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Mating behaviour and fertility of a monandrous insect under thermal stress

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Abstract

Insects are amongst the organisms most affected by rising temperatures, due to negative repercussions on life-history traits and, consequently, on population persistence. In many taxa males become sterile at lower temperatures than females. In these species, females could buffer the negative effect on male fertility improving their own reproductive output by rejecting sterile males or mating with more than one male. However, this rescue is conditioned by the populations' mating system and the mating behaviour of both sexes, which can also be disrupted by temperature. Unfortunately, most work has studied polyandrous species leaving a gap in our knowledge. Thus, understanding if and how monandrous species change their mating behaviour and/or mating system due to heat stress will bring important knowledge on the crucial topic of population persistence under climate warming. Here, we studied the real-time evolution of two bio-geographical distinct populations of a monandrous fruit fly *(Drosophila subobscura)* subjected to a global warming scenario. As such, we could test if selection and population history play a role in the ability to respond to temperature changes. Specifically, we aimed to assess: i) whether adaptation to a warming environment occurs; ii) the impact of high temperatures on male mating behaviour and fertility; and iii) the impact of reduced male fertility on female mating behaviour, fertility, and propensity to remate. We show that adaptation to warming conditions was population-specific and occurred with no evident costs. We also demonstrate that exposure to high temperatures leads to lower performance in males, affecting both behaviour and fertility. Importantly, these males are able to recover a functional mating behaviour through time, but not their reproductive output. Finally, we show that monandrous females remate after exposure to heat-stressed males and that this behaviour is advantageous for the reproductive output of the female leading to a total rescue of their reproductive performance in certain conditions. Ultimately, this project brings new insights on the effects of high temperatures on mating behaviour and fertility in a monandrous species and provide a model to assess how a shift from monandry to polyandry affects species at both the individual and the population levels.

Keywords: Thermal adaptation; Fertility; Mating behaviour; *Drosophila subobscura;* Experimental evolution

Resumo

As alterações climáticas, em particular o aumento da temperatura, são uma das principais preocupações e desafios que enfrentamos atualmente dado o impacto que têm, e continuarão a ter, na biodiversidade. Os animais ectotérmicos, onde estão incluídos os insetos, podem estar entre os organismos mais afetados pelas altas temperaturas, uma vez que não têm a capacidade de regular a sua temperatura interna. Quando expostos a altas temperaturas, o comportamento, a reprodução, a distribuição e, em última instância, a persistência dos ectotérmicos são afetados. Preocupantemente, diversos estudos mostram que estes organismos apresentam pouca capacidade de se adaptarem ao aumento da temperatura, indicando que o potencial evolutivo dos mesmos para responder às alterações climáticas é limitado. Adicionalmente, alguns estudos demonstram que características diferentes apresentam sensibilidades térmicas distintas, sendo que em muitas espécies a fertilidade é negativamente afetada a uma temperatura bastante mais baixa do que a sobrevivência. Além disso, em algumas espécies, machos e fêmeas apresentam diferentes sensibilidades térmicas no que toca à fertilidade, sendo os machos afetados a temperaturas mais baixas quando comparados com as fêmeas. Sendo as fêmeas mais resistentes, estas podem ajudar a reduzir declínios populacionais quando as altas temperaturas afetam a fertilidade dos machos, nomeadamente aumentando a sua predisposição para acasalar com mais machos. No entanto, isto está dependente do sistema de acasalamento da espécie e do comportamento de acasalamento tanto dos machos como das fêmeas.

Neste projeto, usámos como objeto de estudo duas populações de *Drosophila subobscura*, uma espécie monândrica de mosca da fruta, que é considerada um organismo modelo para a biologia evolutiva, principalmente em estudos de adaptação térmica. As populações laboratoriais em estudo têm origens geográficas distintas, uma das populações foi fundada com fêmeas provenientes de Portugal enquanto a outra população foi fundada com fêmeas neerlandesas. Adicionalmente estas populações têm estado a evoluir num regime de aquecimento global há cerca de 50 gerações. Tendo isto em conta, os principais objetivos deste trabalho foram os seguintes: 1) perceber se as populações em estudo estavam adaptadas às altas temperaturas; 2) perceber como é que as altas temperaturas influenciam o comportamento de acasalamento e a fertilidade dos machos, e ainda, 3) perceber como é que a esterilidade dos machos influencia o comportamento de acasalamento e a fertilidade das fêmeas. Este último objetivo foi abordado em muito poucos estudos, além disso esses estudos utilizaram espécies poliândricas, deixando assim uma falha no nosso conhecimento em relação à resposta em espécies monândricas. Adicionalmente, para cada um dos objetivos referidos, foi pretendido perceber quais os efeitos da história populacional, i.e., o efeito dos diferentes fundos genéticos das populações e ainda o efeito da seleção, ou seja, do regime seletivo ao qual as populações foram submetidas.

De forma a atingirmos os objetivos, primeiramente, realizámos testes piloto de forma a estabelecer as melhores condições - temperatura e tempo de exposição - para os ensaios que envolvessem esterilizar os machos e analisar o comportamento de acasalamento tanto dos machos como das fêmeas. Foi selecionado um choque térmico com a duração de 69 horas a 31º C, visto que temperaturas mais baixas não levaram a uma quebra tão grande na fertilidade e temperaturas superiores, nomeadamente 32º C, levaram a uma quebra da sobrevivência superior a 40%.

Para determinar se houve adaptação às altas temperaturas, a fertilidade das populações do regime seletivo de aquecimento global e populações do regime controlo foram analisadas em cada ambiente (ambiente controlo e ambiente de aquecimento global), após 39 gerações de evolução térmica. Observámos que populações dos Países Baixos não estão adaptadas às altas temperaturas, enquanto que populações portuguesas se mostraram adaptadas a essas condições. A resposta adaptativa observada não envolveu quaisquer custos visíveis. É de realçar que, apesar da resposta adaptativa ter acontecido nas populações de Portugal, esta adaptação foi lenta, só se verificando ao fim de bastantes (39) gerações, sendo que um estudo anterior realizado na geração 22 de evolução não mostrou sinais de adaptação para nenhuma das populações.

Para avaliar as consequências das altas temperaturas no comportamento de acasalamento e fertilidade dos machos ao longo de um período considerável da sua vida, um choque térmico de 31º C com a duração de 69 horas (definido com base nos resultados dos testes piloto), foi aplicado aos machos. Este ensaio foi realizado após 45 gerações de evolução térmica e uma vez que se focava nos machos, fêmeas de um *outgroup* foram utilizadas para garantir que diferenças nas respostas dadas por machos de diferentes regimes pudessem ser apenas atribuídas aos mesmos. Posteriormente, os machos tiveram a oportunidade de acasalar com três fêmeas em três momentos distintos, ao longo de oito dias. Durante os acasalamentos vários parâmetros do comportamento de acasalamento formam medidos e, depois de cada acasalamento, a fertilidade dos machos foi avaliada. Assim, foi possível perceber o efeito da temperatura nos machos e se este efeito foi transiente ou permanente. Verificámos que a exposição dos machos ao choque térmico afetou negativamente o seu comportamento de acasalamento e a sua fertilidade. Além disso, ficou demonstrado que os efeitos negativos no comportamento de acasalamento são transientes ao passo que na fertilidade não se verificou qualquer recuperação na performance ao longo do ensaio. Assim sendo, os efeitos na fertilidade podem ser permanentes.

Finalmente, um último ensaio foi realizado para determinar os efeitos da esterilidade dos machos no comportamento de acasalamento e fertilidade das fêmeas. O ensaio foi realizado após 45 gerações de evolução térmica e tinha como alvo de estudo a performance das fêmeas por isso, seguindo o mesmo protocolo do ensaio anterior, machos de uma população *outgroup* foram utilizados para avaliar a resposta das fêmeas. O ensaio envolveu três tratamentos: i) fêmeas tiveram oportunidade de acasalar com dois machos férteis em dois momentos distintos; ii) fêmeas tiveram oportunidade de acasalar com um macho termicamente stressado e posteriormente tiveram oportunidade de acasalar com um macho fértil e, por último; iii) fêmeas tiveram oportunidade de acasalar com dois machos termicamente stressados, em dois momentos diferentes. Durante os acasalamentos vários parâmetros do comportamento de acasalamento formam medidos e, depois de cada acasalamento, a fertilidade das fêmeas foi avaliada. Assim foi possível perceber o efeito da esterilidade dos machos no comportamento de acasalamento e fertilidade das fêmeas, nomeadamente se estas re-acasalavam ou não. Demonstrámos que fêmeas monândricas, exibem um comportamento de re-acasalamento, em resposta a acasalamentos com machos menos férteis. Adicionalmente, verificámos que este comportamento é benéfico para as fêmeas, levando a um resgate total ou parcial da sua fertilidade, dependendo do tratamento e do fundo genético das populações.

Em suma, a falta de resposta adaptativa de umas populações e lenta adaptação da outra suporta a evidencia que os insetos têm pouco potencial evolutivo para responder ao aumento das temperaturas e podem ter extremas dificuldades em lidar com as rápidas alterações climáticas projetadas para as próximas décadas. De facto, os nossos resultados não sugerem que evolução em ambiente de aquecimento global permita uma resposta adaptativa no sentido de minimizar o impacto negativo do

stress térmico no comportamento de acasalamento e na fertilidade dos machos e fêmeas. Em contrapartida, a resposta plástica verificada ao nível da recuperação do comportamento de acasalamento dos machos depois da exposição a altas temperaturas, pode ser um mecanismo para garantir alguma descendência. No entanto, a ausência de recuperação da fertilidade dos machos, indica que o stress térmico continuará a ter consequências negativas para o output reprodutivo dos machos e, possivelmente para a continuidade das populações. Por fim, a resposta plástica observada sob a forma de uma maior propensão para re-acasalar por parte das fêmeas pode atenuar as consequências negativas da esterilidade dos machos, reduzindo repercussões adversas na sua reprodução e, consequentemente, na abundância e persistência das populações.

Palavras-chave: Adaptação térmica; Fertilidade; Comportamento de acasalamento; *Drosophila subobscura;* Evolução experimental

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Introduction

Global climate change, induced mainly by anthropogenic activities, has been one of the most critical issues of the last decades. The rising temperature is a key factor contributing to climate change. The most recent projections point to an increase in average surface temperature between 0.2°C to 0.6°C per decade in the 21st century (IPCC 2022). In addition to the mean temperature rise, an increase in thermal amplitude on daily and seasonal timescales, as well as an intensification of extreme heat events and heatwaves occurrence, is also expected (IPCC 2022). These environmental shifts are negatively affecting biodiversity worldwide (Nunez et al., 2019; Pecl et al., 2017)

In ectotherms, thermoregulation is primarily behavioural as these organisms do not have the ability to regulate their internal body temperature (Davenport, 1992; Kingsolver et al., 2013). For that reason, this group – which is the foremost representative of terrestrial biodiversity (Stork et al., 2015) – where insects are included, could be among the organisms most affected by the rising temperatures. Ectotherms' morphology, physiology, behaviour, and performance are influenced by temperature (Buckley & Huey, 2016; Walsh et al., 2019; Wang & Gunderson, 2022) and changes in these traits will have profound consequences on species distribution, abundance, and interactions (Castañeda et al., 2019; Hoffmann et al., 2003; Root et al., 2003; Zhang et al., 2016). The effects of thermal exposure on organisms' performance depend on several factors, such as the duration and amplitude of exposure (Jørgensen et al., 2006), the life cycle stage at which exposure takes place (e.g., Sales et al., 2021; Walsh et al., 2021), the sex exposed (e.g., David et al., 2005; Iossa, 2019), the mating system of the organisms (Baur et al., 2022; Moiron et al., 2022) and temperature itself (e.g., Vasudeva et al., 2014). Considering this, it is now more important than ever to increase our understanding of the impacts of rising temperatures on ectotherms abundance and distribution.

When behavioural regulation is not sufficient, to prevent population decline or, ultimately, extinction, individuals can adapt to the novel environmental challenges, adjust through different types of phenotypic plasticity, or disperse to new suitable locations (Beldade et al., 2011; Kellermann & van Heerwaarden, 2019). However, in some life cycle stages of many organisms the latter strategy, habitat tracking, can be difficult or even impossible to achieve. For instance, in holometabolous insects, this is particularly true during development, at which time individuals have reduced mobility (Dillon et al., 2009). Therefore, these insects should suffer an exceptionally high pressure to respond to thermal shifts, through plasticity and/or adaptation (Kellermann & van Heerwaarden, 2019; Kingsolver et al., 2013).

Adaptation to new environments is dependent on the genetic architecture of the traits under selection, the standing genetic variation of the populations under study and, the rate of the environmental change itself (Bell, 1997; Hoffmann et al., 2017). During the process of adaptation, antagonistic pleiotropy can play a crucial role, leading to the emergence of trade-offs, and consequently, costs of adaptation (Kawecki & Ebert, 2004). Adaptation to new (warmer) thermal conditions has been reported in some studies in ectotherms (Barghi et al., 2019; Hoffmann et al., 2003; Kellermann & van Heerwaarden, 2019; Perez & Aron, 2020; Tobler et al., 2015). Additionally, intraspecific variation has been shown to be important to respond to temperature; accordingly, Austin and Moehring (2018) showed that different populations of *Drosophila melanogaster* were locally adapted to their own thermal environment. Furthermore, this idea is supported by a plasticity study done by Porcelli et al (2017) in which, *Drosophila subobscura* populations from different latitudes had distinct responses to high temperatures. Despite that, many experimental evolution studies failed to show evolutionary responses to temperature, thus suggesting that the evolutionary potential of ectotherms to respond to climate

change is low (Kellermann et al., 2009, 2015; Kellermann & van Heerwaarden, 2019; Schou et al., 2014). More experimental evolution studies will be important to understand the adaptive capacity of populations evolving under a changing climate, namely under increasingly higher temperatures; moreover, it is crucial to continue to assess if populations with different genetic backgrounds respond similarly to the same selective pressures.

Plasticity is the capacity of a single genotype to produce different phenotypes in response to distinct environmental conditions (Beldade et al., 2011; Pigliucci, 2001). Thermal plasticity can impact fitness-related traits - such as life history and reproductive traits that are critical for a thermal response (Porcelli et al., 2017) - being able to both promote or hinder adaptation (Oostra et al., 2018; Price et al., 2003). Some studies show that stressful thermal conditions during juvenile stages enable a higher adult performance, suggesting positive effects of developmental plasticity (Beaman et al., 2016; Sgrò et al., 2016). On the other hand, other studies demonstrate that warmer developmental temperatures can adversely affect adult traits (Klockmann et al., 2017; Rodrigues et al., 2022; Sales et al., 2018; Santos et al., 2021a; Simões et al., 2020). Furthermore, exposure to high temperatures during the adult stage can also have negative impacts on individuals' fitness. For instance, a study with damselflies (*Ischnura elegans*) showed that heat-stressed adults had reduced body mass, limited ability to fly and a lower immune function than control individuals (Janssens et al., 2014). Furthermore, many Drosophila species and *Tribolium castaneum* exhibited reduced fertility after adult exposure to thermal stress (Canal Domenech & Fricke, 2022; Parratt et al., 2021; Sales et al., 2021). Given these findings, it is essential to deepen our understanding of the impact of stressful temperatures on reproductive success, which is crucial for population persistence.

In the past, studies have mostly focused on the effects of rising temperatures on survival of individuals instead of on their fertility (reviewed in Wash et al., 2019). Recently, it has been reinforced that different traits (viability, survival, fertility) could have contrasting thermal sensitivities, with fertility (defined as the ability to produce offspring) being negatively affected at lower temperatures (Ma et al., 2021; Parratt et al., 2021; Walsh et al., 2019; Zhang et al., 2013). Therefore, future studies should increase focus on the effects of temperature on the individuals' fertility rather than survival. Thermal sensitivity has also been shown to differ between sexes, with males often being more affected by rising temperatures than females (David et al., 2005; Iossa, 2019; Sales et al., 2018; Zwoinska et al., 2020).). In fact, fertility loss in males is also observed in several species when adult individuals are exposed to high temperatures (Sales et al., 2018; Vasudeva et al., 2021). One likely explanation for this phenomenon is that spermatogenesis has a higher thermal sensitivity than oogenesis (David et al., 2005). In addition, recent studies indicate that males' thermal fertility limits are better predictors of species' persistence and distribution than the upper critical thermal limits (CT_{max}) in a global warming scenario (Parratt et al., 2021; van Heerwaarden & Sgrò, 2021). Importantly, in species in which females are more resistant to heat-induced sterility than males, they could function as a buffer against population decline. This effect should be dependent on the species' mating system and the sexes' mating behaviour (Iossa, 2019). Considering this, further research is needed to further assess differences of thermal sensitivity between males and females in different thermal scenarios and understand how these differences affect reproductive output.

To fully grasp the consequences of heat stress on fertility, it is crucial to understand whether heat-induced sterility in males is transient or permanent during the individuals' lifetime. Yet, to my knowledge, few studies have tackled this question. An experiment using the flour beetle *T. castaneum* revealed that males of this species were able to completely recover their fertility after thermal stress exposure in between 15 to 28 days (Sales et al., 2021). This recovery was observed when the stress

was applied in different life cycle stages and with different intensities (Sales et al., 2021). Furthermore, a study performed in *D. melanogaster* showed that a partial recovery of male fertility occurred 6 days after eclosion when heat exposure took place during development (Canal Domenech & Fricke, 2022). In contrast, Parratt et al. (2021) showed that in various species of the Drosophila genus, there was no recovery of fertility seven days following male adult exposure to high temperatures. Additionally, work in *Drosophila virilis* demonstrated that male adult flies exposed to high temperatures had no significant losses in fertility immediately after heat exposure but lost fertility over time and stayed permanently sterile over the rest of the experiment, which lasted until males were 27 days old (Walsh et al., 2021). However, the same work reported that males that were submitted to stress in the pupae stage recovered their fertility throughout the experiment (Walsh et al., 2021). This discrepancies between studies calls for further experiments to better understand the impacts of thermal stress on male fertility during the entire life cycle.

Both pre- and postcopulatory mating behaviours can be affected by rising temperatures (Costa et al., 2022; Farrow et al., 2022; Leith et al., 2020; Sutter et al., 2019; Vasudeva et al., 2021), and are important to populations' response to high temperatures (Gómez-Llano et al., 2021; Sutter et al., 2019; Vasudeva et al., 2021), potentially helping to mitigate the effects of heat-induced male sterility. Studies in some ectotherms' species showed that matings involving heat-exposed males take longer to happen (a precopulatory response; Costa et al., 2022; Leith et al., 2020); which could be linked to female choice between males subjected to different thermal conditions or to a lower eagerness to mate (indicative of male condition). Simultaneously, mating with multiple males (a postcopulatory behaviour) when male sterility is prevalent has been shown to rescue female reproductive success (Sutter et al., 2019; Vasudeva et al., 2021). These studies highlight the importance of polyandry, suggesting that the species mating system plays an important role in the response to rising temperatures (Baur et al., 2022; Moiron et al., 2022).

Indeed, the mating system has enormous repercussions for species at both the individual and the population levels. At the individual level, differences in the mating system could lead to variations in life-history traits highly related to fitness, such as female reproductive output and female longevity (Arnqvist & Nilsson, 2000). At the population level, alternative mating systems may impact on relevant aspects such as inbreeding and effective population size (Holman & Kokko, 2013), the conflict between the sexes (e.g., male harassment or male parental care; Holman & Kokko, 2013) and male-male competition (Lizé et al., 2012). Additionally, distinct mating systems can lead to different responses to heat stress, with a study performed with *T. castaneum* showing that polyandrous females have higher reproductive success than non-stressed females in a heatwave scenario, while heat-stressed monandrous females showed reduced reproductive output when compared to control females (Moiron et al., 2022). Note that the classification of the term mating system has been changing with time, with different researchers' using the term to define distinct situations. For example, when females mate multiple times but there is strong first or last male sperm precedent, some authors consider this species monandrous (Kokko et al., 2014), while others consider them polyandrous. Here we used the latter definition of the term.

Monandrous species should be particularly affected by male sterility and consequently by global warming, since in stressful environments, namely in warmer temperatures, when a monandrous female mates with a less fertile male her reproductive output will be reduced, while if this happens to a polyandrous female, she can mate again, mitigating the reduction of her fitness. Taking this into consideration, the evolution of female remating behaviour and/or the exhibition of a plastic response in which females displayed this behaviour more often at higher temperatures, are possible solutions to

overcome male sterility in monandrous species. Yet, to my knowledge, there are no studies testing both hypotheses, since most studies in this field were done using already polyandrous species (Sutter et al., 2019; Vasudeva et al., 2021). Furthermore, these studies focus on the plastic response of the populations under study and did not analyse the evolution of the behaviour and, consequently, the evolution of the mating system, leaving a gap in our knowledge.

Taking all of this into consideration, understanding if and how monandrous species change their mating behaviour under heat stress will be key to assessing how likely is population persistence under climate warming. To fill this gap, we used the native Palearctic fruit fly *Drosophila subobscura*. This species is exceptional to study thermal adaptation, since this fly has a wide geographical distribution and chromosomic inversions that show pronounced variations in frequency and are locally adapted to spatial and temporal climate trends (Prevosti et al., 1988; Rezende et al., 2010). Additionally, these inversions seem to respond to extreme events like heatwave occurrences (Rodríguez-Trelles et al., 2013) and global warming (Balanyà et al., 2006). Furthermore, temperature changes led to the evolution of higher thermal tolerance and modifications in the (locomotor) behaviour of this fly (Mesas et al., 2021). Contrary to most species of the genus Drosophila, *D. subobscura* is commonly monandrous (Lizé et al., 2012). Indeed, despite evidence of multiple mating under certain circumstances, Fisher et al. (2013) showed that few females (around 14%) exhibit polyandry, indicating that some genetic variation for mating behaviour exists within this species.

Recently, our team has been conducting a thermal experimental evolution study on European populations of *D. subobscura* from different latitudes, - one from low-latitude (Portugal) and other from high-latitude (The Netherlands) –, subjected to a fluctuating warming scenario, to understand how adaptation to an ecologically relevant warming environment occurs. No evidence for an evolutionary response to the warming environment after 9 generations of thermal selection was found (Santos et al., 2021b). On the other hand, Dutch (high-latitude) populations displayed an improved performance at high temperature after 31 generations of evolution (Santos et al., 2023). Nevertheless, these studies were performed at constant temperatures and did not analyze the direct adaptive responses to the fluctuating warming environment in which populations evolved. Furthermore, the male and female behavioral and fertility responses after male exposure to high temperatures were not assessed.

All things considered, this thesis aimed to answer the following questions:

i) Does adaptation to a warming environment occur and does it imply adaptation costs?
ii) How does heat shock during male early life influence male mating behaviour and fertility, and is this effect transient or not?
iii) What are the impacts of heat-induced male sterility on female mating behaviour and fertility?
iv) Within the goals mentioned above we wanted to understand the following question: Does selection and population history play a role in the populations' responses?

For this, we studied the real-time evolution of two historically differentiated *D. subobscura* populations, subjected to a global warming scenario (mentioned above), in a controlled experimental setting. Ultimately, this thesis will provide new insight into the effects of rising temperatures on mating behaviour and fertility in a monandrous insect, allowing to better assess and predict population persistence under a changing climate.

Materials and Methods

1) Experimental populations & Selective regimes

This study involved two historically differentiated European populations of *D. subobscura*, one founded with 213 females from Portugal (Adraga, PT), and another with 170 females from The Netherlands (Groningen, NL) - see Simões et al. (2017). Both populations were kept in similar lab conditions for 70 generations: in large population sizes (census size ranged between 500 and 1000 individuals), with controlled density in the adult (40 individuals per vial) and developmental stage (70 eggs per vial), discrete generations of 28 days (adults were removed from the vials after the eggs were laid), maintained at a constant temperature of 18ºC under a 12:12 light:dark cycle. In 2019, each of these populations was divided and placed under three different regimes differing only in their temperature cycle ("Selection"; see Figure S1): Control (C), Fluctuating (F) and Warming (W), each with three replicate populations. This resulted in a total of 18 populations, nine of Portuguese origin, three kept under the Control regime (PT1-3), three under Fluctuating (FPT1-3), and three under the Warming regime (WPT1-3); and nine of Dutch origin, three kept under the Control regime (NL1-3), three under Fluctuating (FNL1-3), and three under Warming (WNL1-3) (see Figure S1). The Control regime maintained a constant temperature of 18°C throughout the generations. The Fluctuating regime had a circadian temperature cycle ranging between 15° C and 21° C, with no change throughout the generations. Finally, the Warming regime had an initial daily fluctuation between 15°C and 21°C but suffered a progressive increase in both average temperature (0.18ºC) and thermal amplitude (0.54°C) at each generation. Populations are still evolving in these regimes for almost 50 generations. However, populations from the Warming regime had severe crashes when the maximum temperature reached 30ºC. For that reason, the thermal cycle in this regime had to be fixed at the 20th generation of evolution and, from that generation onwards, it oscillates between 13.5° C and 29.4° C with a mean temperature of 21.4ºC (see Figure S2). This study focuses only on the populations from the Control (ancestral state of the Fluctuating and Warming regimes) and Warming regimes, as it aims to understand the populations' responses to a global warming scenario. It covers thermal evolutionary responses from generation 39 to generation 45 of thermal selection.

2) Pilot tests to determine the optimal conditions for the mating assays

2.1) Male dyeing and the dye as a marker for mating occurrence

Two different pilot tests were performed to test whether males could be marked with a dye and if this dye could be used as a marker of mating occurrence, considering the expectation that females would be dyed following mating with a dyed male. In both approaches recently emerged virgin males from all populations of the Control regime (PT1-3, NL1-3) developed at 18°C were anaesthetised with CO_2, separated into groups of five individuals, and maintained at 18°C until being dyed using powder or food dye. In both cases, 5-day-old males were dyed and checked visually to assess if the dye was visible. In *D. subobscura*, courtship involves sparring (taps with the forelegs) and dancing, where males and females stand in front of each other while extending and flapping their wings (Wallace & Dobzhansky, 1946). During the dance, a passage of a (regurgitated) droplet from male to female (nuptial feeding) can happen. It is thought that this droplet is a gift, given from the male to the female to enhance his chances of mating. Feeding from regurgitated gifts is shown to improve female fecundity in certain environmental conditions (Immonen et al., 2009). After the dance, males do a

circular movement around the females to mount them and perform the copula (Wallace & Dobzhansky, 1946).

2.1.1) Powder dye treatment

The powder dye treatment was discarded because the wings of the males were affected by the powder, likely resulting in a decrease in male condition.

