population - ultimately the most important trait for species persistence.

Males from lower latitudes have higher performance under thermal stress

Studies in different species have found variation between populations in adult performance following development under heat stress (e.g. Rohmer et al. 2004, in *D. melanogaster*, Poulet et al. 2015 in *C. elegans*; see also Wang and Gunderson 2022). In our study, we also found that the genetic background has an impact on male thermal performance: low latitude males showed an overall better ability to withstand the stressful environment when compared to the high latitude ones. This tendency was observed regardless of the environment experienced by the female. It is possible that the greater ability of lower latitude males to withstand the warming environment might result from their historical background being shaped by exposure to generally higher (and more variable) developmental temperatures in nature. Although we provide evidence for population differentiation in male responses to heat stress, additional sampling of populations from contrasting latitudes would be needed to accurately attribute the differences that we observe to geographical (latitudinal) variation in nature.

Having said that, and in agreement with our findings, in an independent study Porcelli et al. (2017) found that lower latitude *D. subobscura* populations (from Spain) present higher levels of fertility in response to heat stress during development than their higher latitude, northern counterparts (from Sweden). However, in that study, the impact of heat stress on fertility was not assessed on males and females separately as we have done here. More recent evidence points to the relevance of interspecific variation in male sterility as a major predictor of species response under climate change (Parratt et al. 2021; van Heerwaarden and Sgrò 2021). Our study shows that such interspecific variation in male fertility may build upon variation at the intraspecific level, that is relevant to account for when predicting evolutionary responses to climate change.

Given the lower male fertility under warming conditions in our high latitude ancestral populations, we might expect a higher evolutionary response in the high latitude warming populations relative to the low latitude ones. Nevertheless, this was not the case (see below).

Thermal selection did not shape responses to heat stress

We did not find evidence for an evolutionary response to thermal selection in both low and high latitude populations: males and females of the warming populations do not exhibit higher fertility than controls when exposed to warming conditions during development. This lack of an improvement in fertility as a result of thermal selection agrees with the absence of relevant shifts in thermal reaction norms observed in a short-term evolution study of these populations (generation 9, see (Santos et al. 2021b)). Studies addressing evolution under increasingly warmer environments in other insects have also failed to observe a clear adaptive response (e.g. (Schou et al. 2014; Kinzner et al. 2019, but see Rogell et al. 2014), suggesting limited overall evolutionary potential for fertility changes in response to climate warming under controlled laboratorial conditions. This potential might however occur in natural settings due to higher population sizes, gene flow and the occurrence of ecological features not replicated in the lab.

Conclusions

We here report contrasting sex-specific thermal responses to a warming environment in *Drosophila subobscura* populations. The negative effects of development under warming conditions on reproductive performance were consistently higher in females than in males, calling into question the idea that males are more affected by heat stress.

Furthermore, our results suggest that increased fecundity of females mated with males following exposure to warming conditions is a possible mechanism by which reproductive success can be rescued in the population. In addition, while no beneficial effects of adaptive plasticity and thermal selection were clearly evident, we found that the different historical background of the populations had a relevant role in the stress response by shaping male fertility patterns. Ultimately, considering intra-species variation as well as sex-specific differences in thermal fertility will allow more solid predictions of population persistence and distribution under a changing climate.

References

Araújo, M. B., F. Ferri-Yáñez, F. Bozinovic, P. A. Marquet, F. Valladares, and S. L.Chown. 2013. Heat freezes niche evolution. Ecology Letters 16:1206–1219.

Ashburner, M., and T. F. R. Wright. 1980. The Genetics and Biology of Drosophila. Academic Press Inc.

Bates, D., M. Maechler, B. Bolker, and S. Walker. 2015. Fitting linear mixed-effects models using lme4. J. Stat. Softw. 67:1–48.

Beaman, J. E., C. R. White, and F. Seebacher. 2016. Evolution of Plasticity: Mechanistic Link between Development and Reversible Acclimation. Trends in Ecology and Evolution 31:237–249.

Brooks, M.E., K. Kristensen, K. J. van Benthem, A. Magnusson, C. W. Berg, A. Nielsen, H. J. Skaug, M. Mächler and B. M. Bolker. 2017. The R Journal 9:2, pages 378-400.

Buckley, L. B., and R. B. Huey. 2016. Temperature extremes: geographic patterns, recent changes, and implications for organismal vulnerabilities. Global Change Biology 22:3829–3842.

