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The Impact of Sub-lethal Temperatures on Spider Mite Reproduction

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Abstract

Due to climate change, organisms are being increasingly exposed to longer and more intense periods of heat stress, which critically affect their life-history traits. Reproduction is generally more sensitive to high temperatures than survival, although most studies focus on the latter trait. This suggests that populations will be affected by climate change earlier than predicted by current knowledge. To test the influence of temperature on reproduction, we addressed how sub-lethal high temperatures affect reproductive traits in *Tetranychus urticae*, a haplodiploid agricultural pest of great economic importance. Although several studies have tackled the effect of temperature on this species, and their reproductive behavior, few have addressed the interaction temperature – reproductive behavior.

First, we assessed fertility and survival of both sexes at high temperatures, to identify the temperature that critically impairs fertility but not survival. This is measured in females by decreased fecundity and in males by reduced ratio of female offspring. Results showed that 36ºC best fits this condition. Additionally, male fertility was more affected by temperature in pesticide resistant populations. Then, we measured how temperature affected offspring paternity share, as the pattern of first male sperm precedence seen in this species may change when the first male is sterile due to temperature. Results suggest this is the case. Moreover, females mated with sterilized males had higher remating eagerness, leading to restored offspring sex ratio levels after they remated with fertile males. We also tested if, and how fast, sterilized males regain fertility once placed at an optimal temperature. We show that there is no recovery across five consecutive matings or after two days at an optimal temperature.

This project provides new insights regarding the effects of climate change on reproductive traits in an important crop pest, paving the way to future studies in sexual selection and adaptation to abiotic stresses.

Keywords: *Tetranychus urticae*, temperature, fertility, sperm precedence, mating behavior

Resumo

Devido às alterações climáticas, os organismos têm vindo a ser expostos a períodos de stress térmico mais longos e intensos, o que afeta a sua biologia a vários níveis, desde o comportamento dos indivíduos até à dispersão geográfica das populações. De modo a compreender o efeito que a temperatura tem a longo prazo nas espécies, a maioria dos estudos foca-se na viabilidade dos organismos e usa esses dados para criar modelos preditivos da vulnerabilidade das espécies face ao aumento das temperaturas. No entanto, estes estudos muito provavelmente estão a sobrestimar a capacidade que as espécies têm para lidar com ambientes termicamente stressantes, visto que já foi demonstrado em várias espécies que a fertilidade é afetada a temperaturas menos extremas que a viabilidade dos organismos. Em particular, foi observado em algumas espécies que a fertilidade masculina é mais termicamente sensível que a feminina e, portanto, preferencialmente deveria ser usada como medida para testar os limites térmicos.

De modo a testar a influência da temperatura na reprodução, examinámos como é que altas temperaturas sub-letais afetam várias características reprodutivas do ácaro-aranha *Tetranychus urticae*. Esta espécie é uma praga agrícola que afeta uma grande variedade de plantas globalmente, incluindo espécies de grande valor económico. É também considerado o artrópode com a maior taxa de resistência a pesticidas, sendo, portanto, importante entender como é que esta espécie será afetada pelas alterações climáticas, de modo a melhorar as estratégias de manutenção e controlo desta praga no futuro. O ácaro-aranha é ideal para estudos sobre reprodução devido à sua haplodiploidia. Em particular, ovos fertilizados geram fêmeas diploides, enquanto ovos não fertilizados geram machos haploides, contendo apenas o material genético materno. Isto permite estudar separadamente a fertilidade da fêmea, observada pela fecundidade, e a fertilidade do macho, vista através da proporção de filhas na descendência.

Começámos por testar o efeito de diferentes temperaturas altas na fertilidade, sobrevivência e tempo de desenvolvimento dos ácaros-aranha, de modo a escolher a temperatura sub-letal que afetasse severamente a fertilidade, mas que não tivesse um efeito marcante na sobrevivência dos indivíduos. Foram testadas quatro temperaturas (33, 35, 36 e 37ºC), tendo sido selecionada a temperatura 36ºC para ser aplicada nas experiências seguintes. Isto deveu-se ao facto de a fertilidade e a sobrevivência demonstrarem um aumento até 33ºC seguido de uma diminuição a temperaturas mais altas. O tempo de desenvolvimento de ambos os sexos, pelo contrário, diminuiu com o aumento da temperatura.