2.1.2) Food dye treatment

The protocol of the food dye treatment was as follows. 20 µl of blue or red dye were added to 1 ml of the food medium (axenic medium by David; David, 1962), following an adapted version of the protocol from Verspoor et al. (2015), 5-day-old males from control populations were allowed to feed from the dyed medium for 24 hours. Posteriorly, males were paired with 5-day-old virgin females - maintained at 18ºC since the egg stage - from the same population for three hours at room temperature (21ºC) in a vial without food dye, to guarantee that females could only become dyed from an interaction with the dyed male. To ensure the virginity of both males and females, the separation of individuals using CO_2 anaesthesia was performed 2 or 3 hours after emergence (this procedure was done in all assays). After pairing females' abdomens were checked visually for the presence of the dye every 20 minutes for 3 hours. The food dye did not seem to have an apparent effect on male condition and the colouration lasted approximately 4 to 5 hours. Due to the mating ritual of this species (see 2.1 above for further details) the food dye can be transmitted from male to female in two distinct moments, during the nuptial feeding and/or during the copula. During this pilot the matings were not observed, thus it was not possible to assess when the transmission of the dye from male to female occurred. This was tackled in another pilot test (see 2.3 below).

2.2) Conditions for heat-induced sterility

To inflict a significant negative effect on male fertility, while not producing high mortality (i.e., keeping it below 40%) two different approaches were tested. The first approach used a heatwave applied during part of the developmental stage and the first days of adulthood (see 2.2.1 below), while the second approach used a heat shock during early adult life (see 2.2.2 below).

2.2.1) Heatwave treatment

Individuals from the Control regime were exposed to an 11-day heatwave that had a constant temperature of 26ºC. Prior to the heatwave treatment, vials with 70 eggs were kept at a benign temperature of 18ºC. In the third instar larvae stage (around day 8 of development) the heatwave of 26°C started. Upon emergence individuals were anaesthetised with CO_2 and males were separated into groups of four. Following this, males were placed again at 26°C until their fourth day of adulthood. Males were then placed at 18ºC for approximately 18 hours and then each was paired with a virgin female from their population for 30 hours. These females had developed since egg at 18°C and, upon emergence, were anaesthetized with CO_2, separated into groups of ten and maintained at 18°C until pairing that occurred when females were 5 days old. After the mating period, males were discarded, and females were allowed to lay eggs for 42 hours at 18°C. The number of eggs was counted and 19 days later the number of adult individuals emerged from these eggs was assessed to measure fecundity

(number of laid eggs), offspring viability (ratio between the number of adult offspring and the number of laid eggs), and male reproductive success (number of adult offspring).

This treatment was applied twice, in 16 males from the NL1 population and in males from all populations of the Control regime (PT1-3, NL1-3, 7 males per replicate population).

2.2.2) Heat shock treatment

1-day-old males from the Control (PT1-3, and NL1-3) and Fluctuating (FPT1-3, and FNL1-3) regimes maintained in the conditions of their selective regime since egg stage were subjected to a heat shock. To do so, 24 hours prior to the heat shock, recently emerged virgin males were separated into groups of five using CO_2 anaesthesia. To achieve the combination of temperature and exposure time which induced the highest negative impact on male fertility, various conditions were tested (see Table S1).

After heat stress, each male was paired with a virgin female from the same population. These females had developed since egg stage at 18°C and, upon emergence, were anaesthetized with CO_2, separated into groups of ten and maintained at 18°C until the pairing. To estimate fecundity, offspring viability, and reproductive success, the number of eggs was assessed and 19 days later the number of adult offspring emerged from these eggs was counted.

2.3) Assessing heat-induced sterility and the efficacy of food dye as a marker for mating occurrence

To address the impact of a heatwave on male fertility and mating behaviour as well as the effectiveness of the food dye as a marker for mating occurrence an experiment including four different treatments was designed: 1) Non-stressed, non-dyed males - Males maintained at 18°C, not dyed; 2) Non-stressed, dyed males - Males maintained at 18°C, dyed; 3) Stressed, non-dyed males - Males exposed to a heatwave, not dyed and, finally, 4) Stressed, dyed males - Males exposed to a heatwave, dyed. The dye used to colour the males was the red food dye, which produced the most vibrant colours on males. This assay was performed using individuals from Control populations after 41 generations of thermal evolution and, 32 males (8 per treatment) from each replicate population were tested. Males from all populations and all treatments were present in all experimental racks (of 48 vials) in the same number; furthermore, the position of the males was pseudo-randomized. This assay was performed in two consecutive days; both days had the same number of individuals per population.

The protocol followed to impose a heatwave was described above (section 2.2.1), apart from the following steps: 1) The conditions described for non-stressed females above apply here to both non-stressed males and females; 2) During the recovery period of 18 hours, males from the stressed dyed and the non-stressed dyed treatments were fed with red-dyed medium (see section 2.1.2 above for further details). All males were then paired with virgin females from their population for 24 hours, in a new vial without food dye. In the first two hours mating pairs were maintained at room temperature (~ 21°C) and their mating behaviour was registered. The occurrence of nuptial feeding and the occurrence of copulation were also registered. Additionally, females were checked visually for a dyed abdomen after the nuptial feeding and copula to ascertain the origin of the dyed abdomen (nuptial gift or copula). After the 2 hours of observation, the pairs were maintained at 18°C until the next day. Following the 24 hours of the mating period, the males were discarded, and the females were kept for 48 hours egg laying and then discarded. The number of eggs was counted and 19 days later the

number of adult individuals emerged from these eggs was assessed to measure fecundity, offspring viability and reproductive success.

3) Adaptation to a warming environment

To determine if there was adaptation to warming conditions, both Control and Warming populations were analysed in each environment (control environment and warming environment), after 39 generations of evolution. To eliminate maternal environmental effects that may confound the detection of selective responses (Magalhães et al., 2011), we performed a common garden procedure by maintaining individuals from all populations for one generation in the control environment (constant temperature of 18ºC). Virgin males and females recently emerged from the common garden were anaesthetised with CO_2 and paired. Twenty couples from each replicate population of the two thermal regimes were established and moved to one of the tested environments. These pairs were maintained in these conditions for nine days and transferred to new medium daily. The experiment in each tested environment followed a block design, with block being defined as the set of same-numbered Control and Warming replicate populations assayed in the same pseudo-randomized experimental rack. The three blocks were assayed in synchrony. The eggs were counted between days 6 and 9 after pair formation and the sum of eggs laid between the 6^{th} and 9^{th} day was used to estimate fecundity (total number of eggs laid). To determine offspring viability and reproductive success, the eggs laid on the ninth day were allowed to develop, and 19 days later the number of emerged adult offspring was counted during a period of ten days after the first emergence.

4) Recovery of male mating behaviour and fertility following heat shock

An assay was performed to determine the long-term consequences of high temperatures on male mating behaviour and fertility. The assay monitored the behavioural and reproductive performance of males from the 4^{th} to the 12^{th} day of adult life. This assay focused on males from Control and Warming regimes and was performed after 45 generations of thermal evolution. To remove parental environmental effects, 18 vials (with approximately 70 eggs each) from the Control and Warming populations were maintained in the control environment for two generations before this assay. The common garden lasted two generations instead of one since the Warming populations had a populational crash prior to the assay and were, therefore, transferred earlier to Control conditions to reduce the environmental thermal stress. Virgin males recently emerged from the common garden of each replicate population were anaesthetised with CO_2 and were maintained at 18ºC in groups of five until the heat-shock (stressed males) or until being paired with virgin females (non-stressed males).

One-day-old males from Control and Warming populations were subjected to a heat shock treatment for 69 hours at 31ºC (resulting from the pilot test explained in section 2.2.2 above). After the heat-shock, males were placed at 18ºC for 4 hours to recover from the stress. Then males were paired with virgin females (ranging from 4 to 6 days old) from the Fluctuating regime for approximately 28 hours. Recently emerged virgin females were taken directly from the Fluctuating selection regime and separated into groups of ten until mating using CO_2 at 18ºC. By using non-stressed females from the Fluctuating populations, differences in responses between treatments can be assigned exclusively to differences in males. Mating pairs were assigned taking into consideration the number of the replicate population, e.g., males from PT1 and WPT1 were paired with females from FPT1. In the first two

hours of each mating, the beginning of the courtship, and the beginning and end of copulation were registered. This allowed to estimate behavioural traits, such as the courtship latency, the copulation latency, and the copulation duration. In addition, the occurrence of extra matings was checked every 5 minutes for roughly nine hours. All the observations were done at room temperature (21ºC), and subsequently, the couples were placed at 18ºC (from 9 hours to 28 hours). Following the 28 hours, males were moved to a new vial with new medium. Females were kept in the vial where the mating occurred and were allowed to lay eggs for 44 hours. To assess fecundity, the eggs were counted, and to measure offspring viability and reproductive success the eggs were allowed to develop, and the number of emerged adults was counted during a period of seven days after the first emergence that took place 19 days after egg laying. Three (72 hours) and eight (192 hours) days after the first mating, the same males had the possibility to mate a second and a third time, respectively, with new virgin females (4-6 day old) in new vials, following the same protocol (see Figure 2.1). After the third mating males were discarded.

In this assay, 48 males (24 stressed and 24 non-stressed) from each replicate population of the Control and Warming regimes were tested. The assay was performed in blocks, with each block, i.e., set of same-numbered control and warming replicate populations tested on three separate consecutive days (one day for each block). The same number of males from all populations and treatments were assigned to each rack and within each rack the position of the males was pseudo-randomized.

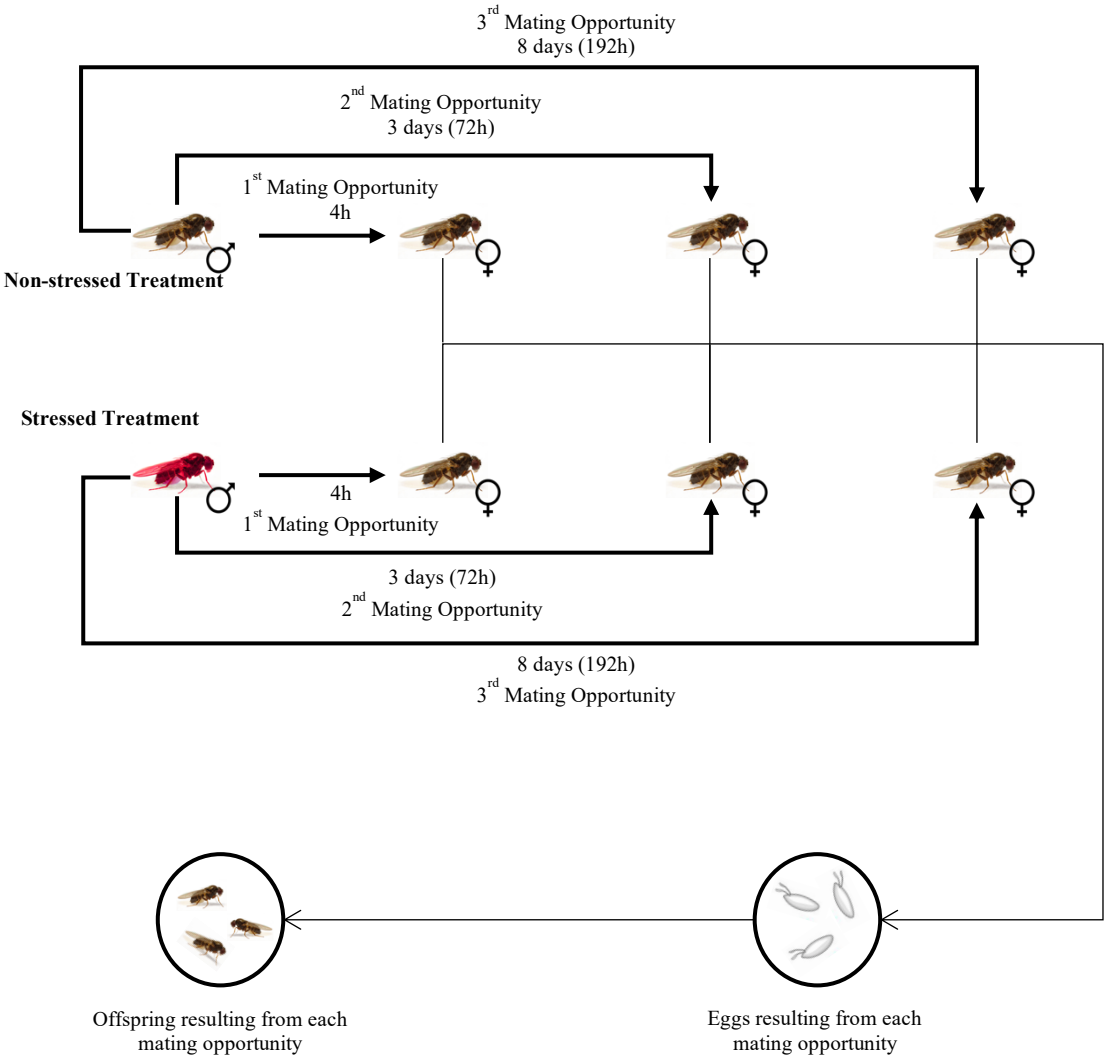


Figure 2.1 – Schematic representation of the protocol used to test the recovery of male mating behaviour and fertility following heat shock. The assay was performed using males from the Control and Warming regimes after 45 generations of thermal evolution. Red flies represent stressed males (exposed to a heat shock of 31ºC for 69 hours), while non-coloured flies represent non-stressed males and females. The time points represent the hours after heat shock when each mating opportunity occurred. In each mating opportunity the courtship latency, copulation latency and copulation duration were registered for 2 hours. Later, for each vial fecundity, offspring viability and reproductive success were assessed by measuring the number of eggs laid and the number of adult offspring produced.

5) Female mating behaviour and fertility following male heat shock

To test whether reduced male fertility affects the female mating behaviour, female fertility, and specifically the female propensity to remate, a remating assay was performed. Three distinct treatments were designed where females were paired with non-stressed and/or stressed males: 1) Non-stressed x non-stressed treatment – females had the opportunity to first mate with a non-stressed male and then with a new non-stressed male; 2) Stressed x non-stressed treatment – females had the opportunity to mate with a stressed male and then with a non-stressed male; 3) Stressed x stressed treatment – females had the opportunity to mate with a stressed male and then with a new stressed male (see Figure 2.2). This assay focused on females from Control and Warming regimes and was

performed after 45 generations of thermal evolution. To remove parental environmental effects, 14 vials (with approximately 70 eggs) from Control and Warming populations were maintained in the control environment (at 18°C) for one full generation prior to the remating assay. Following the same reasoning as in the previous assay, females were crossed with virgin males from an outgroup, the Fluctuating regime, the number of the replicate populations being taken into account when assigning the pairs (e.g., females from PT1 and WPT1 were paired with males from FPT1). Virgin females recently emerged from the common garden of each replicate population were anaesthetised with CO_2 and were maintained at 18ºC in groups of ten until being paired with a male (first mating). Recently emerged virgin males from Fluctuating populations were collected and separated into groups of five using CO_2 anaesthesia. After collection, males were maintained at 18ºC for 4 days until being paired with a female (non-stressed males) or for 24 hours until being exposed to a heat shock treatment for 69 hours at 31ºC (heat-stressed males; see 2.2.2 above for detailed information). Heat-shocked males had the possibility to recover for 4 hours prior to being paired with a female.

In the first mating opportunity females were 4 days old and were paired with 5-day-old males. Posteriorly, the same females had the opportunity to mate again with a new 5-day-old male. Each mating was allowed within a 28-hour period and there was a 3-day interval between the first and the second mating. To assess the courtship and copulation latencies and the copulation duration, the timings of courtship beginning, copulation beginning, and copulation end were registered in the first 2 hours of each mating opportunity. The occurrence of matings kept being checked every 5 minutes for approximately 10 more hours. After each mating, males were discarded, and females were allowed to remain in the vials for 44 hours for additional egg laying. To estimate fecundity, the eggs were counted, and to assess offspring viability and reproductive success the eggs were allowed to develop, and the number of adult offspring was counted. The number of adult offspring was counted during a period of seven days after the emergence of the first individual that took place 19 days after egg laying. It is important to note that female remating was considered if both the first and second copulations were seen or if the female had adult offspring resulting from the first mating and a second copulation was observed.

In this assay, 24 females per treatment and replicate population (within each selection regime) were tested. As in the previous assay, a block experimental design was applied, where same-numbered Control and Warming replicate populations were tested in synchrony, on three consecutive days (one day for each block). Moreover, females from all populations and treatments were assigned to each rack and within each rack their position was pseudo-randomized.

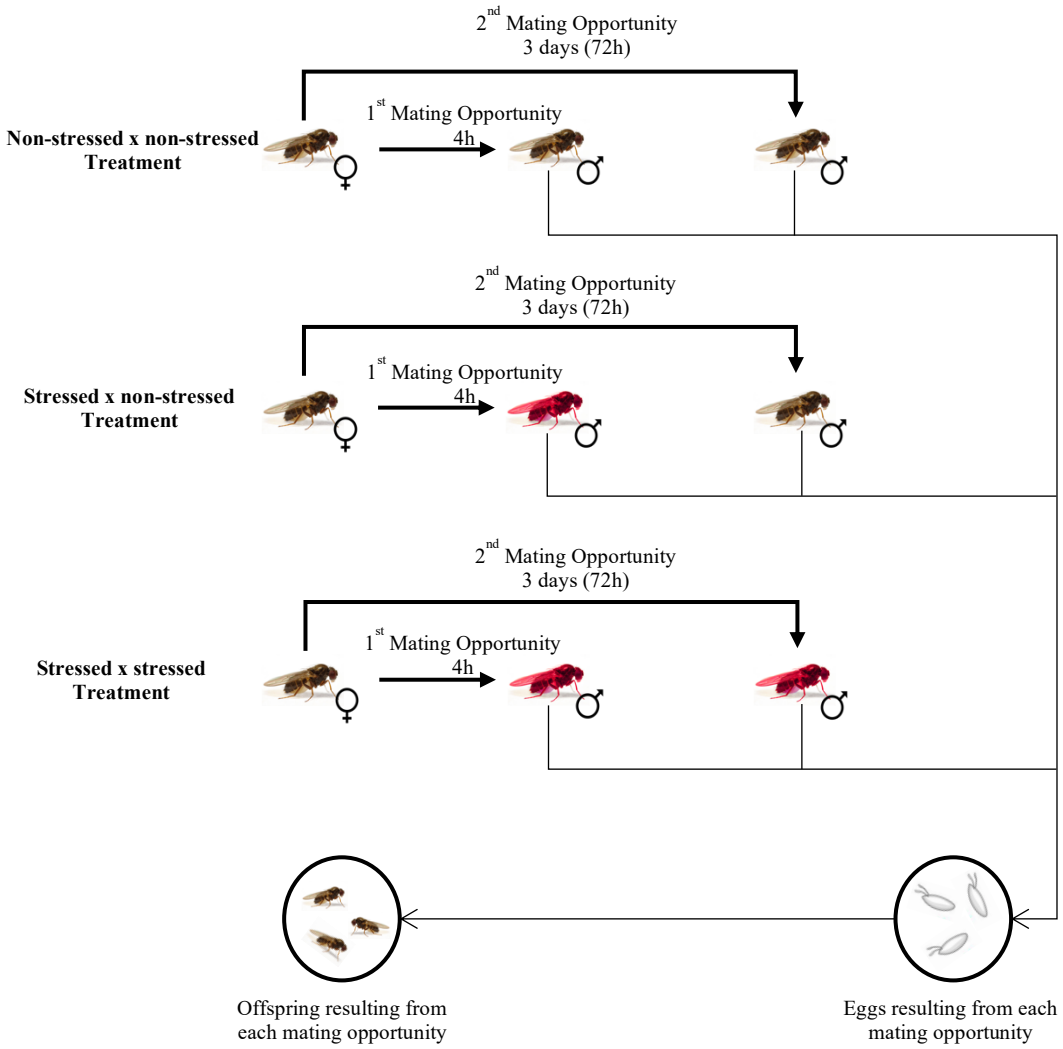


Figure 2.2 – Schematic representation of the protocol used to test the female mating behaviour and fertility following male heat shock. The assay was performed in females from the Control and Warming regimes after 45 generations of thermal evolution. Red flies represent stressed males (exposed to a heat shock of 31ºC for 69 hours), while non-coloured flies represent non-stressed males and females. The time points represent the hours after heat shock when each mating opportunity occurred. In each mating opportunity the courtship latency, copulation latency and copulation duration were registered for 2 hours. Later, for each vial fecundity, offspring viability and reproductive success were assessed by measuring the number of eggs laid and the number of adult offspring produced. Additionally, the female propensity to remate was also estimated.

6) Statistical analyses

All statistical analyses were performed using the software for statistical computing R (R Core Team, 2022, version 2022.7.1.554). Linear models (LM) and linear mixed-effect models (LMM) using the package *lme4* (Bates et al., 2015), mixed-effects Cox models using the package *coxme* (Terry, 2022) and generalized mixed-effects models (GLMM) with *glmmTMB* (Brooks et al., 2017), were performed depending on the dependent variables and the error structure of the data (see Tables S1, S2 and S3). A "sum to zero" contrast option was defined for each factor. Raw individual data was used in the

analysis of all dependent variables. When applicable, normality assumptions and the overdispersion of the data were verified. Maximal models were simplified by removing non-significant interaction terms from the highest- to the lowest-order interaction (see Tables S1, S2 and S3). Model simplification ended when the lowest AIC (Akaike information criterion) value was reached (models with the lowest AICs are those that best fit the data; (Crawley, 2012)). However, the explanatory variables *per se* were never removed from the model, even when their effect was not significant. The significance level of different factors and their interactions were obtained through analyses of variance (Anova; package *car*; Fox & Weisberg, 2019). Furthermore, when the best model contained significant triple interactions or more than one double interaction, additional analyses were performed by subsetting the original dataset into the different levels of a fixed factor present in the model (Tables S1, S2 and S3). The *emmeans* package (Length, 2020) was used to perform *a posteriori* contrasts using Tukey tests. All graphical representations were done with the mean values of each replicate population and were generated using the *ggplot2* package (Wickham, 2016).

6.1) Pilot tests to determine the optimal conditions for the mating assays

6.1.1) No statistical analyses were performed for the 2.1.1; 2.1.2; 2.2.1 and 2.2.2 pilot tests.

6.1.2) Assessing heat-induced sterility and the efficacy of food dye as a marker for mating occurrence

To analyse how a heatwave during a part of the developmental and adult stage influenced males' reproductive output as well as the effectiveness of a food dye as a marker for mating occurrence three traits were studied: Offering of a droplet, copulation occurrence and reproductive success.

A GLMM model with a negative binomial error distribution and a parameter to account for zero-inflated data (ziformula ~1; package *glmmTMB*) was used to analyse reproductive success. The model included History (Portuguese or Dutch), Dye (dyed male or non-dyed male), Treatment (stressed male or non-stressed male), and their interactions as fixed factors. Block, defined as the set of same-numbered replicate populations (1, 2, or 3), was included as a random factor.

6.2) Adaptation to a warming environment

To test for adaptation to warming conditions, three traits were analysed: fecundity, viability, and reproductive success. For fecundity (number of eggs per vial) and reproductive success (number of adult offspring per vial), GLMM models with a quasi-Poisson error distribution and a parameter to account for zero-inflated data (ziformula ~1; package *glmmTMB*) were used. Offspring viability was computed using the function *cbind* with the number of successfully hatched eggs and the number of unhatched eggs as arguments, and a binomial error distribution. The models of all three traits included Selection (Control or Warming), History (Portuguese or Dutch), Environment (control environment or warming environment) and all possible interactions as fixed factors. The Block (defined as the set of same-numbered replicate populations: 1, 2, or 3) was included as a random factor (see Table S1).

Given that a triple interaction between History, Selection, and Environment was present in the model with the lowest AIC, further analyses were performed. So, to better understand the responses of populations with different historical backgrounds, analyses were done for Portuguese and Dutch populations separately. Analyses were similar to those described above with models for each trait

including Selection, Environment, and their interaction as fixed factors, and the Block as a random factor.

6.3) Recovery of Male mating behaviour and fertility following heat shock

To analyse how a heat shock during early life influenced males' mating behaviour and reproductive output throughout most of their lifespan, six traits were studied: Courtship latency, copulation latency and duration, fecundity, viability, and reproductive success (see Table S2).

Courtship latency and copulation latency were analysed using a mixed-effect Cox model with a gaussian error distribution. The data was box cox transformed (courtship latency: $\lambda = 0.03$; copulation latency $\lambda = 0.75$; *MASS* package; Venables & Ripley, 2002) to improve the fit of the model. A LMM model with a gaussian error distribution was used to analyse the copulation duration. The data was also box cox transformed to improve the fit of the model (copulation duration: $\lambda = 1$). The models for these three traits included History (Portuguese or Dutch), Selection (Control or Warming), Treatment (Stressed male or non-stressed male), Mating Opportunity (first, second or third, that corresponded to 4, 72 and 192 hours after heat stress respectively) and their interactions as fixed categorical factors. Block was included as a random factor.

To assess fecundity (number of eggs per vial) and reproductive success (number of adult offspring) GLMM models with a quasi-Poisson error distribution and a parameter to account for zero-inflated data (ziformula ~1; package *glmmTMB*) were used. Offspring viability was computed using the function *cbind* with the number of successfully hatched eggs and the number of unhatched eggs as arguments and was analysed using a GLMM model with a binomial error distribution. The models for these three traits included History, Selection, Treatment, Mating Opportunity, and the interaction between them as fixed factors. Additionally, the Block was incorporated into the model as a random factor. Given that each male was tested multiple times (throughout the three mating opportunities), we accounted for repeated measures (Park et al., 2009) by adding the interaction between male ID (unique identification of each male) and Mating Opportunity as a covariate to the model.

Due to a significant quadruple interaction between all factors affecting fecundity, offspring viability, and reproductive success, analyses were done separately for populations from different bio-geographical origins (factor History) to further investigate the variation in populations' responses. Analyses were similar to those described above with models for each trait including Selection, Treatment, Mating Opportunity, and the interaction between them as fixed factors, the Block as a random factor and the interaction between male ID (unique identification of each male) and Mating Opportunity as a covariate. The error structure of these models was also similar to that of the original models, except for the analysis of fecundity and reproductive success of Dutch populations, where the model used was a GLMM with a Poisson error distribution and a parameter to account for zero inflation (ziformula ~1).

For fecundity and offspring viability, a significant triple interaction with the Treatment factor was obtained, so additional analyses were done separating the different treatments (Non-stressed or Stressed). These models included Selection, Mating Opportunity, and their interaction as fixed factors and Block as a random factor. The models for the analysis of fecundity were GLMM models with a Poisson error distribution and a parameter to account for zero inflation (ziformula ~1), while for offspring viability the models were GLMM models with a binomial error distribution.

6.4) Female mating behaviour and fertility following male heat shock

To analyse how female mating behaviour and fertility were affected by male thermal treatment (stressed or non-stressed), seven traits were studied: courtship latency, copulation latency and duration, female propensity to remate, fecundity, offspring viability, and reproductive success (see Table S3).