Cally, J.G., D. Stuart-Fox, and L. Holman. 2019. Meta-analytic evidence that sexual selection improves population fitness. Nature Communications 10: 2017.

Castañeda, L. E., V. Romero-Soriano, A. Mesas, D. A. Roff, and M. Santos. 2019. Evolutionary potential of thermal preference and heat tolerance in Drosophila subobscura. Journal of Evolutionary Biology 32:818–824.

Chen, P. S., E. Stumm-Zollinger, T. Aigaki, J. Balmer, M. Bienz, and P. Mhlen. 1988. A Male Accessory Gland Peptide That Regulates Reproductive Behavior of Female D. melanogaster. Cell 54:291-298.

Cohen, J. 1988. Statistical Power Analysis for the Behavioral Sciences. Routledge Academic, New York.

David, J. R., L. O. Araripe, M. Chakir, H. Legout, B. Lemos, G. Pétavy, C. Rohmer, D. Joly, and B. Moreteau. 2005. Male sterility at extreme temperatures: A significant but neglected phenomenon for understanding Drosophila climatic adaptations. Journal of Evolutionary Biology 18:838–846.

Fisher, D. N., R. J. Doff, and T. A. R. Price. 2013. True polyandry and pseudopolyandry: Why does a monandrous fly remate? BMC Evolutionary Biology 13:157.

Fox, J., and S. Weisberg. 2019. An {R} Companion to Applied Regression. Third Edit. SAGE, Thousand Oaks CA.

Fragata, I., M. Lopes-Cunha, M. Bárbaro, B. Kellen, M. Lima, M. A. Santos, G. S. Faria, M. Santos, M. Matos, and P. Simões. 2014a. How much can history constrain adaptive evolution? A real-time evolutionary approach of inversion polymorphisms in Drosophila subobscura. Journal of Evolutionary Biology 27:2727–2738.

Fragata, I., P. Simões, M. Lopes-Cunha, M. Lima, B. Kellen, M. Bárbaro, J. Santos, M. R. Rose, M. Santos, and M. Matos. 2014b. Laboratory selection quickly erases historical differentiation. PloS ONE 9:e96227.

Gómez-Llano, M., E. Scott, and E.I. Svensson. 2021. The importance of pre- and postcopulatory sexual selection promoting adaptation to increasing temperatures. Current zoology 67: 321-327.

Iossa, G. 2019. Sex-Specific Differences in Thermal Fertility Limits. Trends in Ecology & Evolution 34:490–492.

IPCC, 2022: Summary for Policymakers [H.-O. Pörtner, D.C. Roberts, E.S. Poloczanska, K. Mintenbeck, M. Tignor, A. Alegría, M. Craig, S. Langsdorf, S. Löschke, V. Möller, A. Okem (eds.)]. In: Climate Change 2022: Impacts, Adaptation and Vulnerability. Contribution of Working Group II to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change [H.-O. Pörtner, D.C. Roberts, M. Tignor, E.S. Poloczanska, K. Mintenbeck, A. Alegría,

M. Craig, S. Langsdorf, S. Löschke, V. Möller, A. Okem, B. Rama (eds.)]. Cambridge University Press, Cambridge, UK and New York, NY, USA, pp. 3–33.

Janowitz, S. A., and K. Fischer. 2011. Opposing effects of heat stress on male versus female reproductive success in Bicyclus anynana butterflies. Journal of Thermal Biology 36:283–287.

Jørgensen, K. T., J. G. Sørensen, and J. Bundgaard. 2006. Heat tolerance and the effect of mild heat stress on reproductive characters in Drosophila buzzatii males. Journal of Thermal Biology 31:280–286.

Kellermann, V., and B. van Heerwaarden. 2019. Terrestrial insects and climate change: adaptive responses in key traits. Physiological Entomology 44:99–115.

Kingsolver, J. G., S. E. Diamond, and L. B. Buckley. 2013. Heat stress and the fitness consequences of climate change for terrestrial ectotherms. Functional Ecology 27:1415–1423.

Kinzner, M. C., A. Gamisch, A. A. Hoffmann, B. Seifert, M. Haider, W. Arthofer, B. C. Schlick-Steiner, and F. M. Steiner. 2019. Major range loss predicted from lack of heat adaptability in an alpine Drosophila species. Science of the Total Environment 695:133753.