Com a temperatura já escolhida, investigámos se haveria uma interação entre resistência a pesticida e a resposta a esta temperatura. Para tal, utilizámos duas populações que apenas diferem num gene que confere resistência ou suscetibilidade ao pesticida Etoxazole. Ambas as populações foram expostas a 25ºC (controlo) ou 36ºC e o efeito da temperatura na fertilidade, sobrevivência e tempo de desenvolvimento foi testado. Não foi encontrada nenhuma diferença no efeito da temperatura entre populações relativamente à sobrevivência e tempo de desenvolvimento dos indivíduos. No entanto, uma diferença entre as populações foi observada na fertilidade, especialmente na fertilidade do macho, indicando que a 36ºC a fertilidade da população resistente apresenta uma redução mais acentuada que a população suscetível ao pesticida. Este custo observado a alta temperatura e relacionado com a resistência ao pesticida pode ser um fator importante para o controlo de *T. urticae* na agricultura face às alterações climáticas.

Esta espécie apresenta um padrão de precedência espermática quase completo, sendo que o primeiro macho a acasalar com a fêmea fertiliza a maioria da sua descendência. De modo a testar se este padrão é afetado a altas temperaturas, utilizámos as duas populações referidas anteriormente e o pesticida Etoxazole. Visto que a resistência a este pesticida é recessiva, foi utilizada como teste de paternidade ao cruzar uma fêmea resistente primeiro com um macho suscetível e de seguida com um macho resistente,

colocando por fim a descendência a desenvolver-se na presença de Etoxazole. Desta forma, tendo em consideração a haplodiploidia da espécie, a descendência poderá consistir em filhos, que apenas contêm o material genético materno, ovos não eclodidos, fecundados pelo primeiro macho (suscetível), e filhas, fecundadas pelo segundo macho (resistente). Quatro cruzamentos foram feitos para o primeiro acasalamento, consistindo nas várias combinações de fêmeas e machos expostos a 25 ou 36ºC durante o desenvolvimento. Os acasalamentos seguintes da fêmea envolveram apenas machos desenvolvidos a 25ºC. Os resultados demonstram que o padrão de precedência espermática foi afetado quando pelo menos um dos indivíduos se desenvolveu a alta temperatura, havendo mais descendência fertilizada pelo segundo acasalamento e acasalamentos seguintes. Em particular, o contributo dos machos subsequentes na paternidade da descendência é maior quando o macho do primeiro acasalamento foi exposto a stress térmico. Esta disrupção do padrão de precedência espermática permite que fêmeas que acasalaram com machos de fertilidade reduzida ao acasalarem novamente possam garantir valores de ovos fertilizados semelhantes a fêmeas que apenas acasalam com machos sem défice na fertilidade.

O comportamento reprodutivo do ácaro-aranha está em geral de acordo com o esperado de espécies onde apenas o primeiro acasalamento é efetivo a fertilizar a descendência da fêmea. Devido a isto, os machos desta espécie tendem a guardar fêmeas nos últimos estádios de desenvolvimento até se tornarem adultas de modo a assegurar o acasalamento, conseguindo também discernir fêmeas virgens de fecundadas através de pistas químicas. Para além disso, as fêmeas tendem a evitar acasalar múltiplas vezes, sendo esperado que acasalamentos posteriores ao primeiro demorem mais a ocorrer e tenham menor duração. Como o padrão de precedência espermática foi alterado quando um dos indivíduos foi exposto a altas temperaturas, testámos se a temperatura também afeta o comportamento de acasalamento. Para tal, cruzámos fêmeas desenvolvidas a 25 ou 36ºC com machos desenvolvidos a uma das temperaturas, e de seguida permitimos que as fêmeas acasalassem múltiplas vezes com machos desenvolvidos a 25ºC. Em todos os acasalamentos medimos o tempo de latência até ao acasalamento e a duração do mesmo. Os resultados obtidos indicam que acasalamentos com machos expostos a alta temperatura são mais curtos e demoram mais tempo a ocorrer, sendo que estas fêmeas mostram uma maior avidez para acasalar novamente. Esta alteração do comportamento de acasalamento é congruente com a disrupção do padrão de precedência espermática referida anteriormente, de modo que fêmeas acasaladas com machos expostos a altas temperaturas não só conseguem utilizar esperma proveniente de acasalamentos subsequentes como também procuram acasalar novamente.