To analyse courtship and copulation latencies mixed-effect Cox models with a gaussian error distribution were used. The data was box cox transformed (courtship latency: $\lambda = 0.27$; copulation latency $\lambda = 0.58$; *MASS* package) to improve the fit of the model. A LMM model with a gaussian error distribution was used to analyse the copulation duration and the data was also box cox transformed to improve the fit of the model (copulation duration: $\lambda = 0.45$). The models for these three traits used History (from Portugal or The Netherlands), Selection (Control or Warming), Treatment (3 different levels: i) Non-stressed x non-stressed; ii) Stressed x non-stressed; iii) Stressed x stressed), Mating Opportunity (first or second) and the interaction between them as fixed factors. Additionally, Block was added as a random factor. *A posteriori* contrasts were performed using Tukey tests to assess the interaction between Treatment and Mating Opportunity in the three traits (courtship latency, copulation latency and duration).

The female propensity to remate (number of matings of females that mated more than once) was analysed using a GLMM model with a binomial (Bernoulli) error distribution. For this trait, the model considered History, Selection, Treatment, and the interactions between them as fixed factors. The Block was used as a random factor.

Fecundity and reproductive success were analysed using GLMM models with a quasi-Poisson error distribution and a parameter to account for zero inflation (ziformula ~1). Offspring viability was computed using the function *cbind* with the number of successfully hatched eggs and the number of unhatched eggs as arguments and was analysed using a GLMM model with a binomial error distribution. The models for these traits included History, Selection, Treatment, Mating Opportunity, and the interaction between them as fixed factors. The Block was included in the model as a random factor. Given that the same females were tested in two different moments (first or second mating opportunities), we accounted for repeated measures by adding the interaction between female ID (unique identification of each female) and Mating Opportunity as covariate to the model.

Due to multiple significant interactions involving the factor History in the analyses of both fecundity and reproductive success, new analyses for these traits were done separately for populations with distinct historical origins. The models for each trait were identical to those described above but including Selection, Treatment, Mating Opportunity, and the interaction between them as fixed factors, and the Block as a random factor. The error structure of these models was also similar to that of the original models, with the exception of the analysis of fecundity of Dutch populations, where the model used was a GLMM with a Poisson error distribution and a parameter to account for zero inflation (ziformula ~1).

Lastly, to assess the effects of the remating behaviour on the trait most related to fitness, reproductive success, an analysis was performed including Remating (No Remating or Remated) as an independent variable. This variable was analysed using a GLMM model with a quasi-Poisson error distribution and a parameter to account for zero inflation (ziformula ~1). Given the high complexity of the model when including History, Selection, Treatment, Mating Opportunity, Remating and the interactions between

them as fixed factors, History and Selection were excluded from the model. Therefore, the model used Treatment, Mating Opportunity, Remating (No Remating or Remated) and the interaction between them as fixed factors. Additionally, the Block was used as a random factor, and once again we accounted for repeated measures by adding the interaction between female ID (unique identification of each female) and Mating Opportunity as covariate to the model. Due to a significant triple interaction between the independent variables, further analyses were performed separating the three different treatments. These models included Mating Opportunity, Remating and the interaction between them as fixed factors, the Block as a random factor and the interaction between the female ID and Mating Opportunity included as a covariate. For the Non-stressed x non-stressed treatment, a GLMM model with a negative binomial error distribution and a parameter to account for zero-inflated data (ziformula ~1) was used. The Stressed x non-stressed treatment was analysed using a GLMM model with a quasi-Poisson error distribution. Finally, for the Stressed x stressed treatment, a GLMM model with a quasi-Poisson error distribution and a parameter to account for zero inflation (ziformula ~1) was used.

Results

Pilot tests to determine the optimal conditions for the mating assays

Male dyeing and the dye as a marker for mating occurrence

The first pilot test indicated that both red and blue food dyes successfully dyed the males throughout ingestion, but the red dye was easier to detect (experiment described in the Material and Methods, section 2.1.2; data not shown). The effect of the red dye was further assessed together with the effect of a heatwave treatment (see Material and Methods, section 2.2.1) and the corresponding results are found below (see results section below).

Conditions for heat-induced sterility

The heatwave treatment showed a very low mortality rate (around 20%), and the male reproductive success (assessed through the number of adult offspring) was zero, so 100% sterility. No visual inspection of male condition was done during this pilot test, so additional tests were needed (see results section below).

In the heat shock treatment, out of the several combinations of temperature and time of exposure employed, the combination that induced a higher reduction in male fertility without seriously compromising survival (remaining above 60%) lasted 69 hours during the early adult stage and had a constant temperature of 31ºC. Under this condition, stressed males from the Control and Fluctuating regimes had on average 33.7 (-53,7%) and 29.9 (-47,6%) fewer eggs than non-stressed males, respectively. Other conditions did not reduce fertility as much or led to a higher mortality rate (see Table S4).

Assessing heat-induced sterility and the efficacy of food dye as a marker for mating occurrence

In the pilot test that combined the effects of the heatwave treatment and the food dye, we found that neither history nor food dye affected male reproductive success (Table S5, Figure S3). Instead, only male thermal treatment influenced the reproductive success of males, as seen before (Heatwave treatment), with males submitted to the heatwave having a drastically reduced fertility (almost zero offspring in all populations) when compared to non-stressed males ($X^2 = 289.496$, $p < 0.001$, see Table S5, Figure S3). The tested conditions visibly affected the wings of the males which became curly and damaged, which can significantly hinder male movement and courtship behaviour. Furthermore, only 50.7% of females that mated with dyed males became dyed themselves, while some females (11.1%) that were not observed mating became dyed as well. This suggested that the colouration is not transmitted during the copula, but in the nuptial feeding and that it is not transmitted every time there is a mating. Given the negative impact of the heatwave on the males' wings and the importance of these during the courtship behaviour (see section 2.1 of the Materials and Methods for additional details), the heatwave treatment was discarded. Moreover, this treatment led to a reduced mating occurrence, with only 43.7% of males subjected to the heatwave being able to mate. Considering that, in one of the assays, the remating rate was a trait of interest, mating occurrence must be higher in the first mating to maximize the possibilities of remating; thus, the heatwave treatment was not appropriate to study the females' remating rates. Additionally, due to the poor efficacy of the food dye as a marker of mating occurrence this method was also discarded.

Altogether, these results helped determining the conditions to be used in following assays: male sterility was induced following a heat-shock protocol lasting 69 hours during the early adult stage at a constant temperature of 31ºC; the occurrence of mating was confirmed visually, without the help of the food dye.

Adaptation to a warming environment

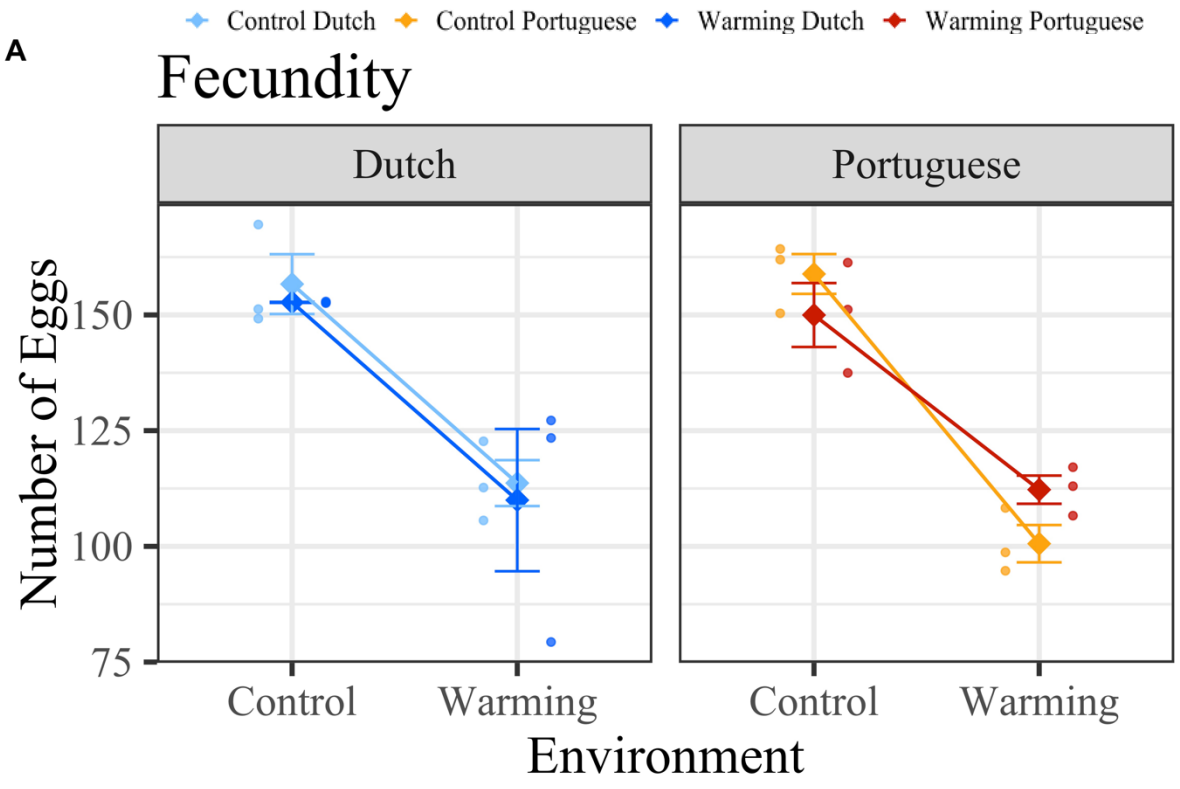
As mentioned above (see Statistical Analyses) the model with the lower AIC included a (marginally significant) triple interaction between History, Selection and Environment (see Table S6). So, to better understand the response of the studied populations to warming, populations with different historical backgrounds were analysed separately.

All traits (fecundity, offspring viability, and reproductive success) of the populations from The Netherlands were only affected by the Environment (X^2 = 61.900, p < 0.001 for fecundity, X^2 = 177.000, p < 0.001 for offspring viability and, X^2 = 99.831, p < 0.001 for reproductive success; see Table 3.1, Figure 3.1), with populations from both selection regimes having lower performances in the warming environment than in control conditions. This poor performance of all Dutch populations in the warming environment, including of the populations from the Warming regime that did not performance better than Control populations in this environment, suggest that they did not adapt to warming (see Figure 3.1).

In the Portuguese populations, fecundity, offspring viability, and reproductive success were significantly shaped by the interaction between Selection and Environment (X^2 = 5.148, p = 0.023; X^2 = 39.230, p < 0.001; X^2 = 11.606, p < 0.001 respectively; see Table 3.1, Figure 3.1). *A posteriori* contrasts done for the trait most related to fitness, reproductive success - which is the product of fecundity and offspring viability - showed that populations from the Warming regime had higher reproductive success in the warming environment relative to Control populations (T ratio = -2.813, p = 0.027; see Table S7, Figure 3.1.C); however, in the control, ancestral environment, there were no significant differences between selection regimes (T ratio = 1.924, p = 0.221; see Table S4). This reveals a pattern of adaptation and indicates that there are no costs of adaptation for reproductive success (Figure 3.1.C).

Table 3.1 – Results from the analyses of variance testing adaptation to a warming environment in the Portuguese and Dutch populations separately. Fecundity: Number of laid eggs between days six and nine; Offspring viability: Ratio between the number of offspring (adult offspring) and the number of laid eggs (total number of eggs) on the ninth day; Reproductive success: number of offspring resulting from eggs laid at day nine. Individuals with one of two bio-geographical origins ("History": The Netherlands or Portugal) that came from one of two selection regimes ("Selection": Control or Warming) were tested in one of two environments ("Environment": control environment or warming environment). "Df": the degrees of freedom. "X^2": the Chi-square value obtained in each analysis. Statistically significant terms are represented in bold.

History	Trait	Independent Variable	*Df*	X^2	*p-value*
The Netherlands	Fecundity	Selection	1	0.152	0.697
		Environment	**1**	**61.900**	**< 0.001**
	Offspring viability	Selection	1	1.305	0.253
		Environment	**1**	**177.000**	**< 0.001**
	Reproductive Success	Selection	1	0.1946	0.660
		Environment	**1**	**99.831**	**< 0.001**
Portugal	Fecundity	Selection	1	0.559	0.455
		Environment	**1**	**79.934**	**< 0.001**
		Selection*Environment	**1**	**5.148**	**0.023**
	Offspring viability	Selection	1	3.047	0.081
		Environment	**1**	**1324.138**	**< 0.001**
		Selection*Environment	**1**	**39.230**	**< 0.001**
	Reproductive Success	Selection	1	1.714	0.191
		Environment	**1**	**115.201**	**< 0.001**
		Selection*Environment	**1**	**11.606**	**< 0.001**



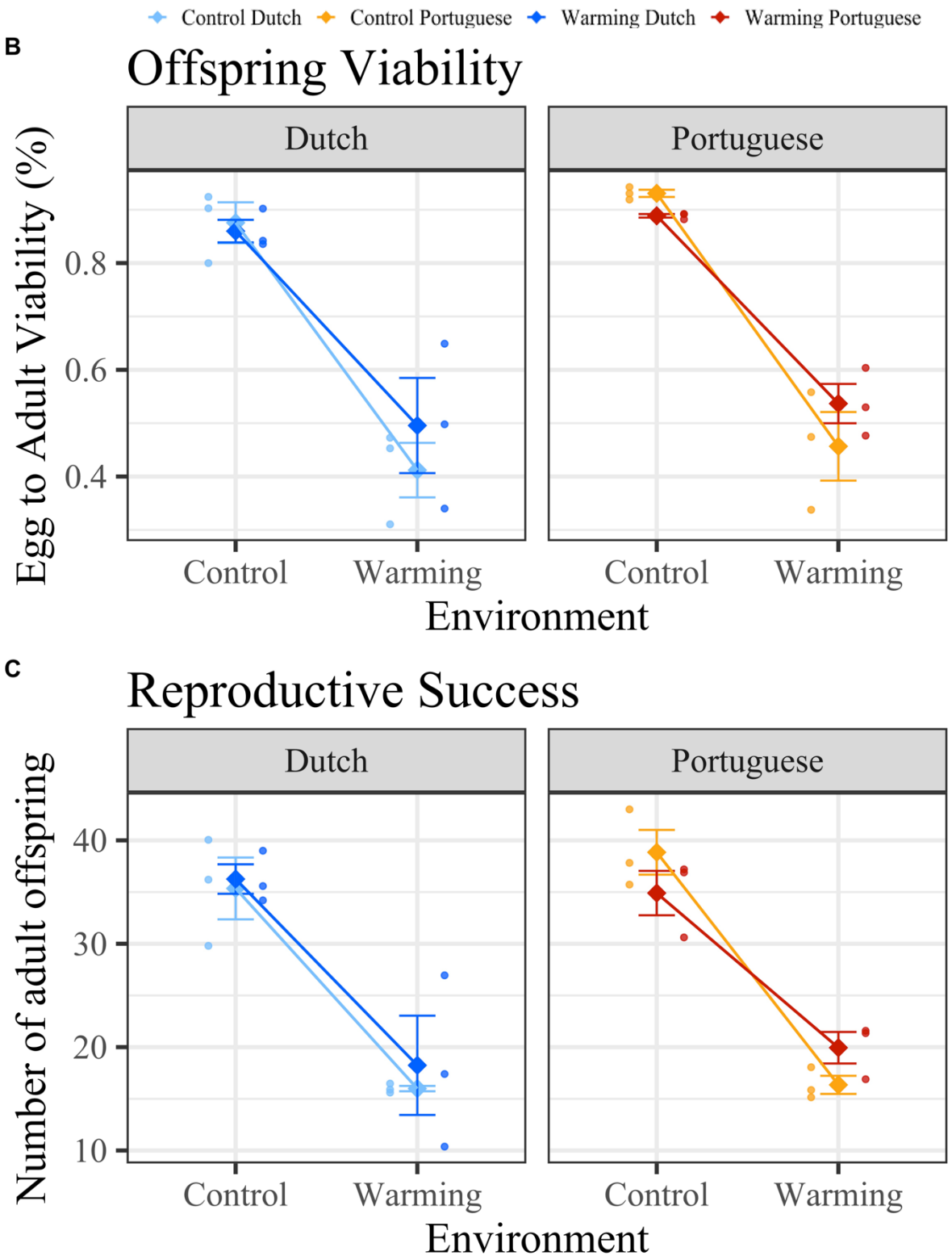


Figure 3.1 – Adaptation to a warming environment after 39 generations of thermal evolution. A) Fecundity: Number of eggs laid from days six to nine; B) Offspring viability: Ratio between the number of adult offspring and the number of eggs laid on the ninth day; C) Reproductive success: number of adult offspring resulting from eggs laid on the ninth day. Colder colours represent Dutch populations, while warmer ones represent Portuguese populations. Lighter tones represent the Control regime, while darker tones represent the Warming one. The small circles represent the mean values of each replicate population, and the big diamonds represent the mean of the three replicate populations. The error bars represent the standard error (variation between replicate populations) of the overall mean.

Recovery of Male mating behaviour and fertility following heat shock

Effect of heat shock during male adulthood on Male mating behaviour

The courtship and copulation latencies showed similar responses, both being affected by an interaction between Treatment and Mating Opportunity (X^2 = 37.469, p < 0.001; X^2 = 33.060, p < 0.001, respectively; Table 3.2, Figure 3.2.A, Figure 3.2.B) but not by Selection or History (Table 3.2). This corresponded to a decrease in courtship and copulation latencies across mating events in Dutch and Portuguese populations from both selection regimes, but this decrease varied with the male treatment. Indeed, both stressed and non-stressed males showed the longest courtship and copulation latencies when offered the first females; however, non-stressed males took the same time before starting to court and copulate with the second and third female they were offered (Z ratio = 0.465, p = 0.997, for courtship latency; Z ratio = 0.210, p = 0.999, for copulation latency see Table S8), while heat shock males were faster when initiating a mating with the third female they were offered (Z ratio = -3.403, p = 0.009, for courtship latency; Z ratio = 3.802, p = 0.002, for copulation latency; see Table S8; Figure 3.2.A, Figure 3.2.B). These results indicate that recently emerged males exposed to high (stressful) temperatures were able to recover a significant part of their behavioural performance in these two traits throughout the duration of the experiment (Figures 3.2.A and 3.2.B), although stressed males still took significantly longer to court and mate relative to non-stressed males by the third mating opportunity (see Table S8).

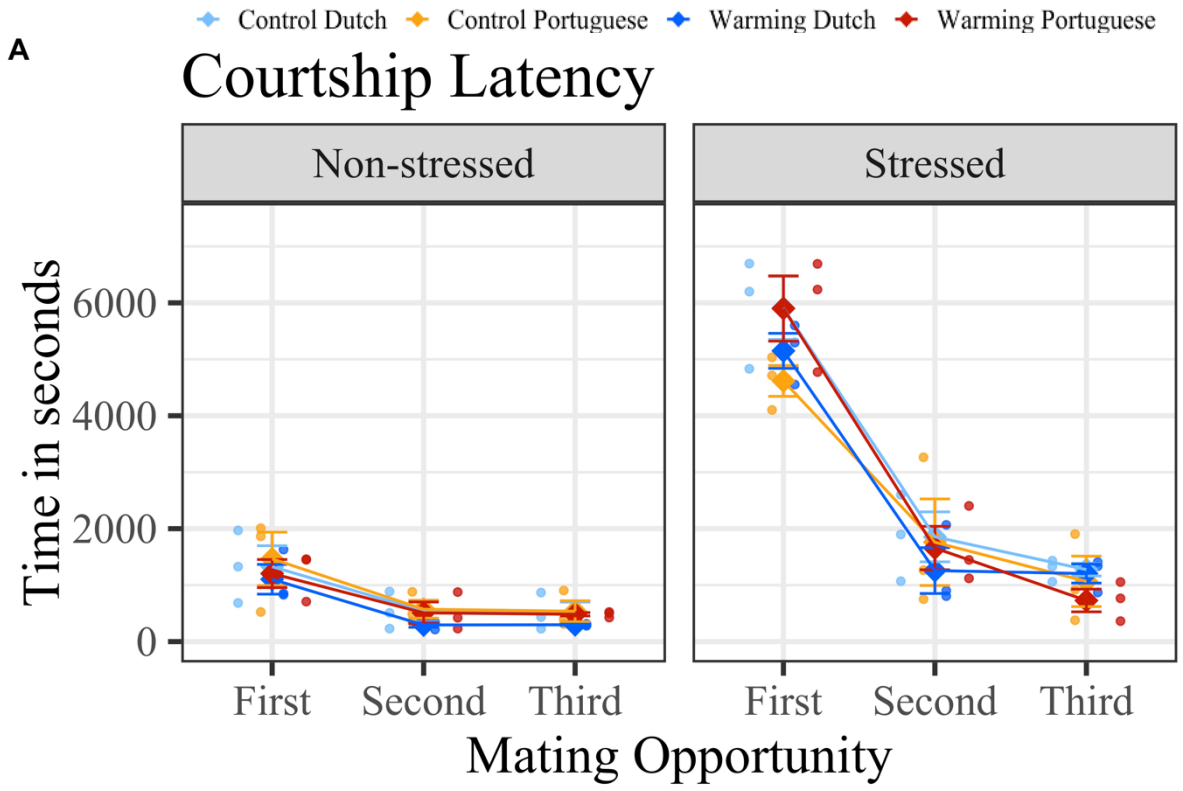
Selection significantly affected copulation duration (X^2 = 33.627, p < 0.001; see Table 3.2, Figure 3.2.C), with Control populations always having longer copulas than populations from the Warming regime. Copulation duration was also affected by an interaction between Treatment and Mating Opportunity (F = 54.228, p < 0.001; see Table 3.2). This results from a distinct response given by males from different treatments across mating opportunities: stressed males had shorter copulas than non-stressed males in the first mating opportunity (T ratio = 3.816, p = 0.002; see Table S8); however, that pattern reversed from the second mating onward, with stressed males having longer copulation durations with increasing mating opportunities, and non-stressed males copulating faster in the second and third matings, with no differences between these last two mating opportunities (see Table S8, Figure 3.2.C). As in courtship and copulation latencies, History had no significant impact on copulation duration.

Table 3.2 – Results from the analyses of variances of the effect of heat shock during male adulthood on male mating behaviour. Courtship Latency: Time elapsed between pairing and beginning of male courtship; Copulation latency: Time elapsed between pairing and copulation beginning; Copulation Duration: Time elapsed between the beginning of the copula and its ending. Males with one of two bio-geographical origins ("History": The Netherlands or Portugal), that came from one of two selection regimes ("Selection": Control or Warming), were subjected to one of two treatments ("Treatment": Non-stressed or Stressed) and were tested in three distinct moments ("Mating Opportunity": First, Second or Third). "Df": the degrees of freedom. Df.res: residual degrees of freedom. "X^2": the Chi-square value obtained in each analysis. "F": F-test obtained in each analysis. Statistically significant terms are represented in bold.

Trait	Independent Variable	*Df (Df.res)*	*Tests statistics*	*p-value*
			X^2	
Courtship Latency	History	1	0.537	0.464
	Selection	1	0.968	0.325
	Treatment	**1**	**247.200**	**< 0.001**
	Mating Opportunity	**2**	**281.521**	**< 0.001**
	Treatment*Mating Opportunity	**2**	**37.469**	**< 0.001**

Table 3.2 – Results from the analyses of variances of the effect of heat shock during male adulthood on male mating behaviour. Continuation.

Trait	Independent Variable	*Df (Df.res)*	*Tests statistics*	*p-value*
			X^2	
Copulation Latency	History	1	1.748	0.186
	Selection	1	0.028	0.868
	Treatment	**1**	**359.925**	**< 0.001**
	Mating Opportunity	**2**	**290.249**	**< 0.001**
	Treatment*Mating Opportunity	**2**	**33.060**	**< 0.001**
			F	
Copulation Duration	History	1 (1245.360)	0.602	0.438
	Selection	**1 (1245.060)**	**33.627**	**< 0.001**
	Treatment	**1 (1246.090)**	**69.773**	**< 0.001**
	Mating Opportunity	**2 (1245.950)**	**15.100**	**< 0.001**
	Treatment*Mating Opportunity	**2 (1245.900)**	**54.228**	**< 0.001**



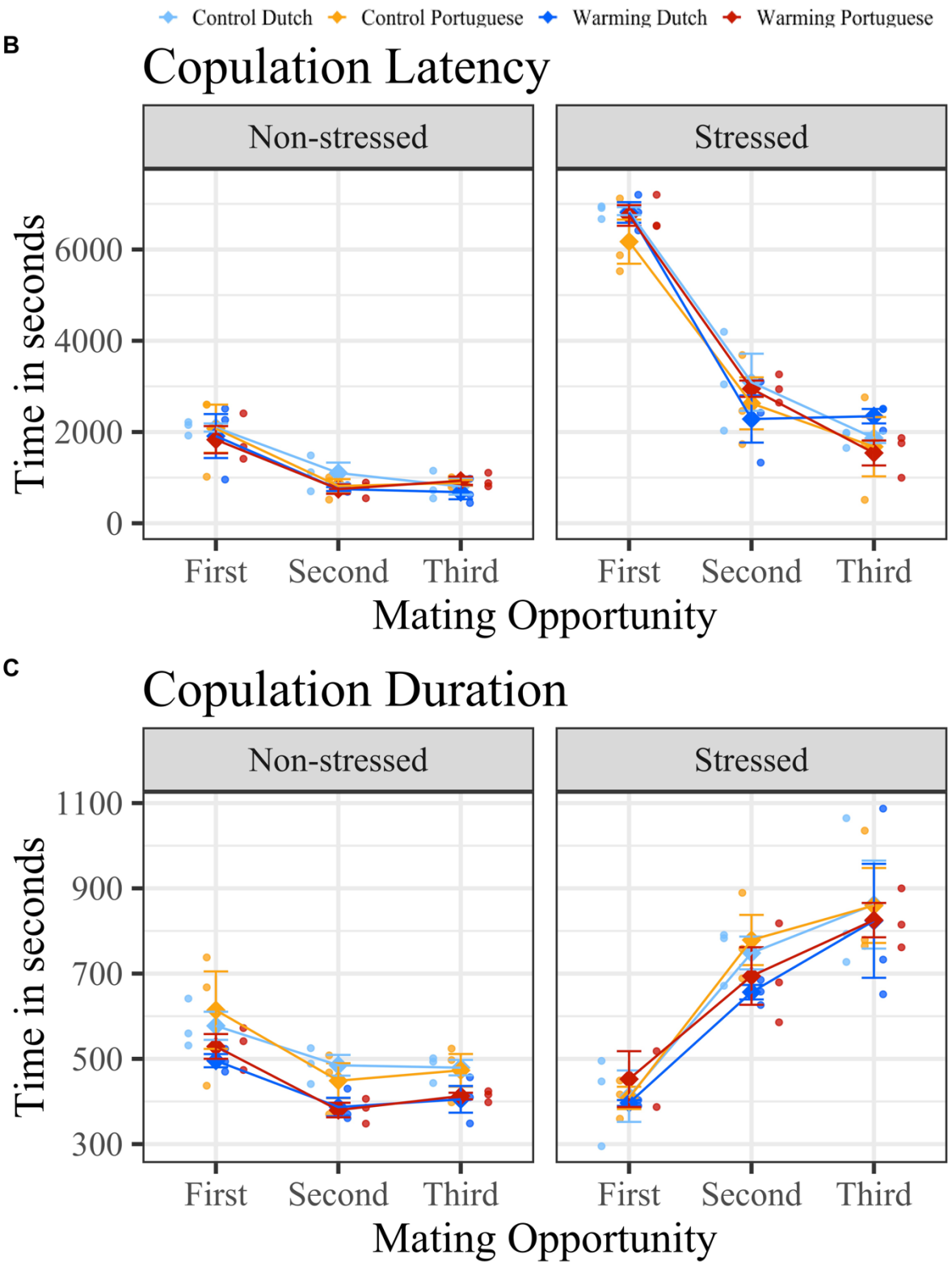


Figure 3.2 – Effect of heat shock during male adulthood on male mating behaviour after 45 generations of thermal evolution. A) Courtship Latency: Time elapsed between pairing and the beginning of male courtship; B) Copulation latency: Time elapsed between pairing and the beginning of copulation; C) Copulation Duration: Time elapsed between the beginning of the copula and its ending. Colder colours represent Dutch populations, while warmer ones represent Portuguese populations. Lighter tones represent the Control regime, while darker tones represent the Warming one. The small circles represent the mean values of each replicate population, and the big diamonds represent the mean of the three replicate populations. The error bars represent the standard error (variation between replicate populations) of the overall mean.