Klockmann, M., F. Kleinschmidt, and K. Fischer. 2017. Carried over: Heat stress in the egg stage reduces subsequent performance in a butterfly. PLoS ONE 12: e0180968.

MacLean, H. J., J. G. Sørensen, T. N. Kristensen, V. Loeschcke, K. Beedholm, V. Kellermann, and J. Overgaard. 2019. Evolution and plasticity of thermal performance: An analysis of variation in thermal tolerance and fitness in 22 Drosophila species. Philosophical Transactions of the Royal Society B: Biological Sciences 374:20180548.

Matos, M., C. Rego, a Levy, H. Teotónio, and M. Rose. 2000. An evolutionary no man's land. Trends Ecol Evol 15:206.

Matos, M., P. Simões, M. A. Santos, S. G. Seabra, G. S. Faria, F. Vala, J. Santos, and I. Fragata. 2015. History, chance and selection during phenotypic and genomic experimental evolution: Replaying the tape of life at different levels. Frontiers in Genetics 6:71.

Mesas, A., A. Jaramillo, and L. E. Castañeda. 2021. Experimental evolution on heat tolerance and thermal performance curves under contrasting thermal selection in Drosophila subobscura. J Evol Biol 34:767–778.

Nguyen, T. M., C. Bressac, and C. Chevrier. 2013. Heat stress affects male reproduction in a parasitoid wasp. J Insect Physiol 59:248–254.

Parratt, S. R., B. S. Walsh, S. Metelmann, N. White, A. Manser, A. J. Bretman, A. A. Hoffmann, R. R. Snook, and T. A. R. Price. 2021. Temperatures that sterilize males better match global species distributions than lethal temperatures. Nature Climate Change 11:481–484.

Porcelli, D., K. J. Gaston, R. K. Butlin, and R. R. Snook. 2017. Local adaptation of reproductive performance during thermal stress. Journal of Evolutionary Biology 30:422–429.

Poullet, N., A. Vielle, C. Gimond, C. Ferrari, and C. Braendle. 2015. Evolutionarily divergent thermal sensitivity of germline development and fertility in hermaphroditic Caenorhabditis nematodes. Evol. Dev. 17, 380–397. Rezende, E., J. Balanyà, F. Rodríguez-Trelles, C. Rego, I. Fragata, M. Matos, L. Serra, and M. Santos. 2010. Climate change and chromosomal inversions in Drosophila subobscura. Climate Research 43:103–114.

Rezende, E.L., F. Bozinovic, A. Szilágyi, and M. Santos. 2020. Predicting temperature mortality and selection in natural Drosophila populations. Science 369:1242-1245.

Rodrigues, L.R., H. A. McDermott, I. Villanueva, J. Djukarić, L. C. Ruf, M. Amcoff, and R. Snook. 2022. Fluctuating heat stress during development exposes reproductive costs and putative benefits. Journal of Animal Ecology 91:391-403.

Rogell, B., W. Widegren, L.R. Hallsson, D. Berger, M. Björklund, and A.A. Maklakov. 2014. Sex-dependent evolution of life-history traits following adaptation to climate warming. Functional Ecology 28: 469–478.

Rohmer, C., J.R. David, B. Moreteau, and D. Joly. 2004. Heat induced male sterility in Drosophila melanogaster: adaptive genetic variations among geographic populations and role of the Y chromosome. Journal of Experimental Biology 207: 2735–2743.

Sales, K., R. Vasudeva, M. E. Dickinson, J. L. Godwin, A. J. Lumley, Ł. Michalczyk, L. Hebberecht, P. Thomas, A. Franco, and M. J. G. Gage. 2018. Experimental heatwaves compromise sperm function and cause transgenerational damage in a model insect. Nature Communications 9:4771.

Sales, K., R. Vasudeva, and M. J. G. Gage. 2021. Fertility and mortality impacts of thermal stress from experimental heatwaves on different life stages and their recovery in a model insect. Royal Society Open Science 8:201717.

Santos, M. A., A. Carromeu-Santos, A. S. Quina, M. Santos, M. Matos, and P. Simões. 2021a. High developmental temperature leads to low reproduction despite adult temperature. Journal of Thermal Biology 95:102794.

Santos, M. A., A. Carromeu-Santos, A. S. Quina, M. Santos, M. Matos, and P. Simões. 2021b. No evidence for short-term evolutionary response to a warming environment in Drosophila. Evolution 75:2816-2829.