Por fim, testámos se a fertilidade de machos expostos a altas temperaturas durante o desenvolvimento pode ser recuperada quando colocados numa temperatura ótima. A recuperação de fertilidade após a aplicação de um stress térmico tem sido observada em várias espécies, enquanto que outras espécies demonstram permanência do efeito da temperatura. No entanto, não existem estudos anteriores que testem a existência de recuperação de fertilidade em *T. urticae*. Começámos então por cruzar machos desenvolvidos a 25ºC com fêmeas da mesma temperatura e machos desenvolvidos a 36ºC com fêmeas desenvolvidas a uma das temperaturas, permitindo que cada macho acasalasse até cinco vezes. Após estes acasalamentos, cada macho foi mantido a 25ºC durante dois dias, podendo por fim acasalar mais uma vez. Os resultados obtidos mostram que, enquanto machos desenvolvidos a 25ºC conseguem fertilizar todas as fêmeas, machos expostos a altas temperaturas durante o desenvolvimento não conseguem fertilizar nenhuma fêmea ao longo de cinco acasalamentos ou após dois dias a uma temperatura ótima. Embora não seja possível indicar se existe recuperação de fertilidade nesta espécie, um período de dois dias de esterilidade pode ser suficiente para afetar negativamente o sucesso reprodutor dos machos, tendo em consideração o tempo de vida curto de *T. urticae*.

Em suma, a redução de fertilidade causada pela exposição a altas temperaturas durante o desenvolvimento pode tornar esta espécie bastante vulnerável às alterações climáticas. Em particular, a perda de fertilidade no macho em populações naturais pode levar a um rácio sexual predominantemente

masculino, impactando a estabilidade e persistência destas populações. No entanto, os resultados aqui descritos demonstram que o padrão de precedência espermática de *Tetranychus urticae* pode ser alterado na presença de stress térmico, permitindo que, após acasalarem com machos de fertilidade reduzida, as fêmeas retornem a valores de descendência fertilizada semelhantes a fêmeas que apenas acasalam com machos férteis. Adicionalmente, a alteração observada no comportamento de acasalamento das fêmeas pode contribuir para a prevenção de uma descida acentuada no crescimento populacional face ao atual aumento das temperaturas globais. Por fim, este projeto proporciona novas ideias relativamente aos efeitos das alterações climáticas em características reprodutivas de uma praga com alta importância agrícola, abrindo espaço para futuros estudos relacionados tanto com seleção sexual como com adaptação a stresses abióticos.

Palavras-chave: *Tetranychus urticae*, temperatura, fertilidade, precedência espermática, comportamento de acasalamento

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Introduction

Climate change is causing average local temperatures to rise and a higher frequency of heatwaves (Kingsolver *et al.*, 2013; Buckley & Huey, 2016). These changes in the global thermal environment affect the biology of organisms at several levels, from behavioral and life-history traits of individuals to geographic range and abundance of populations, thereby posing a severe threat to biodiversity (Parmesan, 2006; Kellermann *et al*., 2012). This may be particularly detrimental to ectotherms, which represent the vast majority of terrestrial biodiversity (Stork *et al*., 2015) as their basic physiology is dependent on ambient temperatures (Hochachka & Somero, 2002). In order to understand the long-term effect of temperature on species, studies have been using viability as a general measure to test thermal limits (Kellermann *et al*., 2012; Geerts *et al*., 2015) and create predictive models based on those limits (Bush *et al.*, 2016; Rezende *et al*., 2020).