Effect of heat shock during male adulthood on Male fertility

The quadruple interaction between History, Selection, Treatment, and Mating Opportunity had a significant effect on fecundity, offspring viability, and reproductive success (X^2 = 9.865, p = 0.007, for fecundity; X^2 = 10.889, p = 0.004, for offspring viability; X^2 = 7.441, p = 0.024, for reproductive success; see Table S9), so additional statistical analyses were done for Portuguese and Dutch populations separately.

The fecundity of Dutch populations was significantly affected by a triple interaction between Selection, Treatment and Mating Opportunity (X^2 = 7.677, p = 0.022; see Table 3.3). Analyses done for each treatment separately showed that non-stressed Dutch males from both selective regimes had a similar response throughout mating opportunities, with Mating opportunity being the only significant factor (X^2 = 13.010, p = 0.001; see Table S10), corresponding to a significant increase in fecundity from the second to the third mating opportunity (T ratio = -3.603, p = 0.001, see Table S11.A, Figure 3.3.A). On the other hand, stressed males from Dutch populations displayed a distinct dynamic between mating opportunities depending on their selection regime (Selection x Mating Opportunity, X^2 = 6.346, p = 0.042; see Table S10, Figure 3.3.A). Despite this significant interaction, *a posteriori* contrasts did not show any differences in fecundity across mating opportunities between populations from different selective regimes (see Table S11.B, Figure 3.3.A). The fecundity of populations from Portugal was significantly affected by the interaction between Treatment and Mating Opportunity (X^2 = 7.102, p = 0.029; see Table 3.3), with males from different treatments showing distinct dynamics across mating opportunities. Indeed, comparing the first and the last (third) mating opportunities, a slight increase in fecundity was observed for stressed males (T ratio = -2.889, p = 0.046; see Table S12) but not for the non-stressed ones (T ratio = -2.023, p = 0.330; see Table S12, Figure 3.3.A). However, differences between males from distinct treatments remained significant in the last mating opportunity (T ratio = 4.247, p = 0.003; see Table S12), with non-stressed males displaying higher levels of fecundity.

The offspring viability of both Dutch and Portuguese populations was affected by a significant interaction between Selection, Treatment and Mating Opportunity (X^2 = 7.808, p = 0.020, for Dutch populations; X^2 = 6.067, p = 0.048, for Portuguese populations; see Table 3.3). Given this triple interaction, additional analyses were performed for each Treatment separately for both the Dutch and Portuguese populations. The offspring viability of both non-stressed and stressed Dutch males, was significantly shaped by the Mating Opportunity (X^2 = 6.112, p = 0.047, for non-stressed males; X^2 = 22.222, p < 0.001, for stressed males; see Table S10), with non-stressed and stressed males presenting a similar pattern. The offspring viability of both types of males was lower in the third relative to the second mating opportunity (T ratio = 2.450, p = 0.039, for non-stressed males; T ratio = 4.711, p < 0.001, for stressed males; see Table S11.A, Figure 3.3.B). For the offspring viability of Portuguese non-stressed males, the Mating Opportunity was once again the only significant factor (X^2 = 8.470, p = 0.014; see Table S10), with a decrease in offspring viability being observed in the last mating opportunity relative to the first one (T ratio = 2.863, p = 0.012, see Table S11.A, Figure 3.3.B). In turn, the offspring viability of Portuguese stressed males was influenced by an interaction between Selection and Mating Opportunity (X^2 = 8.894, p = 0.012; see Table S10): Indeed, stressed males from the Warming regime had a significant increase in offspring viability from the first to the second mating opportunity (T ratio = -3.262, p = 0.015, see Table S11.B), and kept similar levels of offspring viability in the third mating opportunity when compared to the second one. On the other hand, stressed Portuguese males from the Control regime, had similar offspring viability in the first and second

mating opportunities, but a decrease in the final mating opportunity (T ratio = 4.222, p < 0.001, see Table S11.B, Figure 3.3.B).

The reproductive success was significantly shaped by the interaction between Treatment and Mating Opportunity in both the analysis of the Dutch and of the Portuguese populations (X^2 = 7.755, p = 0.021 for Dutch populations; X^2 = 7.996, p = 0.019 for Portuguese populations; see Table 3.3). This significant interaction resulted from stressed and non-stressed males displaying an opposite dynamic across mating opportunities, with stressed males having a slight increase in reproductive success in the second mating contrary to non-stressed males whose reproductive success slightly decreases in that same mating (see Table S12, Figure 3.3.C).

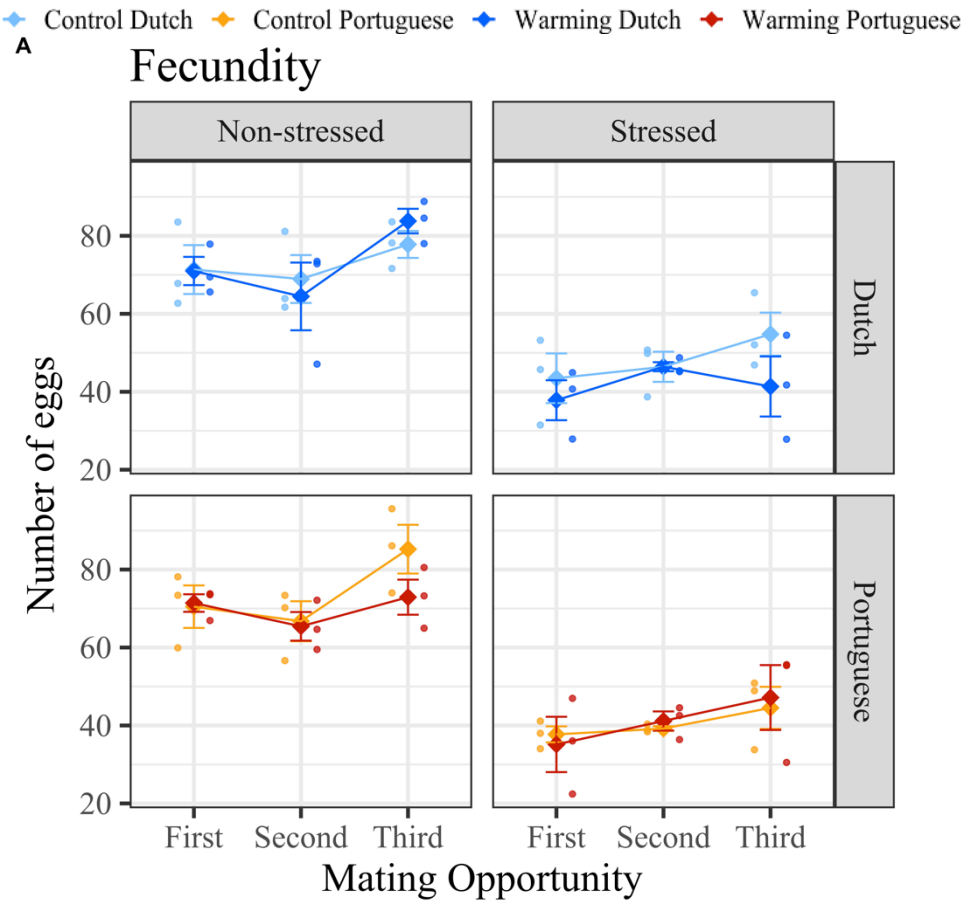
To summarize, while the observation of different temporal dynamics associated with treatments was pervasive across traits, it is important to point out that we did not find any evidence of recovery for the three fertility traits, as clear differences between treatments were observed even in the last mating opportunity (see Figure 3.3).

Table 3.3 – Results from the analyses of variances of the effect of heat shock during male adulthood on male fertility when analysing populations of different history separately. Fecundity: Number of laid eggs; Viability: Ratio between the number of offspring (adult offspring) and the number of laid eggs (total number of eggs); Reproductive success: number of offspring resulting from laid eggs. Males with one of two bio-geographical origins ("History": The Netherlands or Portugal), that came from one of two selection regimes ("Selection": Control or Warming), were subjected to one of two treatments ("Treatment": Non-stressed or Stressed) and were tested in three distinct moments ("Mating Opportunity": First, Second or Third). "Df": the degrees of freedom. "X^2": the Chi-square value obtained in each analysis. Statistically significant terms are represented in bold.

History	Trait	Independent Variable	*Df*	X^2	*p-value*
The Netherlands	Fecundity	Selection	1	0.299	0.585
		Treatment	**1**	**40.770**	**< 0.001**
		Mating Opportunity	**2**	**8.306**	**0.016**
		Selection*Treatment	1	0.792	0.374
		Selection*Mating Opportunity	2	4.098	0.129
		Treatment*Mating Opportunity	2	2.564	0.277
		Selection*Treatment*Mating Opportunity	**2**	**7.677**	**0.022**
	Offspring viability	Selection	1	0.452	0.501
		Treatment	**1**	**52.626**	**< 0.001**
		Mating Opportunity	**2**	**28.135**	**< 0.001**
		Selection*Treatment	1	1.142	0.285
		Selection*Mating Opportunity	2	0.105	0.949
		Treatment*Mating Opportunity	**2**	**8.252**	**0.016**
		Selection*Treatment*Mating Opportunity	**2**	**7.808**	**0.020**
	Reproductive Success	Selection	1	0.003	0.958
		Treatment	**1**	**47.181**	**< 0.001**
		Mating Opportunity	2	0.170	0.919
		Treatment*Mating Opportunity	**2**	**7.755**	**0.021**

Table 3.3 – Results from the analyses of variances of the effect of heat shock during male adulthood on male fertility when analysing populations of different history separately. Continuation.

History	Trait	Independent Variable	*Df*	X^2	*p-value*
Portugal	Fecundity	Selection	1	1.069	0.301
		Treatment	**1**	**36.120**	**< 0.001**
		Mating Opportunity	**2**	**13.794**	**0.001**
		Treatment*Mating Opportunity	**2**	**7.102**	**0.029**
	Offspring viability	Selection	1	1.158	0.282
		Treatment	**1**	**124.425**	**< 0.001**
		Mating Opportunity	**2**	**21.730**	**< 0.001**
		Selection*Treatment	**1**	**4.442**	**0.035**
		Selection*Mating Opportunity	**2**	**13.502**	**0.001**
		Treatment*Mating Opportunity	**2**	**24.379**	**< 0.001**
		Selection*Treatment*Mating Opportunity	**2**	**6.067**	**0.048**
	Reproductive Success	Selection	1	0.040	0.842
		Treatment	**1**	**51.609**	**< 0.001**
		Mating Opportunity	2	3.468	0.177
		Treatment*Mating Opportunity	**2**	**7.966**	**0.019**



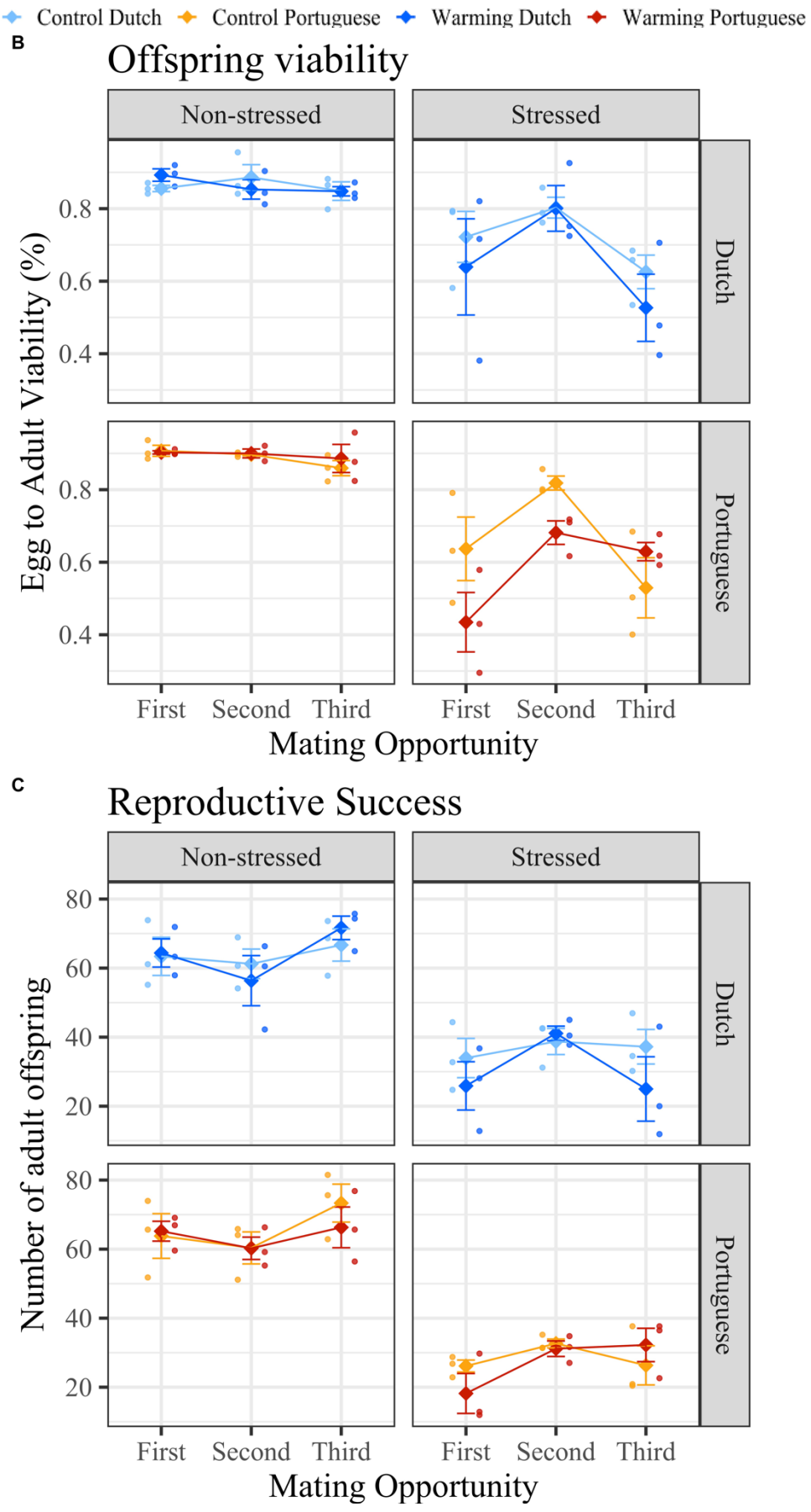

Control Dutch
Control Portuguese
Warming Dutch
Warming Portuguese
B
Offspring viability
Non-stressed
Stressed
Dutch
Portuguese
Egg to Adult Viability (%)
0.8
0.6
0.4
First
Second
Third
Mating Opportunity
C
Reproductive Success
Number of adult offspring
80
60
40
20

Figure 3.3 – Effect of heat-shock during male adulthood on male fertility after 45 generations of thermal evolution. A) Fecundity: Number of laid eggs; B) Offspring viability: Ratio between the number of offspring and the number of laid eggs; C) Reproductive success: number of offspring resulting from laid eggs. Colder colours represent Dutch populations, while warmer ones represent Portuguese populations. Lighter tones represent the Control regime, while darker tones represent the Warming one. The small circles represent the mean values of each replicate population, and the big diamonds represent the mean of the three replicate populations. The error bars represent the standard error (variation between replicate populations) of the overall mean.

Female mating behaviour and fertility following male heat shock

Effect of heat shock during male adulthood on the Female mating behaviour

Treatment, but not History nor Selection, had a significant impact on the female propensity to remate (X^2 = 77.706, p < 0.001; see Table 3.4; see Figure 3.4). Females that first mated with a non-stressed male and then had the opportunity to remate with another non-stressed male exhibited a very low remating rate (3% of the females remated). In opposition, females that were allowed to first mate with a male exposed to heat shock had much higher remating rates: over 40% of the females paired with a non-stressed male remated, while this value decreased significantly to 30% when the females were paired with a stressed male (T ratio = 3.037, p = 0.007; see Table S13; Figure 3.4). These results indicate that commonly monandrous females under benign conditions, can display remating in stressful conditions, namely in response to less fertile males.

Table 3.4 – Results from the analyses of variance of the effect of heat shock during male adulthood on female propensity to remate. Remating: female mates with a different male after already mating once. Females with one of two bio-geographical origins ("History": The Netherlands or Portugal), that came from one of two selection regimes ("Selection": Control or Warming), were subjected to one of three treatments ("Treatment": Non-stressed x non-stressed, Stressed x non-stressed or Stressed x stressed). "Df": the degrees of freedom. "X^2": the Chi-square value obtained in each analysis. Statistically significant terms are represented in bold.

Trait	Independent Variable	*Df*	X^2	*p-value*
Remating	History	1	3.362	0.067
	Selection	1	1.744	0.187
	Treatment	**2**	**77.706**	**< 0.001**

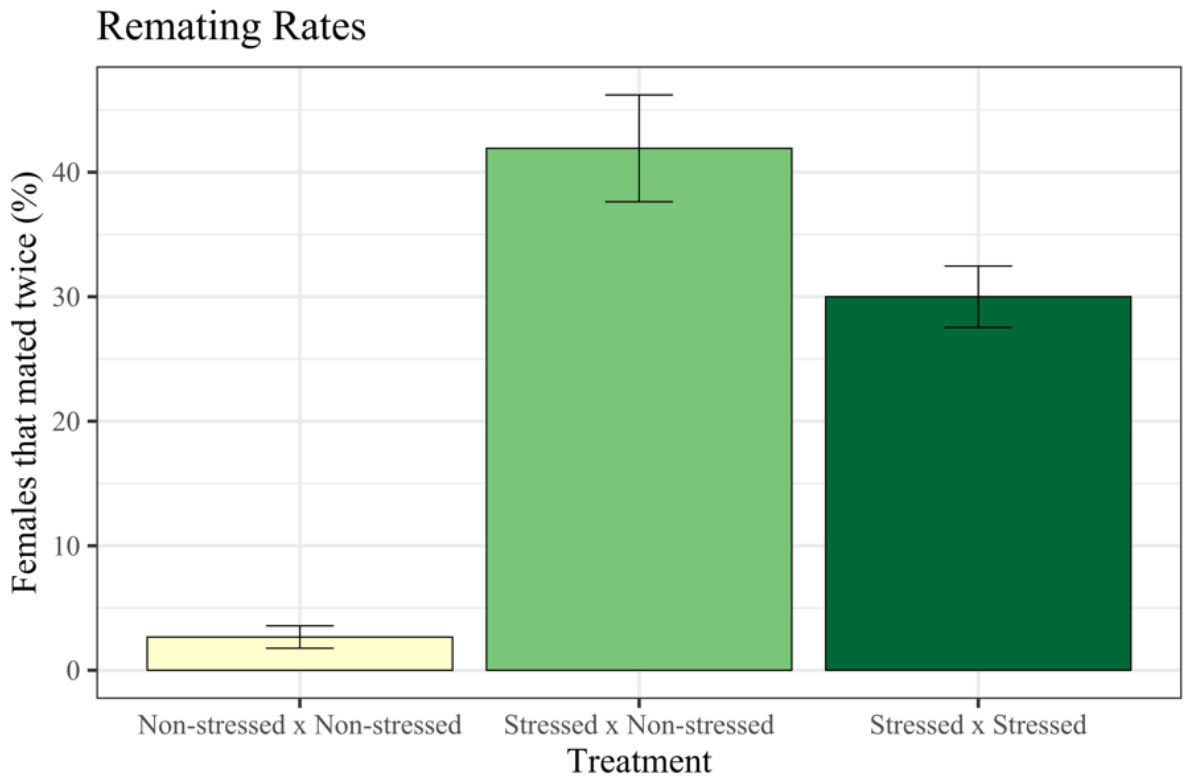


Figure 3.4 – Effect of heat shock during male adulthood on female propensity to remate after 45 generations of thermal evolution. The bars represent the mean values for each treatment. The error bars represent the standard error of the mean.

Courtship and copulation latencies exhibited identical responses, being significantly affected by the interaction between Treatment and Mating Opportunity (Courtship latency: $X^2 = 175.654$, $p < 0.001$; see Table 3.5; see Figure 3.5.A; and Copulation latency: $X^2 = 207.797$, $p < 0.001$; see Table 3.5; see Figure 3.5.B). When the female first mated with a non-stressed male, the latency to courtship and to copulate more than doubled in the second mating opportunity (Z ratio = 13.543, $p < 0.001$, for courtship latency; Z ratio = 15.716, $p < 0.001$, for copulation latency; see Table S14). In turn, when the first mating was with a stressed male, both courtship and copulation latency were much higher than when females first mated with a non-stressed male (see Table S14, Figure 3.5.A and Figure 3.5.B). However, after a first mating with a stressed male, different patterns were exhibited according to the thermal treatment of the second male: when females had the chance to remate with a stressed male, courtship and copulation latencies increased compared to the first mating (Z ratio = 12.633, $p < 0.001$, for courtship latency; Z ratio = 11.812, $p < 0.001$, for copulation latency; see Table S14), but when the second mating opportunity was with a non-stressed male, both the courtship and copulation happened quicker than in the first mating (Z ratio = 3.079, $p = 0.025$, for courtship latency; Z ratio = 3.925, $p = 0.001$, for copulation latency; see Table S14, Figure 3.5.A and Figure 3.5.B, respectively).

The interaction between Treatment and Mating Opportunity also had a significant impact on copulation duration ($F = 12.180$, $p < 0.001$; see Table 3.5; see Figure 3.5.C), although the observed pattern was different from that of both latencies. Copulation duration was similar across mating opportunities when females had the chance to mate with two non-stressed males (Z ratio = 1.123, $p = 0.872$; see Table S14), or with two stressed males (Z ratio = 0.072, $p = 1$; see Table S14). However, in the first mating opportunity, copulation lasted longer when females mated with non-stressed males

than with stressed males (T ratio = 9.978, p < 0.001, for the comparison between Non-stressed x non-stressed and Stressed x non-stressed treatments; T ratio = 9.408, p < 0.001, for the comparison between Non-stressed x non-stressed and Stressed x stressed treatments; see Table S14, Figure 3.5.C). Furthermore, when females had the opportunity to mate first with a stressed male and then with a non-stressed male, the duration of the copula increased between mating opportunities (T ratio = -6.891, p < 0.001; see Table S14), enough for the duration of the second mating to be similar to that of first matings involving a non-stressed male (T ratio = 2.423, p = 0.150; see Table S14, Figure 3.5.C).

Table 3.5 – Results from the analyses of variances of the effect of heat shock during male adulthood on female mating behaviour. Courtship Latency: Time elapsed between pairing and beginning of male courtship; Copulation latency: Time elapsed between pairing and copulation beginning; Copulation Duration: Time elapsed between the beginning of the copula and its ending. Females with one of two bio-geographical origins ("History": The Netherlands or Portugal), that came from one of two selection regimes ("Selection": Control or Warming), were subjected to one of three treatments ("Treatment": Non-stressed x non-stressed, Stressed x non-stressed or Stressed x stressed) and were tested in two distinct moments ("Mating Opportunity": First or Second). "Df": the degrees of freedom. "X^2": the Chi-square value obtained in each analysis. "F": F-test obtained in each analysis. Statistically significant terms are represented in bold.

Trait	Independent Variable	*Df (Df.res)*	*Test statistics*	*p-value*
			X^2	
Courtship Latency	History	1	3.150	0.076
	Selection	1	0.756	0.385
	Treatment	**2**	**54.491**	**< 0.001**
	Mating Opportunity	**1**	**290.333**	**< 0.001**
	Treatment*Mating Opportunity	**2**	**175.654**	**< 0.001**
			X^2	
Copulation Latency	History	1	2.539	0.111
	Selection	1	0.001	0.982
	Treatment	**2**	**48.543**	**< 0.001**
	Mating Opportunity	**1**	**361.207**	**< 0.001**
	Treatment*Mating Opportunity	**2**	**207.797**	**< 0.001**
			F	
Copulation Duration	History	1 (670.640)	0.711	0.400
	Selection	1 (670.690)	0.188	0.665
	Treatment	**2 (670.450)**	**10.190**	**< 0.001**
	Mating Opportunity	1 (671.210)	1.288	0.257
	Treatment*Mating Opportunity	**2 (670.340)**	**12.180**	**< 0.001**

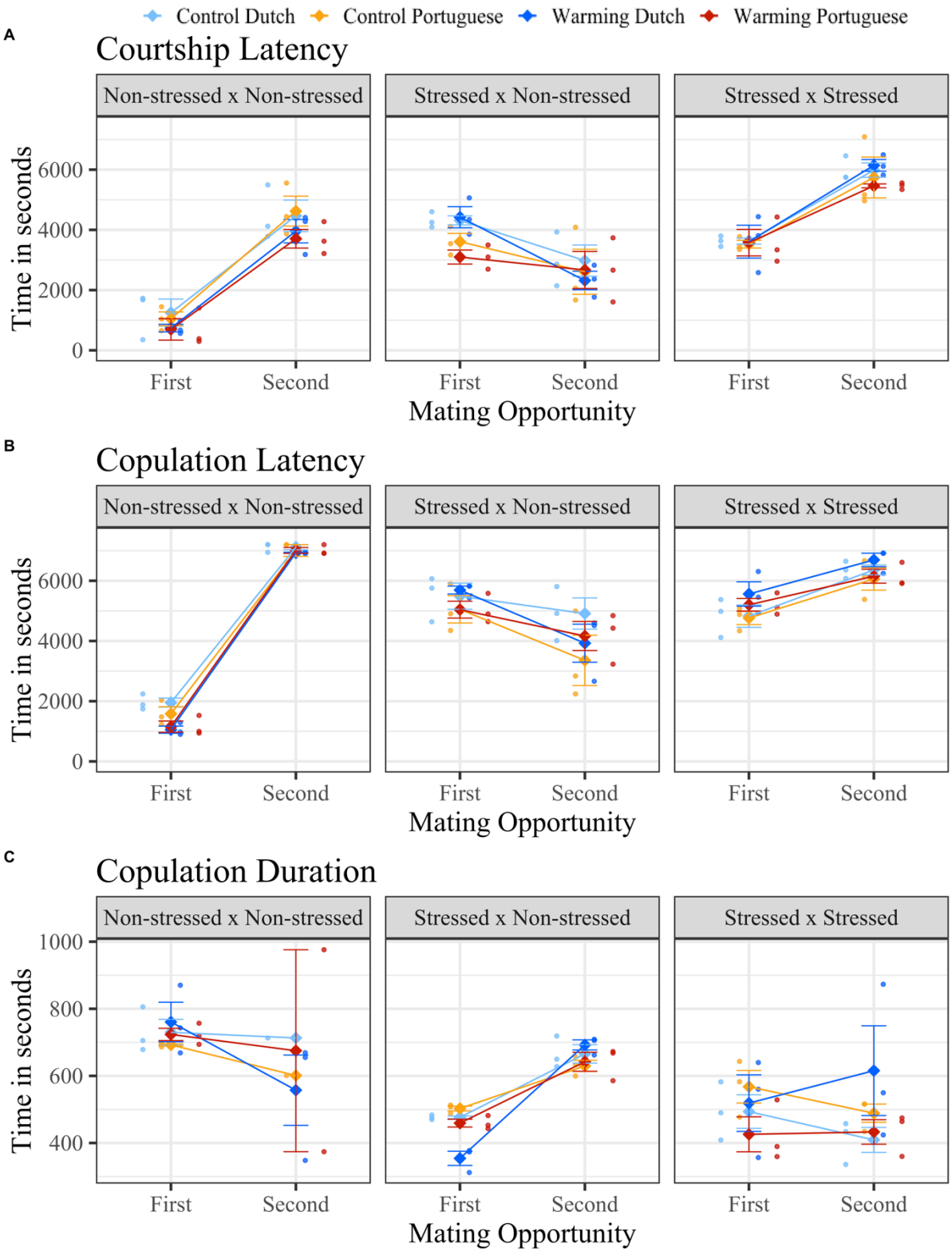


Figure 3.5 – Effect of heat shock during male adulthood on female mating behaviour after 45 generations of thermal evolution. A) Courtship Latency: Time elapsed between pairing and beginning of male courtship; B) Copulation latency: Time elapsed between pairing and copulation beginning; C) Copulation Duration: Time elapsed between the beginning of the copula and its ending. Colder colours represent Dutch populations, while warmer ones represent Portuguese populations. Lighter tones represent the Control regime, while darker tones represent the Warming one. The small circles represent the mean values of each replicate population, and the big diamonds represent the mean of the three replicate populations. The error bars represent the standard error (variation between replicate populations) of the overall mean.