Santos, M. A., A. Carromeu-Santos, A. S. Quina, M. Santos, M. Matos, and P. Simões. 2023. Past history shapes evolution of reproductive success in a global warming scenario. Journal of Thermal Biology 112, 103478.

Schou, M. F., T. N. Kristensen, V. Kellermann, C. Schlötterer, and V. Loeschcke. 2014. A Drosophila laboratory evolution experiment points to low evolutionary potential under increased temperatures likely to be experienced in the future. Journal of Evolutionary Biology 27:1859–1868.

Sgrò, C. M., J. S. Terblanche, and A. A. Hoffmann. 2016. What Can Plasticity Contribute to Insect Responses to Climate Change? Annual Review of Entomology 61:433–451.

Simões, P., I. Fragata, S. Seabra, G. S. Faria, M. Santos, M. R. Rose, M. Santos, and M. Matos. 2017. Predictable phenotypic, but not karyotypic, evolution of historically differentiated populations. Scientific Reports 7: 913.

Simões, P., M. A. Santos, A. Carromeu-Santos, A. S. Quina, M. Santos, and M. Matos. 2020. Beneficial developmental acclimation in reproductive performance under cold but not heat stress. Journal of Thermal Biology 90:102580.

Slotsbo, S., M. F. Schou, T. N. Kristensen, V. Loeschcke, and J. G. Sørensen. 2016. Reversibility of developmental heat and cold plasticity is asymmetric and has long lasting consequences for adult thermal tolerance. Journal of Experimental Biology 219: 2726–2732.Snook, R. R., S. Y. Cleland, M. F. Wolfner, and T. L. Karr. 2000. Offsetting Effects of Wolbachia Infection and Heat Shock on Sperm Production in Drosophila simulans: Analyses of Fecundity, Fertility and Accessory Gland Proteins. Genetics 155:167–178.

van Heerwaarden, B., and C. M. Sgrò. 2021. Male fertility thermal limits predict vulnerability to climate warming. Nature Communications 12:2214.

Vasudeva, R., D. C. Deeming, and P. E. Eady. 2014. Developmental temperature affects the expression of ejaculatory traits and the outcome of sperm competition in Callosobruchus maculatus. Journal of Evolutionary Biology 27:1811–1818.

Vasudeva, R., M. Dickinson, A. Sutter, S. Powell, K. Sales, and M. J. G. Gage. 2021. Facultative polyandry protects females from compromised male fertility caused by heatwave conditions. Animal Behaviour 178:37–48.

Vasudeva, R., A. Sutter, K. Sales, M. E. Dickinson, A. J. Lumley, and M. J. G. Gage. 2019. Adaptive thermal plasticity enhances sperm and egg performance in a model insect. Elife 8:e49452.

Walsh, B. S., S. R. Parratt, A. A. Hoffmann, D. Atkinson, R. R. Snook, A. Bretman, and T. A. R. Price. 2019. The Impact of Climate Change on Fertility. Trends in Ecology and Evolution 34:249–259.

Walsh, B. S., S. R. Parratt, N. L. M. Mannion, R. R. Snook, A. Bretman, and T. A. R. Price. 2021. Plastic responses of survival and fertility following heat stress in pupal and adult Drosophila virilis. Ecology and Evolution 11:18238–18247.

Wang, W. W. Y., and A. R. Gunderson. 2022. The Physiological and Evolutionary Ecology of Sperm Thermal Performance. Front. Physiol. 13:754830.

Zwoinska, M. K., L. R. Rodrigues, J. Slate, and R. R. Snook. 2020. Phenotypic Responses to and Genetic Architecture of Sterility Following Exposure to Sub-Lethal Temperature During Development. Frontiers in Genetics 11:573.

Tables

Table 1 - Analysis of the impact of thermal environment on fertility traits for each historically differentiated population.