Thermal sensitivity appears to be different among the main life-history traits, with fertility being often the most vulnerable to extreme temperatures, followed by development, and lastly viability (Zhao *et al.*, 2014; Ma *et al*., 2015; Zhang *et al.*, 2015a; Ma *et al*., 2020). For example, the moth *Plutella xylostella*, when exposed to high temperatures, experiences negative effects on fertility while survival is not affected (Zhang *et al.*, 2013). Additionally, in the aphid *Sitobion avenae*, fertility has a lower optimal temperature than development (Zhao *et al*., 2014). Because fertility has a major influence on individual fitness and populational growth, studies addressing the response of organisms to climate change by measuring impact of extreme temperatures on viability only may be underestimating their vulnerability and even misidentifying which organisms are most at risk. Recently, there is growing evidence that fertility can be a better predictor for the response of populations to climate change (Walsh *et al*., 2019; Parratt *et al.*, 2021). In particular, several studies noted that male fertility is more thermally sensitive than female fertility, and thus should be preferably used as a predictor (Iossa, 2019; Parratt *et al*., 2021; van Heerwaarden & Sgrò, 2021). Thus, studying the effect of extreme temperatures on fertility is vital to understanding the impact of climate change on biodiversity.

Temperature can also hinder mating activity (Leith *et al*., 2021). These negative effects can be seen in both pre- and postcopulatory mating behaviors (Iossa *et al.*, 2019; Leith *et al*., 2020). For mating to occur, male and female courtship should be coordinated. However, the thermal sensitivity of each sex is not consistently similar, which can create a disparity between the male and female peak of mating activity, leading to one sex constraining the mating success of the other (Macchiano *et al*., 2019; Leith *et al*., 2020). For example, in a species of spiders (*Habronattus clypeatus*) where females only mate once and males produce visual and vibratory signals that only last a few minutes, females become less selective regarding their partners and increase their mating rate at higher temperatures. Consequently, the possibility of mating for males is higher at warmer temperatures. However, at these temperatures male signals become shorter and with lower amplitude, and thus less capable of securing mates (Brandt *et al*., 2018). Similarly, in a species of moths (*Plodia interpunctella*), males are more sensitive to higher temperatures than females, having shorter copulations, lower mating rate and also a reduction in sperm length (Iossa *et al*., 2019). Furthermore, in the cigarette beetle, *Lasioderma serricorne*, postcopulatory behavior is affected by extreme temperatures: by significantly decreasing the number of sperm transferred to the female, the risk of sperm competition is higher (Suzaki *et al.*, 2018).

Mating behavior is often influenced by the pattern of sperm precedence of the organism. These patterns depend on which male sires the offspring of the female, ranging from first- to last-male sperm precedence (Simmons, 2001). In species with first-male sperm precedence, the first male that mates with the female sires most of her offspring, making subsequent matings unsuccessful. Thus, species with this

pattern tend to have pre-copulatory behaviors, such as males distinguishing virgin from mated females or guarding immature females until sexual maturity (Yasui, 1988; Fahey & Elgar, 1997; Stoltz *et al*., 2007; Oku, 2014). In contrast, species with last-male sperm precedence tend to have increased mating frequencies, since only the last mate sires the female offspring (Ridley, 1989). With several aspects of reproduction being hampered by extreme temperatures, these patterns can also be affected. For example, in a species of flies, *Scathophaga stercoraria*, last-male sperm precedence is prevalent in females with three sperm storage compartments, which only rarely have four. However, when facing higher developmental temperatures the frequency of females with four sperm storage compartments increases (Berger *et al*., 2011), which influences their sperm precedence pattern, as females with four compartments tend to have mixed paternity (Ward, 2000).