Effect of heat shock during male adulthood on Female fertility

Multiple interactions with the variable History had a significant effect on fecundity (see Table S15), thus statistical analyses for Dutch and Portuguese populations were performed separately for this trait. The fecundity of both Dutch and Portuguese populations was significantly shaped by the interaction between Treatment and Mating Opportunity (X^2 = 14.311, p < 0.001; X^2 = 10.814, p = 0.004, respectively; see Table 3.6). In the first mating opportunity, females that first mated with males subjected to heat shock had a significant reduction in their fecundity relative to those that mated with non-stressed males (see Table S16.A, Figure 3.6.A). While an increase in fecundity was observed between mating opportunities for all treatments, this increase was significantly steeper in females that first had the chance to mate with stressed males relative to females that were paired with two non-stressed males. In Dutch populations, the fecundity of all females was similar in the second mating opportunity, revealing that Dutch females that first had the opportunity to mate with a male exposed to high temperatures were able to fully rescue their fecundity after a second mating opportunity, regardless of the thermal treatment of the second male (T ratio = 2.548, p = 0.112, for the comparison between Non-stressed x non-stressed and Stressed x non-stressed treatments; T ratio = 2.799, p = 0.059, for the comparison between Non-stressed x non-stressed and Stressed x stressed treatments; see Table S16.A, Figure 3.6.A). However, in Portuguese populations, this increase in fecundity from the first to the second mating, was not enough to reach the fecundity levels of females that were paired with two non-stressed males (T ratio = 4.205, p < 0.001, for the comparison with the Stressed x non-stressed treatment; T ratio = 4.383, p < 0.001, for comparison with the Stressed x stressed treatment; see Table S16.A, Figure 3.6.A). Additionally, the fecundity of Portuguese populations was also affected by an interaction between Selection and Mating Opportunity (X^2 = 9.197, p = 0.002; see Table 3.6), with females from the Control regime having lower fecundity in the first mating opportunity than females from the Warming regime (T ratio = -3.811, p < 0.001; see Table S16.B), but similar levels in the second mating opportunity (T ratio = -1.164, p = 0.650; see Table S16.B, Figure 3.6.A).

The offspring viability of the populations under study was not impacted by either History or Selection (Table 3.7). In contrast this trait was significantly shaped by the Treatment x Mating Opportunity interaction (X^2 = 18.208, p < 0.001; see Table 3.7), with different treatments presenting distinct dynamics across mating opportunities. Indeed, offspring viability was significantly lower in the first mating opportunity when females mated with stressed males relative to females that first mated with a non-stressed male (T ratio = 6.531, p < 0.001, for the comparison between Non-stressed x non-stressed and Stressed x non-stressed treatments; T ratio = 6.157, p < 0.001, for the comparison between Non-stressed x non-stressed and Stressed x stressed treatments; see Table S17, Figure 3.6.B). However, this was not true for the second mating opportunity, with females that had the chance to mate with two non-stressed males having similar offspring viability in both mating opportunities (T ratio = -0.008, p = 1.000 see Table S17), while females that first mated with stressed males significantly increased their offspring viability from the first to the second mating opportunity, regardless of the thermal treatment of the second male. In fact, the offspring viability after the second mating opportunity was similar across all treatments (T ratio = 1.821, p = 0.452, for the comparison between Non-stressed x non-stressed and Stressed x non-stressed treatments; T ratio = 2.550, p = 0.111, for the comparison between Non-stressed x non-stressed and Stressed x stressed treatments; T ratio = 0.796, p = 0.968, for the comparison between Stressed x non-stressed and Stressed x stressed treatments; see Table S17). This pattern shows that a second mating opportunity for females that first mated with a stressed male can rescue their offspring viability (see Figure 3.6.B).

As observed for fecundity, multiple interactions with the variable History had a significant impact on reproductive success (see Table S15), therefore statistical analyses for Dutch and Portuguese populations were done separately once again. The reproductive success of both populations from The Netherlands and from Portugal was significantly affected by the interaction between Treatment and Mating Opportunity (X^2 = 10.351, p = 0.006; X^2 = 14.701, p < 0.001, respectively; see Table 3.6). In the first mating opportunity, both Dutch and Portuguese females that mated with stressed males had lower reproductive success than females that were paired with a non-stressed male (see Table S16A, Figure 3.6.C). From the first to the second mating opportunity, there was an overall increase of the reproductive success, that differed depending on the treatment and the population's historical background. In Dutch populations, females that first mated with stressed males and then were paired with non-stressed males showed similar levels of reproductive success in the second mating opportunity than that of females that were allowed to mate with two non-stressed males (T ratio = 2.546, p = 0.112; see Table S16.A). On the other hand, the reproductive success of the second mating of Dutch females that had the opportunity to mate with two stressed males approached but did not reach the levels of the reproductive success of females that were paired with non-stressed males in the two mating opportunities (T ratio = 3.114, p = 0.023; see Table S16.A, Figure 3.6.C). In Portuguese populations, the reproductive success of the second mating of females that first mated with a stressed male, regardless of the thermal treatment of the second male, continued to be significantly lower than that of females that first mated with non-stressed males (T ratio = 4.107, p < 0.001, for the comparison between Non-stressed x non-stressed and Stressed x non-stressed treatments; T ratio = 4.573, p < 0.001, for the comparison between Non-stressed x non-stressed and Stressed x stressed treatments; see Table S16.A, Figure 3.6.C). In addition, the reproductive success of populations from Portugal was also shaped by the Selection x Mating Opportunity interaction (X^2 = 8.209, p = 0.004; see Table 3.6). Indeed, females from the Control regime had lower reproductive success in the first mating opportunity than females from the Warming regime (T ratio = -3.798, p < 0.001; see Table S16.B), regardless of the male treatment. However, in the second mating opportunity, there were no differences between populations from different selective regimes (T ratio = -1.305, p = 0.560; see Table S16.B, Figure 3.6.C).

Table 3.6 – Results from the analyses of variances of the effect of heat shock during male adulthood on female fertility when analysing populations of different history separately. Fecundity: Number of laid eggs; Reproductive success: number of offspring resulting from laid eggs. Females with one of two bio-geographical origins ("History": The Netherlands or Portugal), that came from one of two selection regimes ("Selection": Control or Warming), were subjected to one of three treatments ("Treatment": Non-stressed x non-stressed, Stressed x non-stressed or Stressed x stressed) and were tested in two distinct moments ("Mating Opportunity": First or Second). "Df": the degrees of freedom. "X^2": the Chi-square value obtained in each analysis. Statistically significant terms are represented in bold.

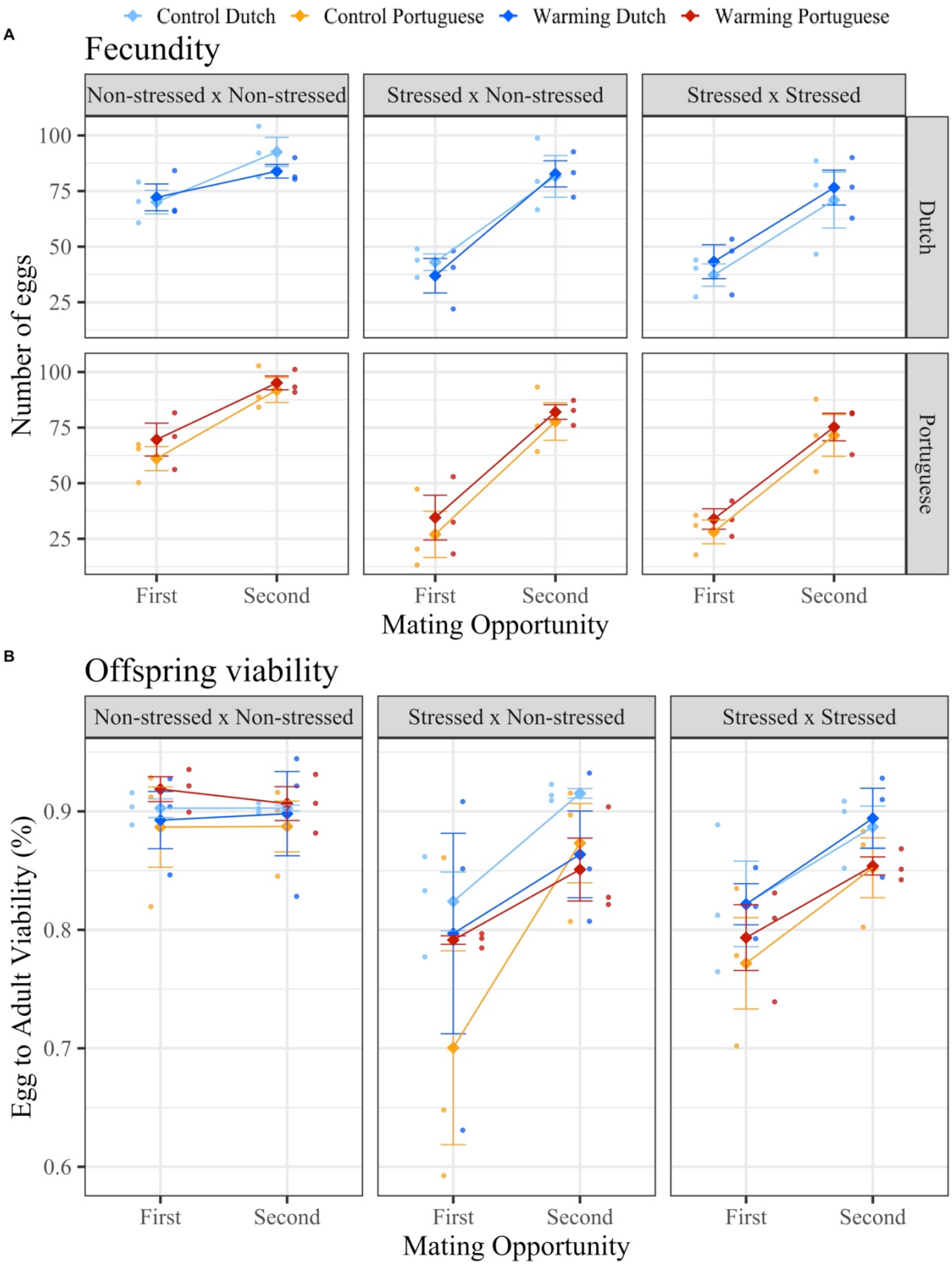
History	Trait	Explanatory Variable	*Df*	X^2	*p-value*
The Netherlands	Fecundity	Selection	1	0.465	0.495
		Treatment	**2**	**33.490**	**< 0.001**
		Mating Opportunity	**1**	**143.136**	**< 0.001**
		Treatment*Mating Opportunity	**2**	**14.311**	**< 0.001**
	Reproductive Success	Selection	1	0.690	0.406
		Treatment	**2**	**35.879**	**< 0.001**
		Mating Opportunity	**1**	**137.913**	**< 0.001**
		Treatment*Mating Opportunity	**2**	**10.351**	**0.006**

Table 3.6 – Results from the analyses of variances of the effect of heat shock during male adulthood on female fertility when analysing populations of different history separately. Continuation.

History	Trait	Explanatory Variable	*Df*	X^2	*p-value*
Portugal	Fecundity	**Selection**	**1**	**12.476**	**< 0.001**
		Treatment	**2**	**54.232**	**< 0.001**
		Mating Opportunity	**1**	**204.534**	**< 0.001**
		Selection*Mating Opportunity	**1**	**9.197**	**0.002**
		Treatment*Mating Opportunity	**2**	**10.814**	**0.004**
	Reproductive Success	**Selection**	**1**	**12.401**	**< 0.001**
		Treatment	**2**	**63.761**	**< 0.001**
		Mating Opportunity	**1**	188.398	**< 0.001**
		Selection*Mating Opportunity	**1**	**8.209**	**0.004**
		Treatment*Mating Opportunity	**2**	**14.701**	**< 0.001**

Table 3.7 – Results from the analyses of variances of the effect of heat shock during male adulthood on female offspring viability. Offspring viability: Ratio between the number of offspring (adult offspring) and the number of laid eggs (total number of eggs). Females with one of two bio-geographical origins ("History": The Netherlands or Portugal), that came from one of two selection regimes ("Selection": Control or Warming), were subjected to one of three treatments ("Treatment": Non-stressed x non-stressed, Stressed x non-stressed or Stressed x stressed) and were tested in two distinct moments ("Mating Opportunity": First or Second). "Df": the degrees of freedom. "X^2": the Chi-square value obtained in each analysis. Statistically significant terms are represented in bold.

Trait	Independent Variable	*Df*	X^2	*p-value*
Offspring viability	History	1	3.400	0.0652
	Selection	1	0.075	0.7836
	Treatment	**2**	**42.661**	**< 0.001**
	Mating Opportunity	**1**	**30.537**	**< 0.001**
	Treatment*Mating Opportunity	**2**	**18.208**	**< 0.001**


A
Control Dutch
Control Portuguese
Warming Dutch
Warming Portuguese
Fecundity
Non-stressed x Non-stressed
Stressed x Non-stressed
Stressed x Stressed
Dutch
Portuguese
Number of eggs
100
75
50
25
First
Second
Mating Opportunity
B
Offspring viability
Egg to Adult Viability (%)
0.9
0.8
0.7
0.6

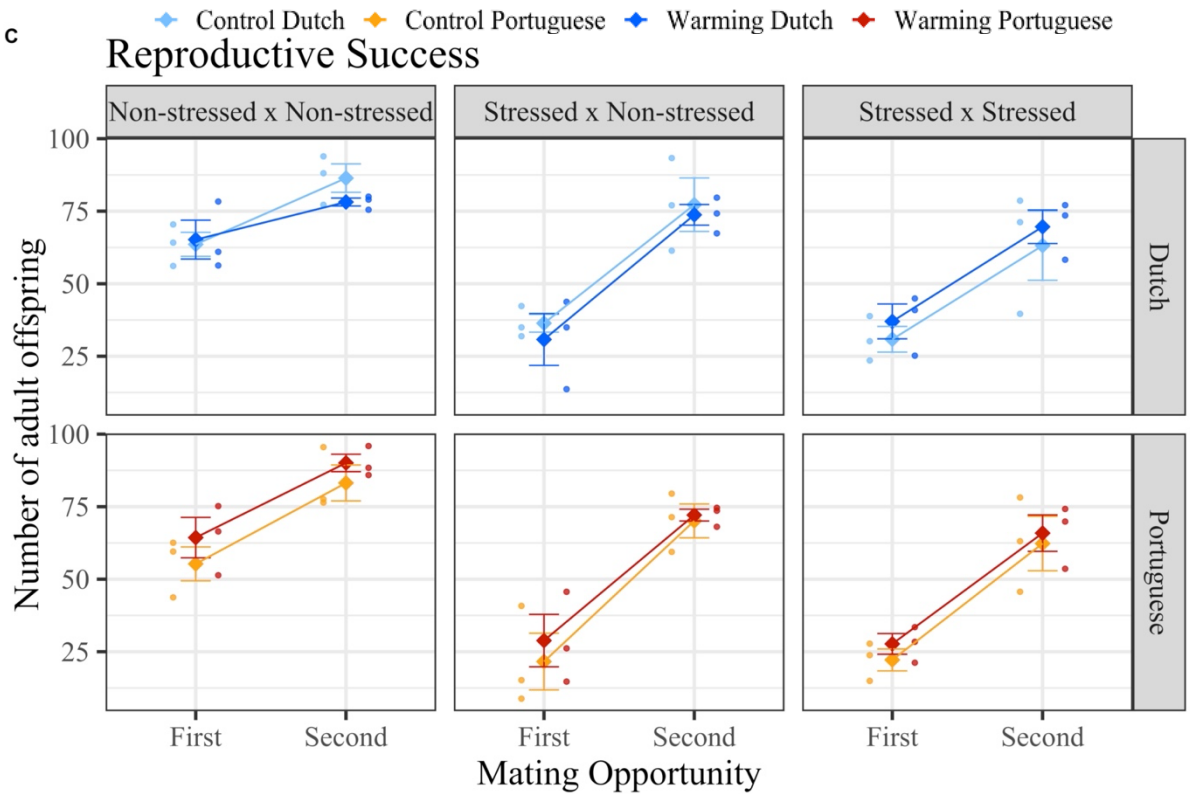


Figure 3.6 – Effect of heat-shock during male adulthood on female fertility after 45 generations of thermal evolution. A) Fecundity: Number of laid eggs; B) Offspring viability: Ratio between the number of offspring and the number of laid eggs; C) Reproductive success: number of offspring resulting from laid eggs. Colder colours represent Dutch populations, while warmer ones represent Portuguese populations. Lighter tones represent the Control regime, while darker tones represent the Warming one. The small circles represent the mean values of each replicate population, and the big diamonds represent the mean of the three replicate populations. The error bars represent the standard error (variation between replicate populations) of the overall mean.

Effect of remating behaviour on Female reproductive success

To directly assess whether remating behaviour was responsible for the rescue of female reproductive performance, an analysis of reproductive success with remating behaviour (remated vs non-remated) as an explanatory variable was performed.

The reproductive success of females was significantly affected by a triple interaction between Treatment, Mating Opportunity, and Remating ($X^2 = 34.411$, $p < 0.001$, see Table S18). Thus, separate analyses were done for each of the three treatments. For females that had the opportunity to mate with two non-stressed males, both Mating Opportunity and Remating had a significant but independent impact on reproductive success ($X^2 = 100.527$, $p < 0.001$; $X^2 = 4.808$, $p = 0.028$, respectively; see Table 3.8). This results from a lower reproductive success in the first mating relative to the second one, and from an overall lower performance of females that remated relative to females that did not remate (see Figure 3.7). For females that first had the opportunity to mate with stressed males their reproductive success was significantly shaped by the interaction between Mating Opportunity and Remating ($X^2 = 40.931$, $p < 0.001$, for the Stressed x non-stressed treatment; $X^2 = 45.504$, $p < 0.001$, for the Stressed x stressed treatment; see Table 3.8), with remated females showing a steeper increase in reproductive success from first to second mating opportunity relative to non-remated females (see Figure 3.7). In these two treatments, remated females had substantially lower reproductive success in

the first mating relative to females that did not remate (T ratio = 7.514, $p < 0.001$, for the Stressed x non-stressed treatment; T ratio = 7.355, $p < 0.001$, for the Stressed x stressed treatment; see Table S19). However, in the second mating opportunity, remated females approached the levels of reproductive success displayed by non-remated females. Despite both treatments involving stressed males as first mates presented such convergence, the pattern varied between them. In fact, remated females from the Stressed x non-stressed treatment approached but did not reach the levels of reproductive success of non-remated females (T ratio = 3.426, $p = 0.004$; see Table S19), while in the Stressed x stressed treatment both remated and non-remated females displayed similar levels of reproductive success by the second mating opportunity (T ratio = 1.870, $p = 0.242$; see Table S19). These results indicate that the remating behaviour is beneficial for females that mated with males exposed to high temperatures, contributing to a (partial) rescue of the female reproductive success.

Table 3.8 – Results from the analyses of variances of the effect of remating behaviour on the female reproductive success. Reproductive success: number of offspring resulting from laid eggs. Females were subjected to one of three treatments ("Treatment": Non-stressed x non-stressed, Stressed x non-stressed or Stressed x stressed) tested in two distinct moments ("Mating Opportunity": First or Second) and displayed remating or not ("Remating": No Remating or Remated). "Df": the degrees of freedom. "X^2": the Chi-square value obtained in each analysis. Statistically significant terms are represented in bold.

Trait	Treatment	Explanatory Variable	*Df*	X^2	*p-value*
Reproductive Success	Non-stressed x non-stressed	**Mating Opportunity**	**1**	**100.527**	**< 0.001**
		Remating	**1**	**4.808**	**0.028**
	Stressed x non-stressed	**Mating Opportunity**	**1**	**289.369**	**< 0.001**
		Remating	**1**	**57.602**	**< 0.001**
		Mating Opportunity*Remating	**1**	**40.931**	**< 0.001**
	Stressed x stressed	**Mating Opportunity**	**1**	**111.949**	**< 0.001**
		Remating	**1**	**55.585**	**< 0.001**
		Mating Opportunity*Remating	**1**	**45.504**	**< 0.001**

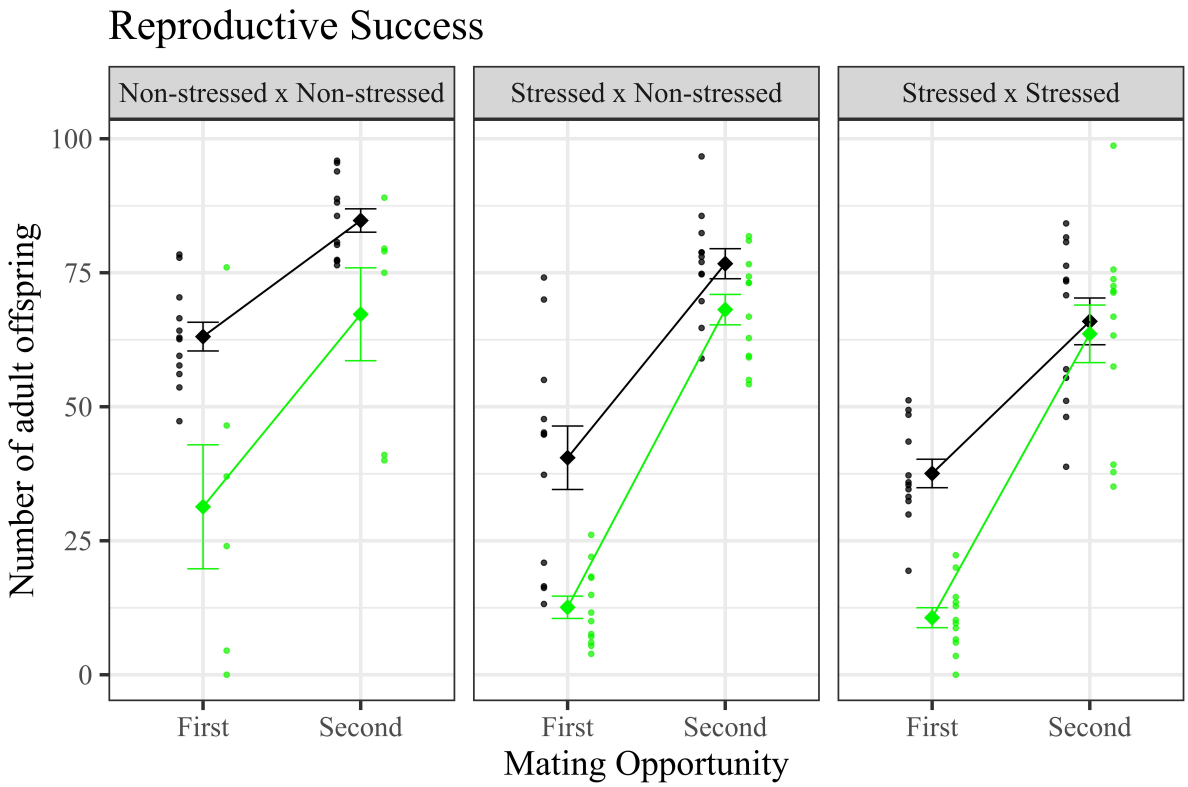


Figure 3.7 – Effect of remating behaviour on the female reproductive success after 45 generations of thermal evolution. Reproductive success: number of offspring resulting from laid eggs. The colour black represents females that did not remate, while the green colour represents remated females. The small circles represent the mean values of each replicate population, and the big diamonds represent the mean of the twelve replicate populations. The error bars represent the standard error (variation between replicate populations) of the overall mean.

Discussion

Slow paced adaptation to a global warming scenario

We observed a pattern of adaptation in low-latitude (Portuguese) populations, with populations that evolved under warming showing higher fertility at warmer conditions. In contrast we found no signs of adaptation to warming conditions in high-latitude (Dutch) populations after 39 generations of thermal evolution. The fact that high latitude populations did not show an adaptive response to warming could be linked to a lack of standing genetic variation or to a different pace of evolution between populations of different locations. It is important to consider that a similar experiment was performed after 22 generations of thermal evolution, and neither low- nor high-latitude populations showed signs of adaptation, indicating that several generations are needed to adapt to warming conditions. The absence of clear changes in thermal reaction norms during short-term (~ 9 generations) evolution in the warming environment also corroborates this idea (Santos et al., 2021b). This absence of an adaptive response is reported in other experimental evolution studies, in which populations evolving under rising temperatures did not show clear-cut evolutionary responses to increasing temperatures (Schou et al., 2014; van Heerwaarden & Sgrò, 2021). The population-specific response of populations from different latitudes is in line with a plasticity study done by Porcelli et al. (2017), where low-latitude *D. subobscura* populations coped better with heat stress during the developmental or adult stage than high-latitude populations. Similarly, in a study with populations of *D. melanogaster* different populations exhibited local adaptation to native temperatures during different stages of the life cycle (Austin & Moehring, 2019).