Trait	Model parameters	Low latitude (χ2 / F)	High latitude (χ2 / F)
Reproductive Success	Selection	1.218 n.s.	2.733 m.s.
	Female Env	3.281 m.s.	13.415 ***
	Male Env	2.280 n.s.	25.132 ***
	Selection*Female Env	2.447 n.s.	0.108 n.s.
	Selection*Male Env	0.003 n.s.	0.085 n.s.
	Male Env*Female Env	0.005 n.s.	0.330 n.s.
	Selection*Male Env*Female Env	0.538 n.s.	1.836 n.s.
Fecundity	Selection	0.515 n.s.	1.741 n.s.
	Female Env	11.249 ***	8.289 **
	Male Env	9.832 **	2.171 n.s.
	Selection*Female Env	2.381 n.s.	2.120 n.s.
	Selection*Male Env	0.012 n.s.	0.645 n.s.
	Male Env*Female Env	5.621 *	0.297 n.s.
	Selection*Male Env*Female Env	2.063 n.s.	3.906 *
Viability	Selection	$F_{1,2.0}$ = 1.155 n.s.	$F_{1,2.0}$ = 0.375 n.s.
	Female Env	$F_{1,2.2}$ = 4.407 n.s.	$F_{1,2.0}$ = 11.773 m.s.
	Male Env	$F_{1,2.2}$ = 1.125 n.s.	$F_{1,2.1}$ = 36.472 *
	Selection*Female Env	$F_{1,2.0}$ = 0.018 n.s.	$F_{1,2.0}$ = 0.316 n.s.
	Selection*Male Env	$F_{1,2.0}$ = 0.098 n.s.	$F_{1,2.0}$ = 0.510 n.s.
	Male Env*Female Env	$F_{1,2.1}$ = 2.354 n.s.	$F_{1,2.0}$ = 0.897 n.s.
	Selection*Male Env*Female Env	$F_{1,2.1}$ = 1.580 n.s.	$F_{1,2.1}$ = 0.206 n.s.

Note: Degrees of freedom for χ2 = 1. Degrees of freedom for the F-statistics are provided in the Table. Significance levels: $p>0.1$ n.s.; $0.1>p>0.05$ m.s.; $0.05>p>0.01$ *; $0.01>p>0.001$ **; $p<0.001$ ***

Figures

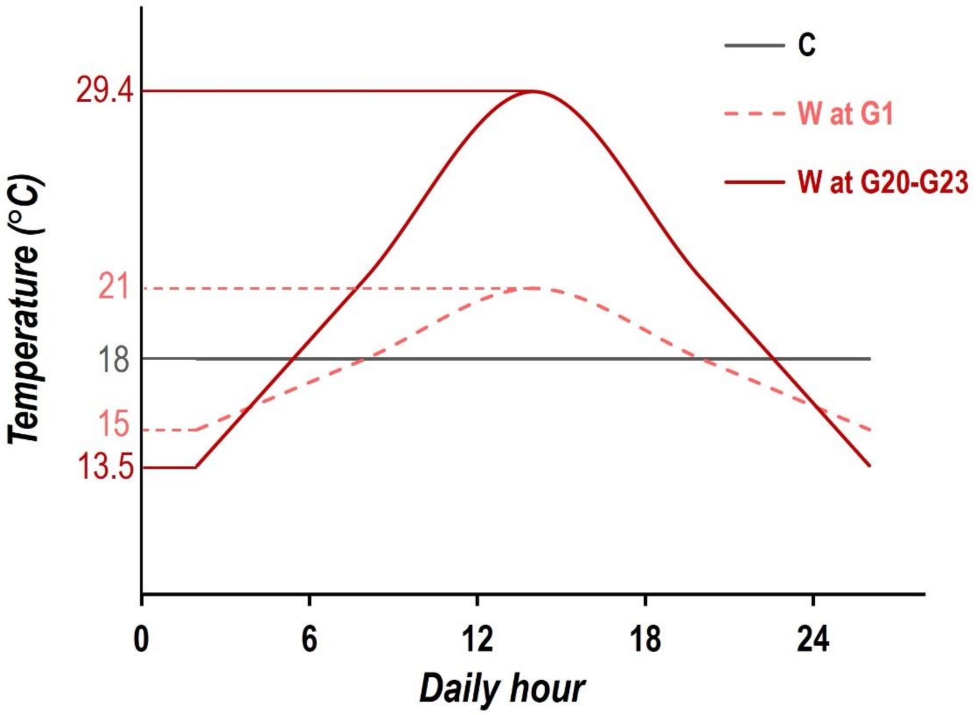

Figure 1. Thermal cycles for control selection regime and warming selection regime at generations 1 and 23.