The thermal sensitivity of fertility depends on several factors, such as the life stage and sex of the individual. It can vary across the life cycle of organisms (Zhang *et al*., 2015b; Ma *et al*., 2020), with juvenile life-stages being expected to be more sensitive to temperature, as they are restricted to certain natural habitats due to their limited food resources and decreased mobility, which prevents them to avoid exposure to high temperatures (Kingsolver *et al*., 2011). In contrast, there seems to be a larger reduction in reproduction when extreme temperatures are experienced closer to and during adulthood (Zani *et al*., 2005; Zhang *et al*., 2015a; Gasparini *et al*., 2018). Moreover, fertility thermal limits are also sex-specific, with males often showing a reduction in fertility at lower high temperatures than females (David *et al*., 2005; Sales *et al*., 2018; Iossa, 2019). For example, in *Drosophila melanogaster*, it has been shown that sublethal temperatures during development have a larger effect on male fertility compared to female fertility (Zwoinska *et al.*, 2020), which David *et al.* (2005) indicates that it is due to spermatogenesis being more thermally sensitive than oogenesis. This heat-induced sterility in males can be temporary in some cases if they are placed at benign temperatures afterwards (Rohmer *et al*., 2004; David *et al*., 2005; Nguyen *et al*., 2013). However, under severe heat stress, and depending on their genetic composition, sterility can be permanent (Vollmer *et al*., 2004; Jørgensen *et al*., 2006). Regardless of whether the male can recover fertility or not after a period of thermal stress, temporary sterility can be enough to significantly affect the male reproductive success in species with short life span.

The two-spotted spider mite *Tetranychus urticae* is a polyphagous mite that occurs on a wide variety of plant species across the world (Migeon *et al*., 2010), including crop plants of great economic significance. For damaging its host plants, and thus entire crops worldwide, it is considered an important agricultural pest. This spider mite can also develop resistance to pesticides rapidly, being considered the species with the highest rate of pesticide resistance among arthropods (van Leeuwen *et al.*, 2010). Studying how climate change affects spider mites is of great importance, so management strategies to control this pest can be improved in the future. This species has a life cycle of around 15 days, from egg to adult, passing through three juvenile stages (larva, protonymph and deutonymph) with quiescent periods in between. Moreover, males reach adulthood before females (Helle & Sabelis, 1985) and the adult sex ratio is generally female biased (Mitchell, 1972). This spider mite simultaneously presents an interesting system to study the impact of sublethal temperatures on reproduction since it has a haplodiploid sex-determination system (Helle & Sabelis, 1985). Indeed, fertilized eggs generate diploid females, while unfertilized eggs generate haploid males, carrying only the genetic material of the mother, which allows disentangling the effects of temperature on males vs females.

Studies on spider mites have shown that extreme high temperatures hinder their life-history traits (Praslička & Huszár, 2004; Riahi *et al*., 2013; Farazmand, 2020). Offspring survival is decreased with rising temperatures (Riahi *et al*., 2013), while development is faster as temperature increases (Praslička & Huszár, 2004). At high temperatures, fertility is also affected, with female oviposition rate having its peak at around 30 ºC and decreasing afterwards (Laing, 1969; Praslička & Huszár, 2004; Riahi *et al*., 2013; Zou *et al*., 2018) and offspring sex ratio of males that are exposed to high temperatures becoming

male-biased (note that male fertility is represented by the proportion of daughters due to haplodiploidy) (Margolies & Wrensch, 1996). While some studies have shown that warmer temperatures reduce female fertility, the effect on males is rarely studied. This is unfortunate because males have shown more sensitivity to extreme temperatures in other taxa, as previously referred. Furthermore, there is no study on *T. urticae* addressing if, and in which circumstances, male sterility due to high temperatures can be reversed.