Given that our populations had evolved for several generations in the Control regime prior to the imposition of the selective regimes, we were able to test if there were any costs of adaptation to warming by comparing the performance in the ancestral (control) environment of Portuguese populations evolving in the Warming regime. Kawecki and Ebert (2004) claim that during the process of local adaptation, trade-offs may arise, implying that no single genotype is better in all habitats. These trade-offs could be linked to antagonistic pleiotropy, where the same alleles have opposite effects on fitness across environments, leading to costs of adaptation (Kawecki & Ebert, 2004). Adaptation costs were reported in a few studies (reviewed in Bono et al., 2017). However, here we found no robust evidence for the existence of trade-offs, i.e., the adaptation to the novel environment did not involve any clear cost on the ancestral environment. This absence of costs is reported in other studies (e.g., Hereford, 2009; Magalhães et al., 2009) and could indicate that in this case, different genes are involved in the adaptation to different environments (control environment and warming environment). Alternatively, in our work, trade-offs can still exist in traits that were not tested (e.g., longevity). Still, it is important to note that, costs of adaptation are more likely to arise during selection to homogenous environments due to selection being blind to costs associated with conditions that are not being experienced in the local environment (Bono et al., 2017). Our populations had been evolving in a temporal heterogeneous environment, where the mean and the thermal amplitude changed between generations, and for that reason, this changing environment may have promoted the evolution of a more generalist performance.

Our finding of population-specific adaptive response with no clear costs involved suggests a possible advantage in the future climatic scenario where warmer seasons are projected to be warmer (IPCC 2022), while populations will need to keep their ability to cope with lower temperatures in colder seasons. However, this is most likely not enough to ensure population persistence, given the slow pace of the evolutionary response of Portuguese populations and the absence of adaptation of the

populations from The Netherlands. This supports the evidence that ectotherms may have limited capability to respond evolutionarily to temperature shifts (Kellermann & van Heerwaarden, 2019) and may struggle to show an adaptive response to a fast-paced global warming.

Male behaviour but not fertility recovers following exposure to high temperatures

Exposing males to high temperatures during early adult stage resulted in changes in mating behaviour, with males taking longer to start courting and mating immediately (4 hours) after the exposure to the stress. The first result - more extended time till courtship beginning - differs from that of Jiao et al. (2009), where wolf spiders (*Pardosa astrigera*) showed a gradual decrease in courtship latency with increasing temperatures. However, the observation of a longer time to start to copulate is in agreement with several studies in arthropods (Canal Domenech & Fricke, 2022; Costa et al., 2022; Leith et al., 2020). There are two non-exclusive hypotheses for the observed higher copulation latency: females avoid mating with heat-stressed males (female choice) and/or the heat-stressed males are less eager to mate. In our case, both lowered male condition and female choice may be playing a role. Indeed, we noted that heat stressed males exhibit a longer latency to courtship, which suggests that exposure to high temperatures reduced male performance, given that in this species the beginning of courtship is mainly male driven (Immonen et al., 2009). In addition, in one of the pilot tests, females more often accepted the nuptial feeding from non-stressed males than from males subjected to heatwave. This decreased preference for the nuptial feeding of stressed males could be reflected in mating avoidance as well, leading to an increase in mating latency. Curiously, a recent meta-analysis did not find clear evidence for an impact of temperature on mating latency and choosiness (Pilakouta & Baillet, 2022). The lack of a consistent effect of temperature on mating behaviour is likely due to differences in the species under study and to different methodologies used (e.g., the magnitude of the temperature, the time of exposure and the life cycle stage studied). The immediate increase in latency to courtship and copula post stress was followed by a progressive decrease, with heat-stressed males being able to court and mate faster three days following exposure to heat stress and even faster eight days after. Even though stressed males were still slower to court and mate compared to non-stressed males in all timepoints, this result shows that males can recover a relevant portion of their behavioural performance within eight days after thermal stress.

Male exposure to high temperatures led to shorter copulas immediately after the stress. The same pattern has been reported in other works (Costa et al., 2022; Jiao et al., 2009; Sutter et al., 2019). In our study, this might, once again, be linked to the poor condition of heat-stressed males and/or to a lower female eagerness to mate for longer periods. It is likely that this poor condition displayed by stressed males does not allow them to withstand such long copulas as non-stressed males. In addition, females could detect that mating with stressed males' results in less effective copulas (Proshold, 1995). It this is true, females should spend less energy with this kind of mating, thus forcing its interruption. Moreover, as in both latencies, the pattern displayed three and eight days later was different compared to the one immediately after the stress. However, while the courtship and copulation latency of heat-stressed males converged to levels shown by non-stressed males, the duration of the copula with stressed males increased three- and eight-days post-stress, surpassing the levels of those with non-stressed males. Higher copulation duration following thermal stress was also found by Sales et al. (2018) after exposure of *Tribolium castaneum* adult males to a five-day heatwave. Additionally, longer copulas have also been observed in response to other stressful scenarios in *D. subobscura*, namely in response to the presence of conspecific rivals (male competition; Fisher et al., 2013; Lizé et al., 2012; see Bretman et al., 2013 for a similar result in *D. melanogaster*). Finding a female that is willing to mate is difficult, being even harder if females are

monandrous. Therefore, sterile or less fertile males may extend the copula as much as possible, maximising their reproductive output from that mating, as they may not find another female available to mate. In addition, the quality and/or the amount of functional sperm in these stressed males is likely lower than usual (e.g., Canal Domenech & Fricke, 2022; Sales et al., 2018), making longer copulas potentially advantageous by allowing for an increased transfer of sperm to guarantee reproductive success. To my knowledge, there is no other study following the behavioural performance of males through time after heat stress, so our results bring new insights into how global warming affects insects.

We found that the exposure to sub-lethal temperatures, not only affected mating behaviour, but also led to a decline in male fertility, as reported in many studies (Parratt et al., 2021; Rodrigues et al., 2022; Sales et al., 2018, 2021; Walsh et al., 2019, 2021). Here we were only interested in understanding the consequences of male heat stress on populations at the ecological and evolutionary levels. For this reason, we did not evaluate how this stress affects the individuals at the functional level, hence it was not possible to find a clear explanation for the reduction of male fertility. However, based on other studies, likely causes might be reductions in sperm quantity and quality (Canal Domenech & Fricke, 2022; Sales et al., 2018; Vasudeva et al., 2014); reviewed in Wang & Gunderson, 2022), reductions in testis volume (Sales et al., 2021) and accessory gland (Canal Domenech & Fricke, 2022). Furthermore, we showed that this fertility loss was permanent or at least long lasting, with the males remaining partially sterile a minimum of eight days post stress. The evidence for recovery in males is variable. On one hand, a study in several species of Drosophila showed no signs of recovery of fertility seven days after adult exposure to high temperatures (Parratt et al., 2021). Furthermore, a study using *Drosophila virilis* also found permanent sterility upon exposure to thermal stress during the adult stage (Walsh et al., 2021). On the other hand, in the same work, Walsh et al. (2021) showed that males subjected to thermal stress during development can recover their reproductive output. Along the same line, Sales et al. (2021), showed that *T. castaneum* males were capable of fully recovering their fertility following heat exposure when the stress was applied in either the developmental or adult stages. Finally, Canal Domenech and Fricke (2022) demonstrated that *Drosophila melanogaster* males subjected to high temperatures during development were able to partially recover their reproductive output, approaching the levels of the control males on the sixth day of the study. All in all, it seems that males are more likely to recover their reproductive performance following thermal stress when the exposure takes place during the developmental stage rather than during the adult stage, although the reasons for this are unclear. Additional studies measuring the effects on fertility over time of heat stress applied separately in juvenile and adult stages are needed to clarify these differences.

It is curious that the recovery of behavioural performance was not accompanied by an improvement in the male reproductive output. Still, the temperature and the time of exposure that males were subjected to, did not lead to full sterility; thus, it is possible that this recovery in mating behaviour confers some competitive advantage to heat-stressed males by allowing them to (partially) fertilize more females simply by being more competent in mating. That would mean that by recovering its behaviour, the reproductive success per mating of a male should remain the same but its overall reproductive success should increase, a hypothesis that remains to be tested. Evidence suggests that, during the warmer seasons, in wild populations most individuals of *D. subobscura* die within a ten-day period, with few individuals living more than three weeks (Begon, 1978). Therefore, even if males were able to recover their fertility after eight days, given the short life span of this species in warmer seasons, a reduction in fertility during this period should have an enormous impact on male reproductive output. Thus, when males are unable to recover their fertility in an ecologically relevant time span, the recovery of the

mating behaviour could be the only mechanism that heat-stress males have to ensure some progeny after exposure to high temperatures, and consequently prevent large population declines under global warming.

Monandrous females display high levels of remating to rescue fertility when male sterility is prevalent

Given that the fertility of males was clearly affected by temperature and males were unable to recover their performance within a considerable time of adulthood, we tested if females, that are often less affected by high temperatures (e.g., David et al., 2005; Iossa, 2019; Zwoinska et al., 2020), could buffer against population decline by changing their mating behaviour. Female preference for fertile males should be under strong selective pressure, especially in monandrous species, since in these species, only one male is responsible for sire all the female offspring (Fisher et al., 2013). However, if females mate with a sub-fertile male, it should be highly advantageous to remate to avoid reduced fitness, even if they are monandrous. Here we showed that monandrous females (with 3% of remating rate when first mated with fertile males) are 10 to 14 times more prone to remate after mating with heat-stressed males (30% of remating rate when the second mating is with another heat-stressed male, and 42% when the second mating is with a fertile male). These results suggest that females display a remating behaviour as a plastic response that is triggered by a first mating with a stressed male. Similar results were reported in two studies with *Drosophila pseudoobscura* and *T. castaneum* (two polyandrous species), where mating with males exposed to high temperatures led to higher levels of female remating behaviour (Sutter et al., 2019; Vasudeva et al., 2021). The tremendous increase (10 to 14-fold) in female remating rate that we found in our study was clearly higher than that observed in polyandrous species (2-fold increase for *D. pseudoobscura* in Sutter et al., 2019; and around 1.3-fold increase for *T. castaneum* in Vasudeva et al., 2021). Additionally, other studies found higher remating rates in response to other causes of male sterility (Friesen et al., 2014; Landeta-Escamilla et al., 2016). It is noteworthy that here, in contrast to other studies, we reported an alteration in the mating system in response to male sterility, with monandrous females shifting from single to multiple paternity (polyandry) after mating with a heat-stressed male.

Several factors can help explain this female plasticity in remating behaviour. First, first matings involving heat-stressed males had longer courtship and copulation latencies as well as, shorter copulas, compared to matings with non-stressed males, all cues that could be used as indicators of male lowered condition. Furthermore, sex pheromones that can also be disturbed by temperature (Savarit & Ferveur, 2002) may play a role in the female post-copulatory behaviour (Everaerts et al., 2010). In addition, it has been shown that females can detect the levels of ejaculate stored in their spermatheca, which can influence remating propensity (Proshold, 1995). These pre, peri and post-copulatory inputs may function as cues that lead females to adjust their remating behaviour. Alternatively, the female propensity to remate can be mediated by seminal fluid proteins that are produced by the accessory gland in males (Canal Domenech & Fricke, 2022; Harmer et al., 2006). Indeed, a recent work points to the possibility that heat-stressed males cannot transfer sufficient amount or functional seminal fluid proteins, due to a reduction in accessory gland size and, for this reason, males are not able to inhibit female remating (Canal Domenech & Fricke, 2022). Future work should explore the relevance of these mechanisms in explaining female remating behaviour and whether they vary across species.

Such an increase in remating resulted in improved female fertility, independently of the thermal treatment of the second male, indicating that females can boost their reproductive output by both remating with a non-stressed or heat-stress male. This suggests that females are able to use sperm from

more than one male, and the sperm of two heat-stressed males is sufficient to restore female fertility. Interestingly, females from populations from distinct latitudes had a slightly different response when the second male they mated with was non-stressed: while females from low-latitude populations showed a partial rescue of their reproductive performance, females from high-latitude populations displayed a total rescue of the reproductive performance. Regardless of the level of the reproductive rescue, we were able to demonstrate that the remating behaviour is driving this rescue. Being able to recover their fertility, totally or partially, should be highly advantageous for females first mated with heat-exposed males. Our results suggest that females from monandrous species – and not only polyandrous ones (see Sutter et al, 2019; Vasudeva et al. 2021) - are able to make dynamic reproductive decisions, varying their mating behaviour accordingly to their environment. Furthermore, this plastic shift from monandry to polyandry in response to male infertility was highly beneficial to female fitness. Male infertility has been reported in many taxa (reviewed in Walsh et al., 2019), thus polyandry may be a way to assure female fertility, explaining why this behaviour is so widespread in animal species (Taylor et al., 2014).

Taking into consideration the fact that the mating system has enormous repercussions for species both at the individual and the population level (Arnqvist & Nilsson, 2000; Holman & Kokko, 2013; Lizé et al., 2012; Moiron et al., 2022), our findings provide an opportunity to better understand the consequences of a shift from single to multiple paternity. Here we showed that this shift has consequences at the individual level, resulting from alterations in life-history traits related to fitness, namely in female reproductive output. Future experiments should continue to assess how multiple paternity in this species affect the individuals by studying female longevity for example, as reproduction and longevity are thought to be strongly connected (review in De Loof, 2011). It would also be interesting to comprehend how this shift affects male-male competition, particularly within the female. Our results suggest that under certain conditions, females use sperm from more than one male to produce progeny, therefore sperm competition should be considered and assessed in upcoming research.

No clear evolutionary responses on male behavioural and fertility recovery, nor on female remating behaviour and fertility rescue

Although we found an adaptive response to warming conditions in low-latitude populations, we did not find any evident effect of adaptation when assessing male behavioural and fertility recovery, nor on female remating behaviour and fertility rescue after heat stress in males.

An evolutionary response was only observed for copulation duration, with males that evolved in a global warming scenario having shorter copulas than males that evolved at benign temperatures, independently of whether they were exposed to heat stress prior to mating. This could be due to an increase in the metabolic rate under warming conditions (Somero, 2012). Indeed, higher temperatures have been correlated to higher metabolic rates and higher activity in insects (Tüzün & Stoks, 2022; see Colinet et al., 2015 for a review). In addition, in our populations subjected to the global warming scenario a reduction of 3 days in developmental time was observed when compared to populations that have been evolving at 18ºC. Thus, rising temperatures could be linked to a fast-paced life (Tüzün & Stoks, 2022) in which some processes can be quicker with mating duration being one of them. However, this pattern did not lead to an improvement in the fertility response of males that evolved under Warming conditions.

Different explanations can be put forth for the general lack of an evolutionary response. First, the discrepancies could be due to the distinct setups of the first assay when compared to the other two assays. In the first assay (i.e., adaptation to a warming environment), the conditions were identical to the conditions of the populations' maintenance during experimental evolution, therefore the experimental setup maximised the possibility of a response from the Warming populations. This was not the case in the other two assays, where males were subjected to a heat shock, in which the temperature was higher than the maximum temperature experienced by the populations during maintenance. Furthermore, in the first assay that directly tested adaptation to warming, both males and females were from the same population, while in the other assays individuals from the Fluctuating selection regime were used as mates, to ensure that the differences in responses could be attributed exclusively to the sex under study. By doing that we are unable to detect adaptive responses that could have co-evolved from the interaction between males and females from the same selection regime. Finally, for the female remating behaviour, the plastic response observed might be sufficient to ensure the female fertility rescue, hindering an evolutionary response in the Warming populations. Furthermore, the fact that this response observed in both Control and Warming populations may indicate that the plasticity in this behaviour was already selected prior to the foundation of the laboratory populations. This suggests that selection might not generally favour polyandrous females, instead favouring females that flexibly adjust their remating behaviour depending on the environmental conditions. In the wild, where environmental changes often occur (fluctuating environment), this flexibility in remating behaviour may be crucial to population maintenance, since the costs and benefits of polyandry are presumably changing dynamically.

Conclusions

This thesis studied the plastic and evolutionary responses of two populations from contrasting locations in Europe subjected to a global warming scenario. We reported that only high-latitude populations showed adaptation to the warming environment. This adaptation was only detected after 39 generations of evolution, being absent after 22 generations of selection (data not shown, paper under revision). Thus, we provide support for the idea that historically differentiated genetic backgrounds have an important impact on the evolutionary potential of populations. However this population-specific and slow-paced adaptation to warming is probably not enough to keep up with the rapid changes associated with the predictable fast-paced global warming (IPCC 2022).

Despite the adaptive response to the warming environment in the fertility of low-latitude populations, we did not find any clear evidence of higher recovery of mating behaviour and fertility nor of higher female remating behaviour and fertility rescue in populations that evolved under the Warming regime. Instead, the observed responses were plastic, resulting from differences between stressed and non-stressed males, regardless of the selection regime.

Exposure to sub-lethal temperatures during the adult stage resulted in male reduced reproductive behaviour and output, the last not being recoverable with time after exposure. This behavioural recovery could be a mechanism that allows males to produce some progeny. However, the lack of fertility recovery is concerning under predicted climate change scenarios, where an increase in mean temperature, as well as an increase in the occurrence of heat extreme events are expected (IPCC 2022). Heat-induced male infertility occurs in many taxa (reviewed in Walsh et al., 2019), with infertility due to temperatures lower than the species CT_{max} constituting not only a conservation concern but also an economic one.

Still, this work shows that commonly monandrous females that mated with males subjected to sub-lethal temperatures during adulthood exhibited an increase in their propensity to remate leading to a partial to total recovery — depending on population history — of their reproductive success. This finding indicates that an increased female propensity to remate — that led to a shift from monandry to polyandry — may buffer the negative consequences of male infertility, reducing adverse repercussions to populations' reproduction and, subsequently, populations' abundance and persistence. It is however important to note that females were not subjected to thermal stress, under the assumption that females are less affected by high temperatures than males, as found in many species (David et al., 2005; Iossa, 2019; Zwoinska et al., 2020). Future research should focus on assessing the influence of high temperatures on both sexes simultaneously, to better predict population subsistence under climate warming.

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Supplementary Material

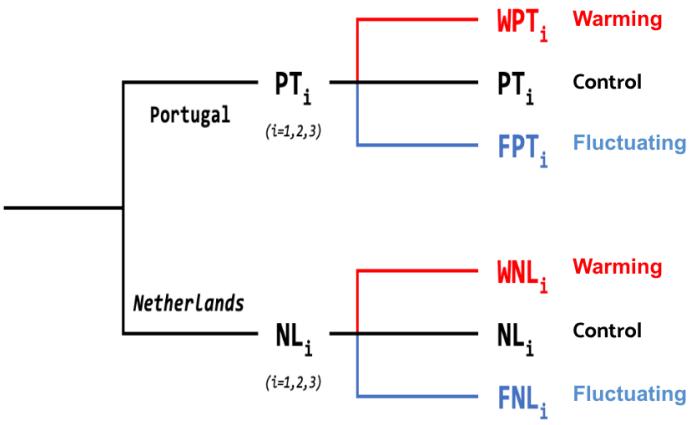

Figure S1 – Population history & Selection regimes. Foundation of two laboratory populations derived from two distinct European locations (Portugal and The Netherlands). After 70 generations of evolution in the lab two new selection regimes were created (Warming and Fluctuating regimes). The black lines represent the Control regime, the red lines the Warming regime and, the blue lines the Fluctuating regime. *i* represents the replicate population number (1, 2 or 3).

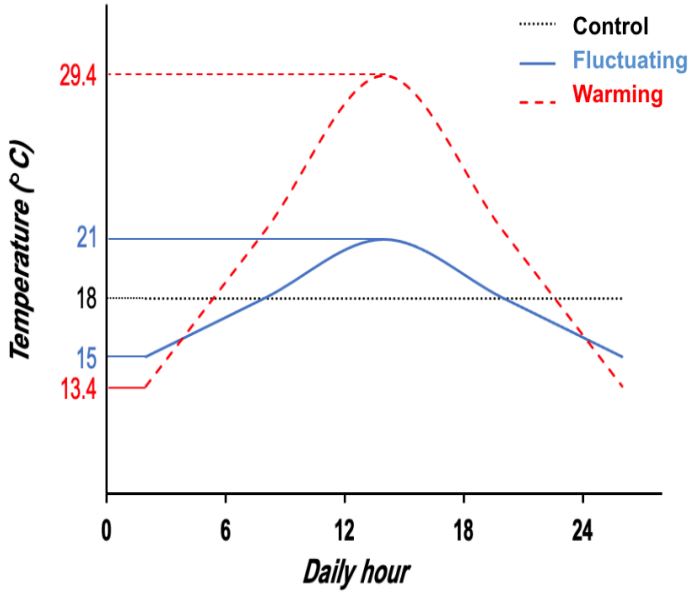

Figure S2 – Daily temperature profile of the three thermal regimes. The black dashed line represents the Control regime, the red dashed line represents the Warming regime (from generation 20 of thermal evolution onwards) and, the blue line the Fluctuating regime.

Table S1 – Description of the statistical models used for data analysis in *Adaptation to a warming environment*. "Response variable": How the variable of interest was coded in the model. "Dataset": Data used to perform the analysis; when needed, the original data was divided into the different levels of a fixed factor present in the model: [a] – subset by History (Dutch or Portuguese). "Sample size": total number of focal individuals included in each analysis. "Maximal model": complete set of explanatory variables included in the model. "Minimal model": model containing only the variables that were statistically significant. Square brackets indicate the error structure used ("qp1": quasi-Poisson, accounting for zero inflation; "b": binomial). "History": different bio-geographical origins of the populations under study (Dutch or Portuguese); "Selection": different thermal selective regimes of the populations under study (Control or Warming); "Environment" different conditions where populations were assayed (control or warming); "Block": different sets of the same-numbered replicate populations (1, 2, or 3). Fecundity: Number of laid eggs between days six and nine; Offspring viability: Ratio between the number of offspring (adult offspring) and the number of laid eggs (total number of eggs) on the ninth day; Reproductive success: number of offspring resulting from eggs laid at day nine.

Var. of interest	Response variable	Dataset	Sample size	Maximal model	Minimal model	R subroutine [err struct.]
Fecundity	Number of eggs	All	470	History * Selection * Environment + (1|Block)	History * Selection * Environment + (1|Block)	glmmTMB [qp1]
		Dutch [a]	236	Selection * Environment + (1|Block)	Selection + Environment + (1|Block)	glmmTMB [qp1]
		Portuguese [a]	234	Selection * Environment + (1|Block)	Selection * Environment + (1|Block)	glmmTMB [qp1]
Offspring viability	cbind(Number of adult offspring, unhatched eggs)	All	448	History * Selection * Environment + (1|Block)	History * Selection * Environment + (1|Block)	glmmTMB [qp1]
		Dutch [a]	227	Selection * Environment + (1|Block)	Selection + Environment + (1|Block)	glmmTMB [qp1]
		Portuguese [a]	221	Selection * Environment + (1|Block)	Selection * Environment + (1|Block)	glmmTMB [qp1]
Reproductive Success	Number of adult offspring	All	448	History * Selection * Environment + (1|Block)	History * Selection * Environment + (1|Block)	glmmTMB [b]
		Dutch [a]	227	Selection * Environment + (1|Block)	Selection + Environment + (1|Block)	glmmTMB [b]
		Portuguese [a]	221	Selection * Environment + (1|Block)	Selection * Environment + (1|Block)	glmmTMB [b]

Table S2 – Description of the statistical models used for data analysis in *Recovery of male mating behaviour and fertility following heat shock*. "Response variable": How the variable of interest was coded in the model. "Dataset": Data used to perform the analysis; when needed, the original data was divided into the different levels of a fixed factor present in the model: [a] – subset by History (Dutch or Portuguese). [b] – subset by Treatment (Non-stressed or Stressed). "Sample size": total number of replicates included in each analysis. [c] - Number of individuals per Mating Opportunity (First/Second/Third). "Maximal model": complete set of explanatory variables included in the model. "Minimal model": model containing only the variables that were statistically significant. Square brackets indicate the error structure used ("g": gaussian; "qp1": quasi-Poisson, accounting for zero inflation; "p1": Poisson, accounting for zero inflation; "b": binomial). "History": different bio-geographical origins of the populations under study (Dutch or Portuguese); "Selection": different thermal selective regimes of the populations under study (Control or Warming); "Treatment": different thermal treatments applied to the males under study (Non-stressed or Stressed); "Mating Opportunity": different mating events (First, Second or Third); "Block": different sets of same-numbered replicate populations (1, 2, or 3); "id": unique identification of each male. Courtship Latency: Time elapsed between pairing and beginning of male courtship; Copulation latency: Time elapsed between pairing and copulation beginning; Copulation Duration: Time elapsed between the beginning of the copula and its ending. Fecundity: Number of laid eggs; Viability: Ratio between the number of offspring (adult offspring) and the number of laid eggs (total number of eggs); Reproductive success: number of offspring resulting from laid eggs.

Var. of interest	Response variable	Dataset	Sample size	Maximal model	Minimal model	R subroutine [err struct.]
Courtship latency	Surv(Courtship latency, sensor)	All	516/480/48 [c]	History * Selection * Treatment * Mating Opportunity + (1|Block)	History + Selection + Treatment + Mating Opportunity + (Treatment:Mating Opportunity) + (1|Block)	coxme [g]
Copulation latency	Surv(Copulation latency, sensor)	All	563/543/534 [c]	History * Selection * Treatment * Mating Opportunity + (1|Block)	History + Selection + Treatment + Mating Opportunity + (Treatment:Mating Opportunity) + (1|Block)	coxme [g]
Copulation duration	Copulation duration	All	279/474/502 [c]	History * Selection * Treatment * Mating Opportunity + (1|Block)	History + Selection + Treatment + Mating Opportunity + (Treatment:Mating Opportunity) + (1|Block)	lmer [g]
Fecundity	Number of eggs	All	562/542/533 [c]	History * Selection * Treatment * Mating Opportunity + (1|Block) + (Mating Opportunity|id)	History * Selection * Treatment * Mating Opportunity + (1|Block) + (Mating Opportunity|id)	glmmTMB [qp1]
		Dutch [a]	277/263/259 [c]	Selection * Treatment * Mating Opportunity + (1|Block) + (Mating Opportunity|id)	Selection * Treatment * Mating Opportunity + (1|Block) + (Mating Opportunity|id)	glmmTMB [p1]
		Dutch [a] Non-stressed [b]	143/139/136 [c]	Selection * Mating Opportunity + (1|Block) + (Mating Opportunity|id)	Selection + Mating Opportunity + (1|Block) + (Mating Opportunity|id)	glmmTMB [p1]
		Dutch [a] Stressed [b]	134/124/123 [c]	Selection * Mating Opportunity + (1|Block) + (Mating Opportunity|id)	Selection * Mating Opportunity + (1|Block) + (Mating Opportunity|id)	glmmTMB [p1]
		Portuguese [a]	285/279/274 [c]	Selection * Treatment * Mating Opportunity + (1|Block) + (Mating Opportunity|id)	Selection + Treatment + Mating Opportunity + (Treatment:Mating Opportunity) + (1|Block) + (Mating Opportunity|id)	glmmTMB [qp1]

Table S2 – Description of the statistical models used for data analysis in *Recovery of male mating behaviour and fertility following heat shock*. Continuation.