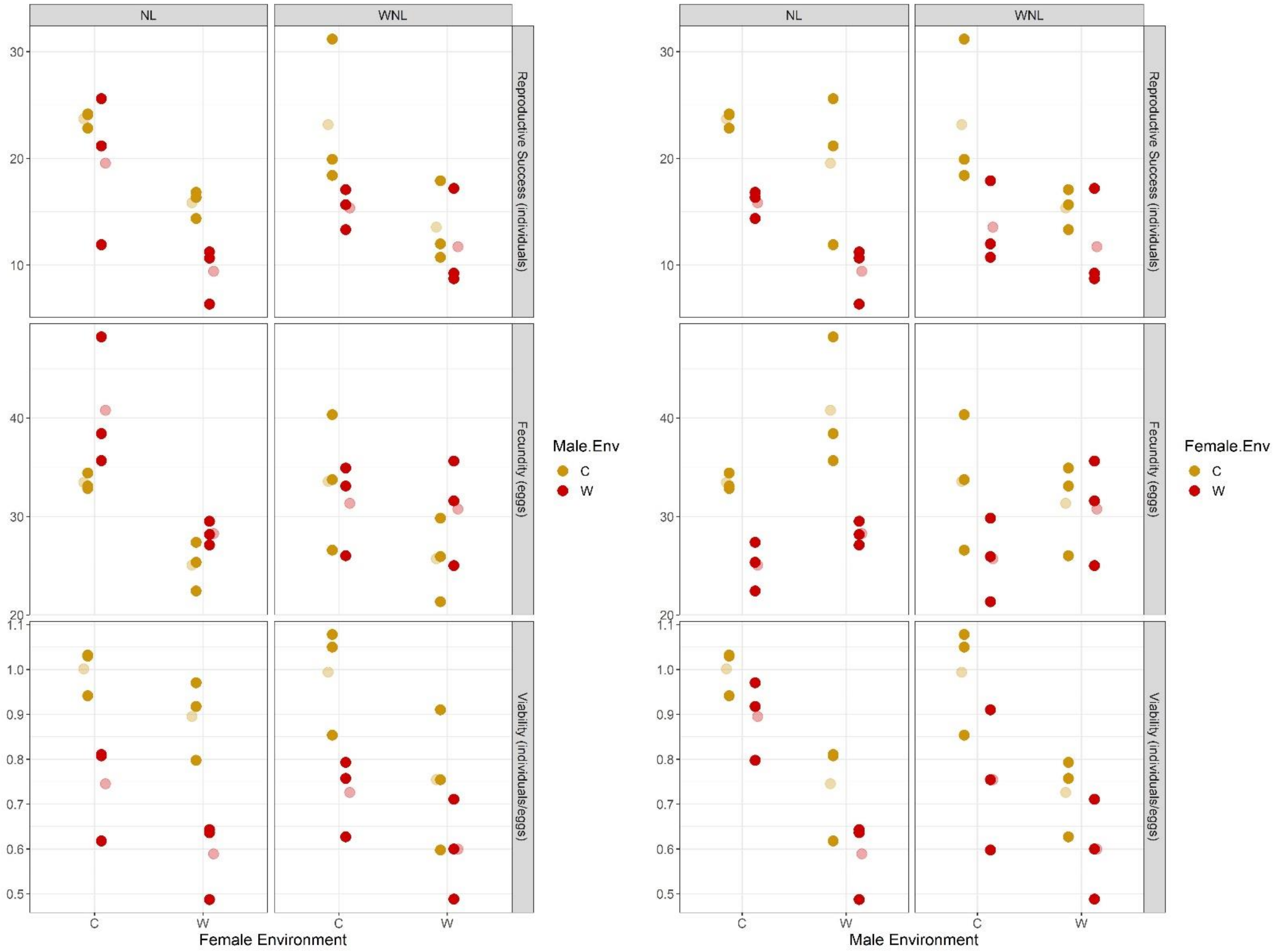

Figure 2. Fertility in High latitude Control (NL) and Warming (WNL) populations after exposure to control or warming developmental temperatures. Legend: Darker colored dots represent the average value for each of the three replicate populations of a thermal regime with a similar origin (i.e. NL_{1-3} and WNL_{1-3}). Light colored dots represent the average of the three replicate values with a similar origin. Reproductive Success (number of offspring); Fecundity (number of eggs); Viability (ratio of reproductive success and fecundity, arcsine transformed).