T. urticae presents a pattern of first-male sperm precedence (Helle, 1967), that is, females of this species fertilize their eggs mostly with sperm from the first mating, making subsequent matings inefficient (Rodrigues *et al*., 2020). The mating behavior of spider mites follows the expected behavior of species with this pattern, with males actively guarding quiescent females to mate with them as soon as they reach adulthood and are still virgin (Potter *et al.*, 1976). In addition, males extend matings by guarding the female after sperm transfer to ensure sperm precedence (Satoh *et al*., 2001). Males also prefer to mate with virgin females than mated ones, using chemical cues to distinguish them (Rodrigues *et al*., 2017). There are currently no studies testing if the sperm precedence pattern in this species can be disrupted due to exposure to high temperatures. If this is the case, females can recover from mating with a sterile male (as shown for instance in *Drosophila pseudoobscura*; Sutter *et al*., 2019), using sperm stored from subsequent matings to fertilize their offspring. Otherwise, mating with sterile males can cause females to have male-biased offspring, leading to a reduction in populational growth. If this pattern changes, it can affect the mating behavior described previously, which may lead to, for example, females seeking remating after an unsuccessful mating.

The goal of this project is to further understand the impact of sublethal temperatures on the reproduction of spider mites, using the model species *Tetranychus urticae*. Firstly, several high temperatures will be applied to test their effect on various life-history traits, to ultimately choose one that best damages fertility while not greatly affecting survival. Then, four questions will be addressed: do high sublethal temperatures affect i) life-history traits in populations with different pesticide resistance; ii) the pattern of sperm precedence; iii) and the mating behavior of spider mites; and lastly iv) is there recovery of male fertility after being exposed to high sublethal temperatures. Ultimately, this project will provide new insight on the effects of climate change on reproductive traits in an important crop pest, paving the way to future studies in both sexual selection and adaptation to abiotic stresses.

Materials and Methods

Spider mite maintenance and rearing

Populations

Two populations of spider mites (red form of *Tetranychus urticae*), one resistant and one susceptible to the pesticide Etoxazole, were used in these experiments.

The susceptible population is referred to as WuSS (uninfected with *Wolbachia*, susceptible to the pesticide) and was originated by merging six populations originally collected in the region of Lisbon, Portugal, in 2013. The resistant population is referred to as WuRR (uninfected with *Wolbachia* and resistant to the pesticide), having gained its resistance by introgressing WuSS with a resistant strain. Both populations were replicated five times, and these replicates were merged in September 2020 to create the base populations used in the following experiments. Therefore, the two populations differ in the allele that confers resistance, CHS1 (van Leeuwen *et al.*, 2012).

Both populations were used in *Interaction between pesticide resistance and response to temperature* to determine if each population was affected differently by high temperatures, and also in *Effect of temperature on the pattern of sperm precedence* where the resistance to Etoxazole was used to assess the paternity share between the first and the subsequent males. Since there were no major differences between populations when testing the temperatures effect on fertility and survival, only one population was used in the remaining experiments. The selected population was WuRR, since females from that population had to be used as focal individuals when testing the offspring paternity share (see *Effect of temperature on the pattern of sperm precedence*).

Rearing conditions

The base populations were maintained in large numbers (> 2000) under continuous generations from September 2020 to January 2021, then in smaller numbers (> 1000) and with discrete generations from January 2021 onwards. Mites were kept on bean leaves (*Phaseolus vulgaris*, variety Contender, provided by Germisem, Portugal) under controlled conditions (25 ºC; 16h Light: 8h Dark photoperiod).

Cohorts

To ensure that a large number of individuals of the same age was available simultaneously, cohorts were produced. Females were collected from the base populations and placed to oviposit for two days in large bean leaf patches placed in a Petri dish filled with water-soaked cotton, after which they were discarded. As spider mites are haplodiploid, males develop from unfertilized eggs and females from fertilized ones. Thus, cohorts were made with quiescent, hence virgin, females to obtain male offspring. Isolating mated females led to a cohort with offspring of both sexes, from which quiescent, hence virgin, female offspring could be isolated. Cohorts were made at different temperatures (25, 33, 35, 36 or 37 ºC) depending on the protocol of each experiment. The individuals isolated from the cohorts were maintained at the same temperature as the one experienced in the cohorts until the start of the experiment.