Var. of interest	Response variable	Dataset	Sample size	Maximal model	Minimal model	R subroutine [err struct.]
Offspring viability	cbind(Number of adult offspring, unhatched eggs)	All	462/462/467 [c]	History * Selection * Treatment * Mating Opportunity + (1|Block) + (Mating Opportunity|id)	History*Selection*Treatment*Mating Opportunity+(1|Block)+(Mating Opportunity|id)	glmmTMB [b]
		Dutch [a]	232/234/235 [c]	Selection * Treatment * Mating Opportunity + (1|Block) + (Mating Opportunity|id)	Selection * Treatment * Mating Opportunity + (1|Block) + (Mating Opportunity|id)	glmmTMB [b]
		Dutch [a] Non-stressed [b]	135/136/135 [c]	Selection * Mating Opportunity + (1|Block) + (Mating Opportunity|id)	Selection * Mating Opportunity + (1|Block) + (Mating Opportunity|id)	glmmTMB [b]
		Dutch [a] Stressed [b]	97/98/100 [c]	Selection * Mating Opportunity + (1|Block) + (Mating Opportunity|id)	Selection + Mating Opportunity + (1|Block) + (Mating Opportunity|id)	glmmTMB [b]
		Portuguese [a]	230/228/232 [c]	Selection * Treatment * Mating Opportunity + (1|Block) + (Mating Opportunity|id)	Selection * Treatment * Mating Opportunity + (1|Block) + (Mating Opportunity|id)	glmmTMB [b]
		Portuguese [a] Non-stressed [b]	135/137/137 [c]	Selection * Mating Opportunity + (1|Block) + (Mating Opportunity|id)	Selection + Mating Opportunity + (1|Block) + (Mating Opportunity|id)	glmmTMB [b]
		Portuguese [a] Stressed [b]	95/91/95 [c]	Selection * Mating Opportunity + (1|Block) + (Mating Opportunity|id)	Selection * Mating Opportunity + (1|Block) + (Mating Opportunity|id)	glmmTMB [b]
Reproductive Success	Number of adult offspring	All	562/542/533 [c]	History * Selection * Treatment * Mating Opportunity + (1|Block) + (Mating Opportunity|id)	History * Selection * Treatment * Mating Opportunity + (1|Block) + (Mating Opportunity|id)	glmmTMB [qp1]
		Dutch [a]	277/263/259 [c]	Selection * Treatment * Mating Opportunity + (1|Block) + (Mating Opportunity|id)	Selection + Treatment + Mating Opportunity + (Treatment:Mating Opportunity) + (1|Block) + (Mating Opportunity|id)	glmmTMB [p1]
		Portuguese [a]	285/279/274 [c]	Selection * Treatment * Mating Opportunity + (1|Block) + (Mating Opportunity|id)	Selection + Treatment + Mating Opportunity + (Treatment:Mating Opportunity) + (1|Block) + (Mating Opportunity|id)	glmmTMB [qp1]

Table S3 – Description of the statistical models used for data analysis in *Female mating behaviour and fertility following male heat shock*. "Response variable": How the variable of interest was coded in the model. "Dataset": Data used to perform the analysis; when needed, the original data was divided into the different levels of a fixed factor present in the model: [a] – subset by History (Dutch or Portuguese). [b] – subset by Treatment (Non-stressed or Stressed). "Sample size": total number of replicates included in each analysis. [c] - number of individuals per Mating Opportunity (First/Second). "Maximal model": complete set of explanatory variables included in the model. "Minimal model": model containing only the variables that were statistically significant. Square brackets indicate the error structure used ("g": gaussian; "qp1": quasi-Poisson, accounting for zero inflation; "p1": Poisson, accounting for zero inflation; "b": binomial; "nb1"; negative binomial, accounting for zero inflation "qp": quasi-Poisson). "History": different bio-geographical origins of the populations under study (Dutch or Portuguese); "Selection": different thermal selective regimes of the populations under study (Control or Warming); "Treatment": different combinations of males paired with the females under study (Non-stressed x non-stressed, Stressed x non-stressed or Stressed x stressed); "Mating Opportunity": different mating events (First or Second); "Block": different sets of same-numbered replicate populations (1, 2, or 3); "id": unique identification of each female; "Remating" difference between non-remated and remated females (No Remating or Remated). Remating: Female mates with a different male after already mating once. Courtship Latency: Time elapsed between pairing and beginning of male courtship; Copulation latency: Time elapsed between pairing and copulation beginning; Copulation Duration: Time elapsed between the beginning of the copula and its ending. Fecundity: Number of laid eggs; Viability: Ratio between the number of offspring (adult offspring) and the number of laid eggs (total number of eggs); Reproductive success: number of offspring resulting from laid eggs.

Var. of interest	Response variable	Dataset	Sample size	Maximal model	Minimal model	R subroutine [err struct.]
Remating	Remating	All	834	History * Selection * Treatment + (1|Block)	History + Selection + Treatment + (1|Block)	glmmTMB [b]
Courtship latency	Surv(Courtship latency, sensor)	All	795/794 [c]	History * Selection * Treatment * Mating Opportunity + (1|Block)	History + Selection + Treatment + Mating Opportunity + (Treatment:Mating Opportunity) + (1|Block)	coxme [g]
Copulation latency	Surv(Copulation latency, sensor)	All	849/833 [c]	History * Selection * Treatment*Mating Opportunity + (1|Block)	History + Selection + Treatment + Mating Opportunity + (Treatment:Mating Opportunity) + (1|Block)	coxme [g]
Copulation duration	Copulation duration	All	489/191 [c]	History * Selection * Treatment * Mating Opportunity + (1|Block)	History + Selection + Treatment + Mating Opportunity + (Treatment:Mating Opportunity) + (1|Block)	lmer [g]
Fecundity	Number of eggs	All	841/832 [c]	History * Selection * Treatment * Mating Opportunity + (1|Block) + (Mating Opportunity|id)	History + Selection + Treatment + Mating Opportunity + (History:Selection) + (History:Mating Opportunity) + (Selection:Mating Opportunity) + (Treatment:Mating Opportunity) + (1|Block) + (Mating Opportunity|id)	glmmTMB [qp1]
		Dutch [a]	418/414 [c]	Selection * Treatment * Mating Opportunity + (1|Block) + (Mating Opportunity|id)	Selection + Treatment + Mating Opportunity + (Treatment:Mating Opportunity) + (1|Block) + (Mating Opportunity|id)	glmmTMB [p1]
		Portuguese [a]	423/418 [c]	Selection * Treatment * Mating Opportunity + (1|Block) + (Mating Opportunity|id)	Selection + Treatment + Mating Opportunity + (Selection:Mating Opportunity) + (Treatment:Mating Opportunity) + (1|Block) + (Mating Opportunity|id)	glmmTMB [qp1]
Offspring viability	cbind(Number of adult offspring, unhatched eggs)	All	643/736 [c]	History * Selection * Treatment * Mating Opportunity + (1|Block) + (Mating Opportunity|id)	History + Selection + Treatment + Mating Opportunity + (History:Selection) + (History:Mating Opportunity) + (Selection:Mating Opportunity) + (Treatment:Mating Opportunity) + (1|Block) + (Mating Opportunity|id)	glmmTMB [b]

Table S3 – Description of the statistical models used for data analysis in *Female mating behaviour, and fertility following male heat shock*. Continuation.

Var. of interest	Response variable	Dataset	Sample size	Maximal model	Minimal model	R subroutine [err struct.]
Reproductive Success	Number of adult offspring	All	842/833 [c]	History * Selection * Treatment * Mating Opportunity + (1|Block) + (Mating Opportunity|id)	History + Selection + Treatment + Mating Opportunity + (Treatmentt:Mating Opportunity) + (1|Block) + (Mating Opportunity|id)	glmmTMB [qp1]
		Dutch [a]	419/415 [c]	Selection * Treatment * Mating Opportunity + (1|Block) + (Mating Opportunity|id)	Selection + Treatment + Mating Opportunity + (Treatment:Mating Opportunity)+ (1|Block) + (Mating Opportunity|id)	glmmTMB [qp1]
		Portuguese [a]	423/418 [c]	Selection * Treatment * Mating Opportunity + (1|Block) + (Mating Opportunity|id)	Selection + Treatment + Mating Opportunity + (Selection:Mating Opportunity) + (Treatment:Mating Opportunity) + (1|Block)+(Mating Opportunity|id)	glmmTMB [qp1]
		All	842/833 [c]	Treatment * Mating Opportunity * Remating + (1|Block) + (Mating Opportunity|id)	Treatment * Mating Opportunity * Remating + (1|Block) + (Mating Opportunity|id)	glmmTMB [qp1]
		Non-stressed x non-stressed [b]	286/284 [c]	Mating Opportunity * Remating + (1|Block) + (Mating Opportunity|id)	Mating Opportunity + Remating + (1|Block) + (Mating Opportunity|id)	glmmTMB [nb1]
		Stressed x non-stressed [b]	287/285 [c]	Mating Opportunity * Remating + (1|Block) + (Mating Opportunity|id)	Mating Opportunity * Remating + (1|Block) + (Mating Opportunity|id)	glmmTMB [qp]
		Stressed x stressed [b]	269/264 [c]	Mating Opportunity * Remating + (1|Block) + (Mating Opportunity|id)	Mating Opportunity * Remating + (1|Block) + (Mating Opportunity|id)	glmmTMB [qp1]

Table S4 – Experimental conditions and results of the heat shock treatment pilot test. Males: Selective regime of the males that were subjected to heat shock. Temperature: Constant temperature applied during the heat shock. Exposure time: Time elapsed between the beginning and the end of the heat shock. Recovery time: Time elapsed between the end of the heat shock and paring with a virgin female. Differences to the control: differences in fecundity (number of laid eggs) between control and heat-stressed males from the same population. The experimental conditions that produced a higher reduction in male fertility are represented in bold. * The heat shock at 32°C resulted in over 40% of male mortality and for that reason we excluded this condition.

Males	Temperature	Exposure Time	Sample size	Recovery time	Differences to control (fecundity)
Control Regime	27°C	24h	10	24h	-4.0
		48h	10	24h	+5.3
	28°C	24h	10	24h	-4.7
		48h	10	24h	-1.3
	30°C	12h	12	24h	-5.2
		24h	12	24h	+2.9
	31°C	12h	12	24h	+9.6
		24h	12	24h	-18.1
			48	40h	-15.3
		58h	48	15h	-20.8
		69h	**48**	**4h**	**-33.7**
Fluctuating Regime	31°C	**69h**	**48**	**4h**	**-29.9**
	32°C	2.5h	48	*	*
		3h	48	*	*

Table S5 – Results from the analyses of variance of the effect of a heatwave during developmental and adult stages and the effect of a food dye on male reproductive success. Reproductive success: number of adult offspring. Males with one of two biogeographical origins ("History": The Netherlands or Portugal), dyed or not ("Dye": Non-dyed or Dyed), were subjected to one of two treatments ("Treatment": Non-stressed or Stressed). "Df": the degrees of freedom. "X^2": the Chi-square value obtained in each analysis. Statistically significant terms are represented in bold.

Trait	Independent Variable	Df	X^2	p-value
Reproductive Success	History	1	0.6325	0.426
	Dye	1	0.0578	0.810
	Treatment	**1**	**289.496**	**< 0.001**

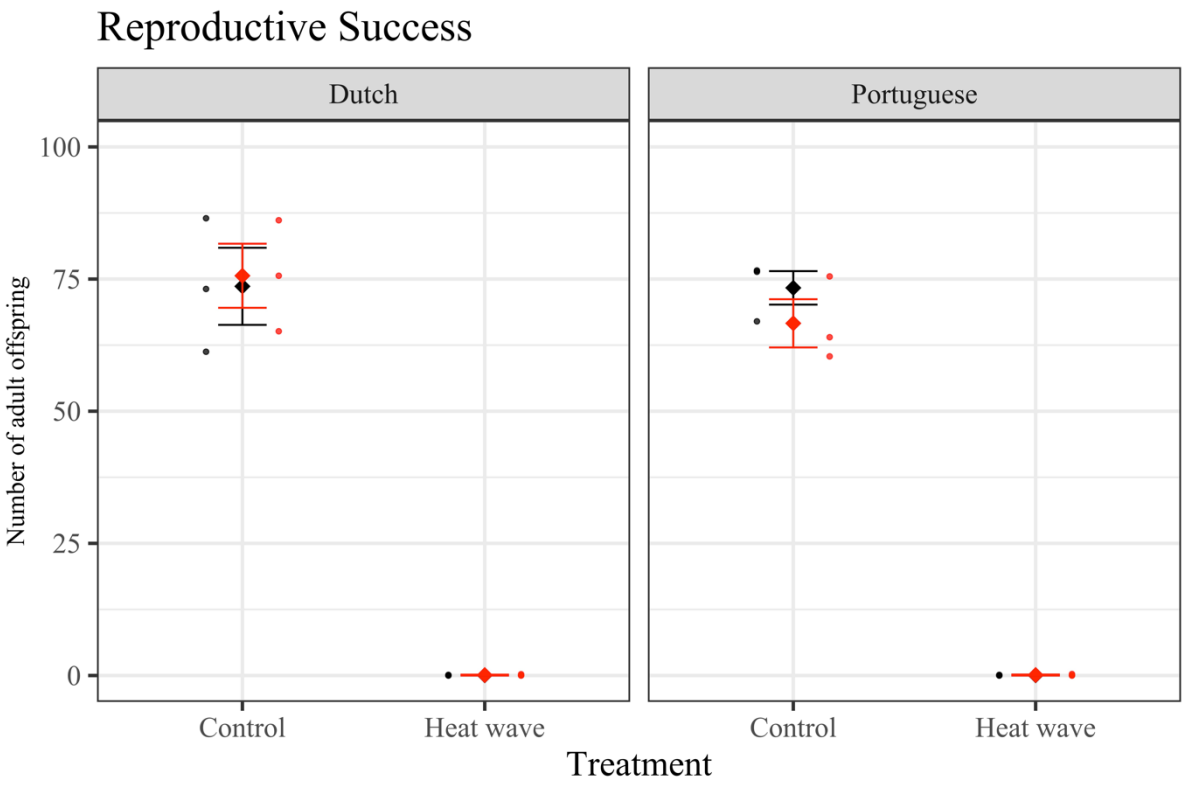

Figure S3 – Effect of a heatwave during developmental and adult stages and the effect of a food dye on male reproductive success after 41 generations of thermal evolution. Reproductive success: number of offspring resulting from laid eggs. The colour black represents males that were not dyed, while the red colour represents dyed males. The circles represent the mean values of each replicate population, and the diamonds represent the mean of the three replicate populations. The error bars represent the standard error (variation between replicate populations) of the overall mean.

Table S6 – Results from the analyses of variance of Adaptation to a warming environment. Fecundity: Number of laid eggs between days six and nine; Offspring viability: Ratio between the number of offspring (adult offspring) and the number of laid eggs (total number of eggs) on the ninth day; Reproductive success: number of offspring resulting from eggs laid at day nine. Individuals with one of two bio-geographical origins ("History": The Netherlands or Portugal) that came from one of two selection regimes ("Selection": Control or Warming) were tested in one of two environments ("Environment": control environment or warming environment). "Df": the degrees of freedom. "X^2": the Chi-square value obtained in each analysis. Statistically significant terms are represented in bold.

Trait	Independent Variable	*Df*	X^2	*p-value*
Fecundity	History	1	2.918	0.089
	Selection	1	0.108	0.742
	Environment	**1**	**142.710**	**< 0.001**
	History*Selection	1	0.644	0.422
	History*Environment	1	2.818	0.093
	Selection*Environment	1	2.653	0.103
	History*Selection*Environment	1	3.382	0.066
Offspring viability	**History**	**1**	**37.091**	**< 0.001**
	Selection	1	0.112	0.738
	Environment	**1**	**2823.276**	**< 0.001**
	History*Selection	**1**	**6.004**	**0.014**
	History*Environment	1	1.409	0.235
	Selection*Environment	**1**	**66.032**	**< 0.001**
	History*Selection*Environment	1	1.722	0.189
Reproductive Success	History	1	0.648	0.4209
	Selection	1	2.221	0.136
	Environment	**1**	**217.241**	**< 0.001**
	History*Selection	1	0.010	0.752
	History*Environment	1	0.031	0.860
	Selection*Environment	**1**	**8.859**	**0.003**
	History*Selection*Environment	1	2.850	0.091

Table S7 – *A posteriori* contrasts of reproductive success of Portuguese population in Adaptation to a warming environment. Reproductive Success: Number of adult offspring. "T ratio": the T-test value obtained in each comparison. Comparison: Interaction between Selection (Control or Warming) and Environment (control environment or warming environment). Statistically significant terms are represented in bold.

Trait	History	Comparison	*T ratio*	*p-value*
Reproductive Success	Portugal	Control x control environment - Warming x control environment	1.924	0.221
		Control x control environment - Control x warming environment	**9.262**	**< 0.001**
		Control x control environment - Warming x warming environment	**7.400**	**< 0.001**
		Warming x control environment - Control x warming environment	**7.804**	**< 0.001**
		Warming x control environment - Warming x warming environment	**5.663**	**< 0.001**
		Control x warming environment - Warming x warming environment	**-2.813**	**0.027**

Table S8 – *A posteriori* contrasts for the effect of heat shock during male adulthood on male mating behaviour. Courtship Latency: Time elapsed between pairing and beginning of male courtship; Copulation latency: Time elapsed between pairing and copulation beginning; Copulation Duration: Time elapsed between the beginning of the copula and its ending. *A posteriori* tukey contrasts. "Z or T ratio": the T-test value obtained in each comparison. Comparison: Interaction between Treatment (Stressed or Non-stressed) and Mating Opportunity (First, Second or Third). Statistically significant terms are represented in bold.

Trait	Comparison	*Z or T ratio*	*p-value*
		Z ratio	
Courtship Latency	**Non-stressed x First - Stressed x First**	**13.427**	**<0.001**
	Non-stressed x First - Stressed x Second	1.533	0.643
	Non-stressed x First - Stressed x Third	-1.923	0.388
	Non-stressed x First - Non-stressed x Second	**-7.778**	**<0.001**
	Non-stressed x First - Non-stressed x Third	**-7.297**	**<0.001**
	Non-stressed x Second - Stressed x First	**-20.018**	**<0.001**
	Non-stressed x Second - Stressed x Second	**9.096**	**<0.001**
	Non-stressed x Second - Stressed x Third	**5.816**	**<0.001**
	Non-stressed x Second - Non-stressed x Third	0.465	0.997
	Non-stressed x Third - Stressed x First	**-19.424**	**<0.001**
	Non-stressed x Third - Stressed x Second	**-8.615**	**<0.001**
	Non-stressed x Third - Stressed x Third	**5.357**	**<0.001**
	Stressed x First - Stressed x Second	**-11.906**	**<0.001**
	Stressed x First - Stressed x Third	**-14.894**	**<0.001**
	Stressed x Second - Stressed x Third	**-3.403**	**0.009**
		Z ratio	
Copulation Latency	**Non-stressed x First - Stressed x First**	**14.902**	**<0.001**
	Non-stressed x First - Stressed x Second	**3.635**	**0.004**
	Non-stressed x First - Stressed x Third	-0.284	0.999
	Non-stressed x First - Non-stressed x Second	**-8.206**	**<0.001**
	Non-stressed x First - Non-stressed x Third	**-7.899**	**<0.001**
	Non-stressed x Second - Stressed x First	**-21.717**	**<0.001**
	Non-stressed x Second - Stressed x Second	**11.453**	**<0.001**
	Non-stressed x Second - Stressed x Third	**7.684**	**<0.001**
	Non-stressed x Second - Non-stressed x Third	0.210	0.999
	Non-stressed x Third - Stressed x First	**-21.142**	**<0.001**
	Non-stressed x Third - Stressed x Second	**-11.114**	**<0.001**
	Non-stressed x Third - Stressed x Third	**7.408**	**<0.001**
	Stressed x First - Stressed x Second	**-11.292**	**<0.001**
	Stressed x First - Stressed x Third	**-14.729**	**<0.001**
	Stressed x Second - Stressed x Third	**3.802**	**0.002**
		T ratio	
Copulation Duration	**Non-stressed x First - Stressed x First**	**3.816**	**0.002**
	Non-stressed x First - Stressed x Second	**-5.902**	**<0.001**
	Non-stressed x First - Stressed x Third	**-9.690**	**<0.001**
	Non-stressed x First - Non-stressed x Second	**6.642**	**<0.001**
	Non-stressed x First - Non-stressed x Third	**5.595**	**<0.001**
	Non-stressed x Second - Stressed x First	-0.555	0.994
	Non-stressed x Second - Stressed x Second	**-12.367**	**<0.001**
	Non-stressed x Second - Stressed x Third	**-16.485**	**<0.001**
	Non-stressed x Second - Non-stressed x Third	-1.083	0.888
	Non-stressed x Third - Stressed x First	-1.075	0.891
	Non-stressed x Third - Stressed x Second	**11.375**	**<0.001**
	Non-stressed x Third - Stressed x Third	**-15.464**	**<0.001**
	Stressed x First - Stressed x Second	**-6.852**	**<0.001**
	Stressed x First - Stressed x Third	**-8.746**	**<0.001**
	Stressed x Second - Stressed x Third	**-3.433**	**0.008**

Table S9 – Results from the analyses of variances of the effect of heat shock during male adulthood on male fertility. Fecundity: Number of laid eggs; Offspring viability: Ratio between the number of offspring (adult offspring) and the number of laid eggs (total number of eggs); Reproductive success: number of offspring resulting from laid eggs. Males with one of two bio-geographical origins ("History": The Netherlands or Portugal), that came from one of two selection regimes ("Selection": Control or Warming), were subjected to one of two treatments ("Treatment": Non-stressed or Stressed) in three distinct moments ("Mating Opportunity": First, Second or Third). "Df": the degrees of freedom. "X^2": the Chi-square value obtained in each analysis. Statistically significant terms are represented in bold.

Trait	Independent Variable	*Df*	X^2	*p-value*
Fecundity	History	1	0.080	0.777
	Selection	1	1.076	0.299
	Treatment	**1**	**80.083**	**< 0.001**
	Mating Opportunity	**2**	**18.648**	**< 0.001**
	History*Selection	1	0	0.999
	History*Treatment	1	0.055	0.814
	Selection*Treatment	1	0.370	0.543
	History*Mating Opportunity	2	1.894	0.388
	Selection*Mating Opportunity	2	2.194	0.334
	Treatment*Mating Opportunity	**2**	**9.322**	**0.009**
	History*Selection*Treatment	1	0.549	0.459
	History*Selection*Mating Opportunity	2	2.266	0.322
	History*Treatment*Mating Opportunity	2	1.910	0.385
	Selection*Treatment*Mating Opportunity	2	0.746	0.689
	History*Selection*Treatment*Mating Opportunity	**2**	**9.865**	**0.007**
Offspring viability	**History**	**1**	**3.897**	**0.048**
	Selection	1	1.523	0.738
	Treatment	**1**	**174.501**	**< 0.001**
	Mating Opportunity	**2**	**47.182**	**< 0.001**
	History*Selection	1	0.154	0.695
	History*Treatment	**1**	**13.615**	**< 0.001**
	Selection*Treatment	**1**	**5.456**	**0.020**
	History*Mating Opportunity	2	1.521	0.468
	Selection*Mating Opportunity	**2**	**6.250**	**0.044**
	Treatment*Mating Opportunity	**2**	**24.103**	**< 0.001**
	History*Selection*Treatment	1	0.800	0.371
	History*Selection*Mating Opportunity	**2**	**8.238**	**0.016**
	History*Treatment*Mating Opportunity	**2**	**7.634**	**0.022**
	Selection*Treatment*Mating Opportunity	2	3.794	0.150
	History*Selection*Treatment*Mating Opportunity	**2**	**10.889**	**0.004**
Reproductive Success	History	1	0	0.997
	Selection	1	0.243	0.622
	Treatment	**1**	**105.811**	**< 0.001**
	Mating Opportunity	2	2.381	0.304
	History*Selection	1	0.024	0.877
	History*Treatment	1	0.080	0.778
	Selection*Treatment	1	0.193	0.661
	History*Mating Opportunity	2	2.871	0.237
	Selection*Mating Opportunity	2	2.367	0.306
	Treatment*Mating Opportunity	**2**	**17.554**	**< 0.001**
	History*Selection*Treatment	1	0.002	0.965
	History*Selection*Mating Opportunity	2	1.863	0.394
	History*Treatment*Mating Opportunity	2	2.366	0.306
	Selection*Treatment*Mating Opportunity	2	2.588	0.274
	History*Selection*Treatment*Mating Opportunity	**2**	**7.441**	**0.024**

Table S10 –Results from the analysis of variance of the effect of heat shock during male adulthood on male fertility for Dutch and Portuguese populations separated by treatment (Non-stressed or Stressed). Fecundity: Number of laid eggs; Offspring viability: Ratio between the number of offspring (adult offspring) and the number of laid eggs (total number of eggs). Males with one of two bio-geographical origins ("History": The Netherlands or Portugal), that came from one of two selection regimes ("Selection": Control or Warming), were subjected to one of two treatments ("Treatment": Non-stressed or Stressed) in three distinct moments ("Mating Opportunity": First, Second or Third). "Df": the degrees of freedom. "X^2": the Chi-square value obtained in each analysis. Statistically significant terms are represented in bold.

History	Treatment	Trait	Explanatory Variable	*Df*	X^2	*p-value*
The Netherlands	Non-Stressed	Fecundity	Selection	1	0.045	0.832
			Mating Opportunity	**2**	**13.010**	**0.001**
		Offspring viability	Selection	1	0.033	0.857
			Mating Opportunity	**2**	**6.112**	**0.047**
			Selection*Mating Opportunity	2	5.132	0.077
	Stressed	Fecundity	Selection	1	0.456	0.499
			Mating Opportunity	2	0.663	0.718
			Selection*Mating Opportunity	**2**	**6.346**	**0.042**
		Offspring viability	Selection	1	0.220	0.640
			Mating Opportunity	**2**	**22.222**	**< 0.001**
Portugal	Non-Stressed	Offspring viability	Selection	1	3.757	0.053
			Mating Opportunity	**2**	**8.470**	**0.014**
	Stressed	Offspring viability	Selection	1	2.589	0.108
			Mating Opportunity	**2**	**21.317**	**< 0.001**
			Selection*Mating Opportunity	**2**	**8.894**	**0.012**

Table S11 –*A posteriori* contrasts of the effect of heat shock during male adulthood on male fecundity and offspring viability for Dutch and Portuguese populations separated by treatment (Non-stressed or Stressed). Fecundity: Number of laid eggs; Offspring viability: Ratio between the number of offspring (adult offspring) and the number of laid eggs (total number of eggs). *A posteriori* tukey contrasts. "T ratio": the T-test value obtained in each comparison. A) Comparison between Mating Opportunities (First, Second or Third). B) Comparison: Interaction between Selection (Control or Warming) and Mating Opportunity (First, Second or Third). Statistically significant terms are represented in bold.