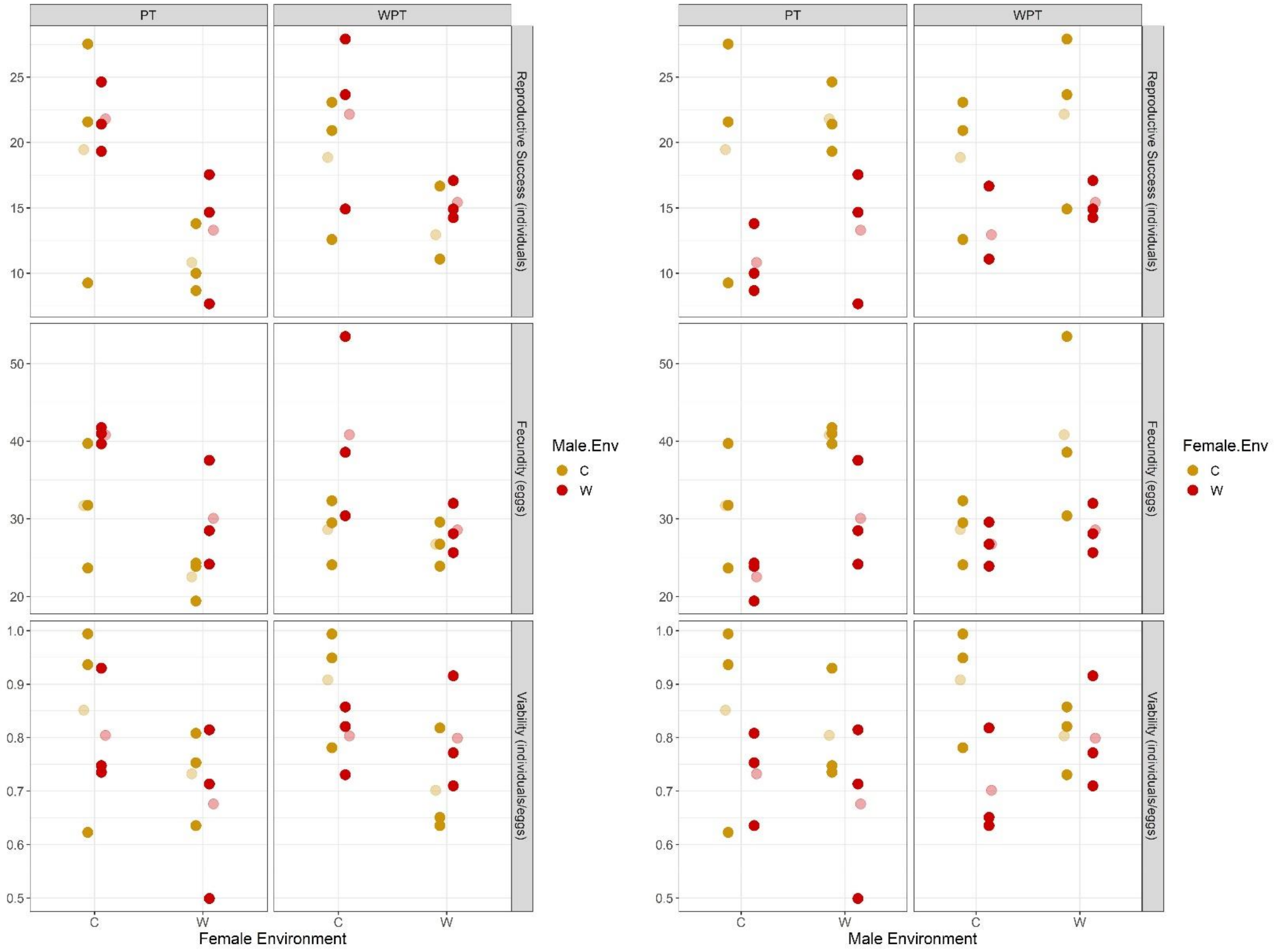

Figure 3. Fertility in Low latitude Control (PT) and Warming (WPT) populations after exposure to control or warming developmental temperatures. Legend: Darker colored dots represent the average value for each of the three replicate populations of a thermal regime with a similar origin (i.e. PT$_{1\text{-}3}$ and WPT$_{1\text{-}3}$). Lighter colored dots represent the average of the three replicate values with a similar origin. Reproductive Success (number of offspring); Fecundity (number of eggs); Viability (ratio of reproductive success and fecundity, arcsine transformed).