A)

History	Treatment	Trait	Comparison	*T ratio*	*p-value*
The Netherlands	Non-Stressed	Fecundity	First - Second	2.181	0.076
			First - Third	-2.028	0.107
			Second - Third	**-3.603**	**0.001**
		Offspring viability	First - Second	-1.820	0.165
			First - Third	1.034	0.556
			Second - Third	**2.450**	**0.039**
	Stressed	Offspring viability	First - Second	-1.643	0.229
			First - Third	2.297	0.057
			Second - Third	**4.711**	**< 0.001**
Portugal	Non-stressed	Offspring viability	First - Second	1.158	0.479
			First - Third	**2.863**	**0.012**
			Second - Third	1.960	0.124

Table S11 –*A posteriori* contrasts of the effect of heat shock during male adulthood on male fecundity and offspring viability for Dutch and Portuguese populations separated by treatment (Non-stressed or Stressed). Continuation.

B)

History	Treatment	Trait	Comparison	*T ratio*	*p-value*
The Netherlands	Stressed	Fecundity	Control x First - Warming x First	-0.594	0.991
			Control x First - Warming x Second	-0.448	0.998
			Control x First - Warming x Third	0.432	0.998
			Control x First - Control x Second	-0.107	1.000
			Control x First - Control x Third	-1.919	0.392
			Control x Second - Warming x First	0.501	0.996
			Control x Second - Warming x Second	-0.355	0.999
			Control x Second - Warming x Third	0.528	0.995
			Control x Second - Control x Third	-2.305	0.1947
			Control x Third - Warming x First	-1.243	0.816
			Control x Third - Warming x Second	-1.465	0.687
			Control x Third - Warming x Third	2.425	0.150
			Warming x First - Warming x Second	0.174	1.000
			Warming x First - Warming x Third	0.970	0.927
			Warming x Second - Warming x Third	1.041	0.904
Portugal	Stressed	Offspring viability	Control x First - Warming x First	2.370	0.169
			Control x First - Warming x Second	-0.250	1.000
			Control x First - Warming x Third	0.488	0.997
			Control x First - Control x Second	-2.170	0.254
			Control x First - Control x Third	1.692	0.538
			Control x Second - Warming x First	**-4.455**	**< 0.001**
			Control x Second - Warming x Second	2.124	0.277
			Control x Second - Warming x Third	2.638	0.090
			Control x Second - Control x Third	**4.222**	**< 0.001**
			Control x Third - Warming x First	-0.976	0.925
			Control x Third - Warming x Second	2.087	0.296
			Control x Third - Warming x Third	-1.114	0.876
			Warming x First - Warming x Second	**-3.262**	**0.015**
			Warming x First - Warming x Third	-2.204	0.238
			Warming x Second - Warming x Third	0.946	0.934

Table S12 – *A posteriori* contrasts for the effect of heat shock during male adulthood on male fertility for Dutch and Portuguese populations separately. Fecundity: Number of laid eggs; Reproductive success: number of offspring resulting from laid eggs. *A posteriori* tukey contrasts. "T ratio": the T-test value obtained in each comparison. Comparison: Interaction between Treatment (Stressed or Non-stressed) and Mating Opportunity (First, Second or Third). Statistically significant terms are represented in bold.

History	Trait	Comparison	*T ratio*	*p-value*
The Netherlands	Reproductive Success	**Non-stressed x First - Stressed x First**	**5.109**	**<0.001**
		Non-stressed x First - Stressed x Second	**3.919**	**0.001**
		Non-stressed x First - Stressed x Third	**5.759**	**<0.001**
		Non-stressed x First - Non-stressed x Second	1.869	0.422
		Non-stressed x First - Non-stressed x Third	-0.737	0.977
		Non-stressed x Second - Stressed x First	**-3.370**	**0.010**
		Non-stressed x Second - Stressed x Second	2.309	0.192
		Non-stressed x Second - Stressed x Third	**3.989**	**0.001**
		Non-stressed x Second - Non-stressed x Third	-2.447	0.142
		Non-stressed x Third - Stressed x First	**-5.662**	**<0.001**
		Non-stressed x Third - Stressed x Second	**-4.456**	**<0.001**
		Non-stressed x Third - Stressed x Third	**6.321**	**<0.001**
		Stressed x First - Stressed x Second	-0.969	0.928
		Stressed x First - Stressed x Third	0.636	0.988
		Stressed x Second - Stressed x Third	1.547	0.634
Portugal	Fecundity	**Non-stressed x First - Stressed x First**	**5.050**	**<0.001**
		Non-stressed x First - Stressed x Second	2.804	0.058
		Non-stressed x First - Stressed x Third	2.345	0.177
		Non-stressed x First - Non-stressed x Second	1.529	0.646
		Non-stressed x First - Non-stressed x Third	-2.023	0.330
		Non-stressed x Second - Stressed x First	**-3.813**	**0.002**
		Non-stressed x Second - Stressed x Second	1.524	0.649
		Non-stressed x Second - Stressed x Third	0.966	0.929
		Non-stressed x Second - Non-stressed x Third	**-3.494**	**0.007**
		Non-stressed x Third - Stressed x First	**-6.812**	**<0.001**
		Non-stressed x Third - Stressed x Second	**-4.575**	**0.001**
		Non-stressed x Third - Stressed x Third	**4.247**	**0.003**
		Stressed x First - Stressed x Second	-2.158	0.259
		Stressed x First - Stressed x Third	**-2.889**	**0.046**
		Stressed x Second - Stressed x Third	-0.582	0.992
	Reproductive Success	**Non-stressed x First - Stressed x First**	**5.745**	**<0.001**
		Non-stressed x First - Stressed x Second	**3.616**	**0.004**
		Non-stressed x First - Stressed x Third	**4.201**	**<0.001**
		Non-stressed x First - Non-stressed x Second	1.388	0.735
		Non-stressed x First - Non-stressed x Third	-0.852	0.958
		Non-stressed x Second - Stressed x First	**-4.721**	**<0.001**
		Non-stressed x Second - Stressed x Second	2.499	0.125
		Non-stressed x Second - Stressed x Third	**3.118**	**0.023**
		Non-stressed x Second - Non-stressed x Third	-2.323	0.186
		Non-stressed x Third - Stressed x First	**-6.303**	**<0.001**
		Non-stressed x Third - Stressed x Second	**-4.267**	**<0.001**
		Non-stressed x Third - Stressed x Third	**4.839**	**<0.001**
		Stressed x First - Stressed x Second	-2.332	0.182
		Stressed x First - Stressed x Third	-1.651	0.565
		Stressed x Second - Stressed x Third	0.678	0.984

Table S13 –*A posteriori* contrasts of the effect of heat shock during male adulthood on female propensity to remate. Remating: female mates with a different male after already mating once. *A posteriori* tukey contrasts. "T ratio": the T-test value obtained in each comparison. A) Comparison between Treatments (Non-stressed x non-stressed, Stressed x non-stressed or Stressed x stressed). Statistically significant terms are represented in bold.

Trait	Comparison	*T ratio*	*p-value*
Remating	**Non-stressed x non-stressed - Stressed x non-stressed**	**-8.736**	**< 0.001**
	Non-stressed x non-stressed - Stressed x stressed	**-7.191**	**< 0.001**
	Stressed x non-stressed - Stressed x stressed	**3.037**	**0.007**

Table S14 – *A posteriori* contrasts for the effect of heat shock during male adulthood on female mating behaviour. Courtship Latency: Time elapsed between pairing and beginning of male courtship; Copulation latency: Time elapsed between pairing and copulation beginning; Copulation Duration: Time elapsed between the beginning of the copula and its ending. *A posteriori* tukey contrasts. "Z or T ratio": the T-test value obtained in each comparison. Comparison: Interaction between Treatment (Non-stressed x non-stressed, Stressed x non-stressed or Stressed x stressed) and Mating Opportunity (First or Second). Statistically significant terms are represented in bold.

Trait	Comparison	*Z or T ratio*	*p-value*
		Z ratio	
Courtship Latency	**Non-stressed x non-stressed x First – Stressed x non-stressed x First**	**12.519**	**< 0.001**
	Non-stressed x non-stressed x First – Stressed x non-stressed x Second	**13.798**	**< 0.001**
	Non-stressed x non-stressed x First – Stressed x stressed x First	**10.787**	**< 0.001**
	Non-stressed x non-stressed x First – Stressed x stressed x Second	**20.049**	**< 0.001**
	Non-stressed x non-stressed x First - Non-stressed x non-stressed x Second	**13.543**	**< 0.001**
	Non-stressed x non-stressed x Second – Stressed x non-stressed x First	**10.315**	**< 0.001**
	Non-stressed x non-stressed x Second – Stressed x non-stressed x Second	**-9.292**	**< 0.001**
	Non-stressed x non-stressed x Second – Stressed x stressed x First	**10.817**	**< 0.001**
	Non-stressed x non-stressed x Second – Stressed x stressed x Second	**-6.224**	**< 0.001**
	Stressed x non-stressed x First – Stressed x stressed x First	-1.961	0.365
	Stressed x non-stressed x First – Stressed x stressed x Second	**11.203**	**< 0.001**
	Stressed x non-stressed x First – Stressed x non-stressed x Second	**3.079**	**0.025**
	Stressed x non-stressed x Second – Stressed x stressed x First	**4.767**	**< 0.001**
	Stressed x non-stressed x Second – Stressed x stressed x Second	**7.901**	**< 0.001**
	Stressed x stressed x First – Stressed x stressed x Second	**12.633**	**< 0.001**
		Z ratio	
Copulation Latency	**Non-stressed x non-stressed x First – Stressed x non-stressed x First**	**14.245**	**< 0.001**
	Non-stressed x non-stressed x First – Stressed x non-stressed x Second	**16.352**	**< 0.001**
	Non-stressed x non-stressed x First – Stressed x stressed x First	**12.339**	**< 0.001**
	Non-stressed x non-stressed x First – Stressed x stressed x Second	**20.637**	**< 0.001**
	Non-stressed x non-stressed x First - Non-stressed x non-stressed x Second	**15.716**	**< 0.001**
	Non-stressed x non-stressed x Second – Stressed x non-stressed x First	**11.780**	**< 0.001**
	Non-stressed x non-stressed x Second – Stressed x non-stressed x Second	**-10.461**	**< 0.001**
	Non-stressed x non-stressed x Second – Stressed x stressed x First	**12.354**	**< 0.001**
	Non-stressed x non-stressed x Second – Stressed x stressed x Second	**-7.748**	**< 0.001**
	Stressed x non-stressed x First – Stressed x stressed x First	-2.091	0.292
	Stressed x non-stressed x First – Stressed x stressed x Second	**10.264**	**< 0.001**
	Stressed x non-stressed x First – Stressed x non-stressed x Second	**3.925**	**0.001**
	Stressed x non-stressed x Second – Stressed x stressed x First	**5.764**	**< 0.001**
	Stressed x non-stressed x Second – Stressed x stressed x Second	**6.548**	**< 0.001**
	Stressed x stressed x First – Stressed x stressed x Second	**11.812**	**< 0.001**

Table S14 – *A posteriori* contrasts for the effect of heat shock during male adulthood on female mating behaviour. Continuation.

Trait	Comparison	*Z or T ratio*	*p-value*
		T ratio	
Copulation Duration	**Non-stressed x non-stressed x First – Stressed x non-stressed x First**	**9.978**	**< 0.001**
	Non-stressed x non-stressed x First – Stressed x non-stressed x Second	2.423	0.150
	Non-stressed x non-stressed x First – Stressed x stressed x First	**9.408**	**< 0.001**
	Non-stressed x non-stressed x First – Stressed x stressed x Second	**6.439**	**< 0.001**
	Non-stressed x non-stressed x First - Non-stressed x non-stressed x Second	1.123	0.872
	Non-stressed x non-stressed x Second – Stressed x non-stressed x First	-2.037	0.322
	Non-stressed x non-stressed x Second – Stressed x non-stressed x Second	-0.403	0.999
	Non-stressed x non-stressed x Second – Stressed x stressed x First	-1.714	0.523
	Non-stressed x non-stressed x Second – Stressed x stressed x Second	1.665	0.556
	Stressed x non-stressed x First – Stressed x stressed x First	-0.918	0.942
	Stressed x non-stressed x First – Stressed x stressed x Second	-0.611	0.990
	Stressed x non-stressed x First – Stressed x non-stressed x Second	**-6.891**	**< 0.001**
	Stressed x non-stressed x Second – Stressed x stressed x First	**-6.188**	**< 0.001**
	Stressed x non-stressed x Second – Stressed x stressed x Second	**4.579**	**0.001**
	Stressed x stressed x First – Stressed x stressed x Second	0.072	1

Table S15 – Results from the analyses of variances of the effect of heat shock during male adulthood on female fertility. Fecundity: Number of laid eggs; Reproductive success: number of offspring resulting from laid eggs. Females with one of two bio-geographical origins ("History": The Netherlands or Portugal), that came from one of two selection regimes ("Selection": Control or Warming), were subjected to one of three treatments ("Treatment": Non-stressed x non-stressed, Stressed x non-stressed or Stressed x stressed) and were tested in two distinct moments ("Mating Opportunity": First or Second). "Df": the degrees of freedom. "X^2": the Chi-square value obtained in each analysis. Statistically significant terms are represented in bold.

Trait	Explanatory Variable	*Df*	X^2	*p-value*
Fecundity	History	1	0.814	0.367
	Selection	**1**	**6.304**	**0.012**
	Treatment	**2**	**87.430**	**< 0.001**
	Mating Opportunity	**1**	**357.253**	**< 0.001**
	History*Selection	**1**	**5.497**	**0.019**
	History*Mating Opportunity	**1**	**10.035**	**0.002**
	Selection*Mating Opportunity	**1**	**10.559**	**0.001**
	Treatment*Mating Opportunity	**2**	**20.950**	**< 0.001**
Reproductive Success	**History**	**1**	**6.668**	**0.010**
	Selection	**1**	**5.196**	**0.023**
	Treatment	**2**	**97.506**	**< 0.001**
	Mating Opportunity	**1**	**335.746**	**< 0.001**
	History*Selection	**1**	**6.660**	**0.010**
	History*Mating Opportunity	**1**	**7.980**	**0.010**
	Selection*Mating Opportunity	**1**	**8.686**	**0.005**
	Treatment*Mating Opportunity	**2**	**22.884**	**< 0.001**

Table S16 – *A posteriori* contrasts for the effect of heat shock during male adulthood on female fertility for Dutch and Portuguese populations. Fecundity: Number of laid eggs; Reproductive success: number of offspring resulting from laid eggs. *A posteriori* tukey contrasts. "T ratio": the T-test value obtained in each comparison. A) Comparison: Interaction between Treatment (Non-stressed x non-stressed, Stressed x non-stressed or Stressed x stressed) and Mating Opportunity (First or Second). B) Comparison: Interaction between Selection (Control or Warming) and Mating Opportunity (First or Second). Statistically significant terms are represented in bold.

A)

History	Trait	Comparison	*T ratio*	*p-value*
The Netherlands	Fecundity	**Non-stressed x non-stressed x First – Stressed x non-stressed x First**	**4.989**	**< 0.001**
		Non-stressed x non-stressed x First – Stressed x non-stressed x Second	-1.738	0.507
		Non-stressed x non-stressed x First – Stressed x stressed x First	**4.817**	**< 0.001**
		Non-stressed x non-stressed x First – Stressed x stressed x Second	-1.424	0.713
		Non-stressed x non-stressed x First - Non-stressed x non-stressed x Second	**-4.626**	**< 0.001**
		Non-stressed x non-stressed x Second – Stressed x non-stressed x First	**-9.000**	**< 0.001**
		Non-stressed x non-stressed x Second – Stressed x non-stressed x Second	2.548	0.112
		Non-stressed x non-stressed x Second – Stressed x stressed x First	**-8.703**	**< 0.001**
		Non-stressed x non-stressed x Second - Stressed x stressed x Second	2.799	0.059
		Stressed x non-stressed x First – Stressed x stressed x First	-0.079	1.000
		Stressed x non-stressed x First – Stressed x stressed x Second	**-6.772**	**< 0.001**
		Stressed x non-stressed x First – Stressed x non-stressed x Second	**-8.268**	**< 0.001**
		Stressed x non-stressed x Second – Stressed x stressed x First	**-6.891**	**< 0.001**
		Stressed x non-stressed x Second – Stressed x stressed x Second	0.335	0.999
		Stressed x stressed x First – Stressed x stressed x Second	**-7.616**	**< 0.001**
	Reproductive Success	**Non-stressed x non-stressed x First – Stressed x non-stressed x First**	**5.038**	**< 0.001**
		Non-stressed x non-stressed x First – Stressed x non-stressed x Second	-2.329	0.184
		Non-stressed x non-stressed x First – Stressed x stressed x First	**4.741**	**< 0.001**
		Non-stressed x non-stressed x First – Stressed x stressed x Second	-1.648	0.567
		Non-stressed x non-stressed x First - Non-stressed x non-stressed x Second	**-5.689**	**< 0.001**
		Non-stressed x non-stressed x Second – Stressed x non-stressed x First	**-9.194**	**< 0.001**
		Non-stressed x non-stressed x Second – Stressed x non-stressed x Second	2.546	0.112
		Non-stressed x non-stressed x Second – Stressed x stressed x First	**-8.804**	**< 0.001**
		Non-stressed x non-stressed x Second - Stressed x stressed x Second	**3.114**	**0.023**
		Stressed x non-stressed x First – Stressed x stressed x First	-0.217	1.000
		Stressed x non-stressed x First – Stressed x stressed x Second	**-6.478**	**< 0.001**
		Stressed x non-stressed x First – Stressed x non-stressed x Second	**-8.012**	**< 0.001**
		Stressed x non-stressed x Second – Stressed x stressed x First	**-6.789**	**< 0.001**
		Stressed x non-stressed x Second – Stressed x stressed x Second	0.649	0.987
		Stressed x stressed x First – Stressed x stressed x Second	**-6.901**	**< 0.001**

Table S16 – *A posteriori* contrasts for the effect of heat shock during male adulthood on female fertility for Dutch and Portuguese populations. Continuation.

A)

History	Trait	Comparison	*T ratio*	*p-value*
Portugal	Fecundity	**Non-stressed x non-stressed x First – Stressed x non-stressed x First**	**4.914**	**< 0.001**
		Non-stressed x non-stressed x First – Stressed x non-stressed x Second	**-3.417**	**0.009**
		Non-stressed x non-stressed x First – Stressed x stressed x First	**5.543**	**< 0.001**
		Non-stressed x non-stressed x First – Stressed x stressed x Second	**-2.935**	**0.040**
		Non-stressed x non-stressed x First - Non-stressed x non-stressed x Second	**-8.016**	**< 0.001**
		Non-stressed x non-stressed x Second – Stressed x non-stressed x First	**-10.736**	**< 0.001**
		Non-stressed x non-stressed x Second – Stressed x non-stressed x Second	**4.205**	**< 0.001**
		Non-stressed x non-stressed x Second – Stressed x stressed x First	**-11.201**	**< 0.001**
		Non-stressed x non-stressed x Second - Stressed x stressed x Second	**4.383**	**< 0.001**
		Stressed x non-stressed x First – Stressed x stressed x First	0.674	0.985
		Stressed x non-stressed x First – Stressed x stressed x Second	**-7.535**	**< 0.001**
		Stressed x non-stressed x First – Stressed x non-stressed x Second	**-8.685**	**< 0.001**
		Stressed x non-stressed x Second – Stressed x stressed x First	**-8.559**	**< 0.001**
		Stressed x non-stressed x Second – Stressed x stressed x Second	0.420	0.998
		Stressed x stressed x First – Stressed x stressed x Second	**-8.817**	**< 0.001**
	Reproductive Success	**Non-stressed x non-stressed x First – Stressed x non-stressed x First**	**4.983**	**< 0.001**
		Non-stressed x non-stressed x First – Stressed x non-stressed x Second	**-2.835**	**0.009**
		Non-stressed x non-stressed x First – Stressed x stressed x First	**6.561**	**< 0.001**
		Non-stressed x non-stressed x First – Stressed x stressed x Second	-2.097	0.290
		Non-stressed x non-stressed x First - Non-stressed x non-stressed x Second	**-7.738**	**< 0.001**
		Non-stressed x non-stressed x Second – Stressed x non-stressed x First	**-10.107**	**< 0.001**
		Non-stressed x non-stressed x Second – Stressed x non-stressed x Second	**4.107**	**< 0.001**
		Non-stressed x non-stressed x Second – Stressed x stressed x First	**-11.580**	**< 0.001**
		Non-stressed x non-stressed x Second - Stressed x stressed x Second	**4.573**	**< 0.001**
		Stressed x non-stressed x First – Stressed x stressed x First	1.508	0.659
		Stressed x non-stressed x First – Stressed x stressed x Second	**-6.700**	**< 0.001**
		Stressed x non-stressed x First – Stressed x non-stressed x Second	**-8.018**	**< 0.001**
		Stressed x non-stressed x Second – Stressed x stressed x First	**-8.910**	**< 0.001**
		Stressed x non-stressed x Second – Stressed x stressed x Second	0.693	0.998
		Stressed x stressed x First – Stressed x stressed x Second	**-9.037**	**< 0.001**

Table S16 – *A posteriori* contrasts for the effect of heat shock during male adulthood on female fertility for Dutch and Portuguese populations. Continuation.

B)

History	Trait	Comparison	T ratio	p-value
Portugal	Fecundity	**Control x First - Warming x First**	**-3.811**	**< 0.001**
		Control x First - Control x Second	**-12.125**	**< 0.001**
		Control x First - Warming x Second	**-11.838**	**< 0.001**
		Warming x First - Control Second	**-7.094**	**< 0.001**
		Warming x First - Warming x Second	**-8.925**	**< 0.001**
		Control x Second - Warming x Second	-1.164	0.650
	Reproductive Success	**Control x First - Warming x First**	**-3.798**	**< 0.001**
		Control x First - Control x Second	**-11.663**	**< 0.001**
		Control x First - Warming x Second	**-11.449**	**< 0.001**
		Warming x First - Control Second	**-6.814**	**< 0.001**
		Warming x First - Warming x Second	**-8.889**	**< 0.001**
		Control x Second - Warming x Second	-1.305	0.560

Table S17 – *A posteriori* contrasts for the effect of heat shock during male adulthood on female offspring viability. Offspring viability: Ratio between the number of offspring (adult offspring) and the number of laid eggs (total number of eggs). *A posteriori* tukey contrasts. "T ratio": the T-test value obtained in each comparison. Comparison: Interaction between Treatment (Non-stressed x non-stressed, Stressed x non-stressed or Stressed x stressed) and Mating Opportunity (First or Second). Statistically significant terms are represented in bold.

Trait	Comparison	*T ratio*	*p-value*
Offspring viability	**Non-stressed x non-stressed x First – Stressed x non-stressed x First**	**6.531**	**< 0.001**
	Non-stressed x non-stressed x First – Stressed x non-stressed x Second	1.816	0.455
	Non-stressed x non-stressed x First – Stressed x stressed x First	**6.157**	**< 0.001**
	Non-stressed x non-stressed x First – Stressed x stressed x Second	2.546	0.112
	Non-stressed x non-stressed x First - Non-stressed x non-stressed x Second	-0.008	1.000
	Non-stressed x non-stressed x Second – Stressed x non-stressed x First	**-6.445**	**< 0.001**
	Non-stressed x non-stressed x Second – Stressed x non-stressed x Second	1.821	0.452
	Non-stressed x non-stressed x Second – Stressed x stressed x First	**-6.076**	**< 0.001**
	Non-stressed x non-stressed x Second - Stressed x stressed x Second	2.550	0.111
	Stressed x non-stressed x First – Stressed x stressed x First	-0.301	1.00
	Stressed x non-stressed x First – Stressed x stressed x Second	**-3.971**	**0.001**
	Stressed x non-stressed x First – Stressed x non-stressed x Second	**-5.403**	**< 0.001**
	Stressed x non-stressed x Second – Stressed x stressed x First	**-4.471**	**0.001**
	Stressed x non-stressed x Second – Stressed x stressed x Second	0.796	0.968
	Stressed x stressed x First – Stressed x stressed x Second	**-4.108**	**< 0.001**

Table S18 – Results from the analyses of variances of the effect of remating behaviour on the female reproductive success. Reproductive success: number of offspring resulting from laid eggs. Females were subjected to one of three treatments ("Treatment": Non-stressed x non-stressed, Stressed x non-stressed or Stressed x stressed) tested in two distinct moments ("Mating Opportunity": First or Second) and displayed remating or not ("Remating": No Remating or Remated). "Df": the degrees of freedom. "X^2": the Chi-square value obtained in each analysis. Statistically significant terms are represented in bold.

Trait	Explanatory Variable	*Df*	X^2	*p-value*
Reproductive Success	**Treatment**	**2**	**59.732**	**< 0.001**
	Mating Opportunity	**1**	**271.638**	**< 0.001**
	Remating	**1**	**161.840**	**< 0.001**
	Treatment*Mating Opportunity	**2**	**42.999**	**< 0.001**
	Treatment*Remating	**2**	**30.161**	**< 0.001**
	Mating Opportunity*Remating	**1**	**124.169**	**< 0.001**
	Treatment*Mating Opportunity*Remating	**2**	**34.411**	**< 0.001**

Table S19 – *A posteriori* contrasts for the effect of remating behaviour on the female reproductive success. Reproductive success: number of offspring resulting from laid eggs. *A posteriori* tukey contrasts. "T ratio": the T-test value obtained in each comparison. Comparison: Interaction between Remating (No Remating or Remated) and Mating Opportunity (First or Second). Statistically significant terms are represented in bold.

Trait	Treatment	Comparison	*T ratio*	*p-value*
Reproductive Success	Stressed x non-stressed	**No Remating x First - No Remating x Second**	**-10.772**	**< 0.001**
		No Remating x First - Remated x First	**7.514**	**< 0.001**
		No Remating x First - Remated x Second	**-7.077**	**< 0.001**
		No Remating x Second - Remated x First	**14.815**	**< 0.001**
		No Remating x Second - Remated x Second	**3.426**	**0.004**
		Remated x First - Remated x Second	**-14.139**	**< 0.001**
	Stressed x stressed	**No Remating x First - No Remating x Second**	**-7.100**	**< 0.001**
		No Remating x First - Remated x First	**7.355**	**< 0.001**
		No Remating x First - Remated x Second	**-3.999**	**0.004**
		No Remating x Second - Remated x First	**9.478**	**< 0.001**
		No Remating x Second - Remated x Second	1.870	0.242
		Remated x First - Remated x Second	**-8.938**	**< 0.001**