Effect of high temperatures on spider mite life-history traits and its interaction with pesticide resistance

To establish the appropriate sublethal temperature to apply in the following experiments, the impact of four high temperatures (33, 35, 36 and 37 ºC) on life-history traits was characterized by testing their effects on survival, developmental time and fertility of spider mites. The ideal sublethal temperature should affect spider-mite fertility, but not its survival, so the effects registered can be attributed to the temperature effect on reproduction and there can be enough viable individuals to manifest those effects. Moreover, the selected temperature was applied during the development of individuals from both populations to test whether it impacted differentially the life history traits of individuals with different resistance status.

Due to logistic constraints, temperatures were not tested simultaneously. Instead, several assays were performed, always including the control temperature (i.e., 25°C): i) 25 ºC, 33 ºC and 37 ºC; ii) 25 ºC and 35 ºC; iii) 25 ºC and 36 ºC.

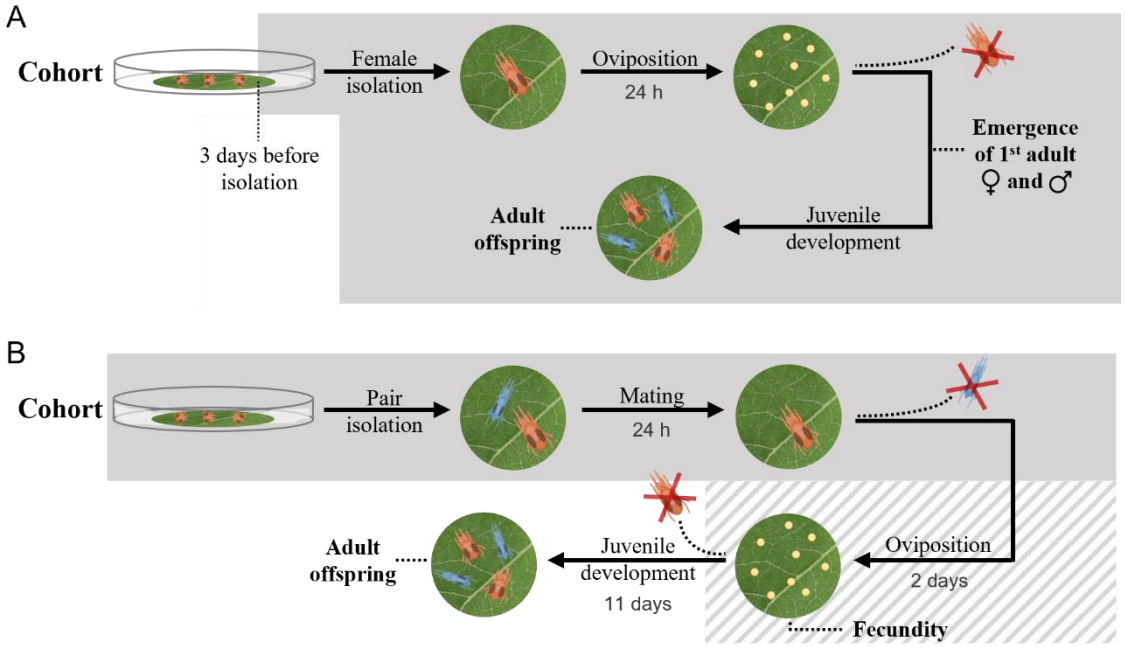

Figure 2.1 – Schematic representation of the protocols used to measure the effect of high temperatures on life-history traits. A) Protocol used to measure developmental time and survival of spider mites developed at high temperatures. B) Protocol used to measure fertility of spider mites developed at high temperatures. Steps included in the grey area take place at the tested temperature, in the white area at 25 ºC, and in the grey-stripped area at the developmental temperature of the female. Controls were entirely done at 25 ºC. The orange and blue mites represent females and males, respectively.

Effect of temperature on life-history traits

To measure the effect of temperature on developmental time and survival of spider mites, cohorts were established at 25 ºC until 3 days prior to the isolation of adult mated focal females. Only then, each cohort was placed at the tested temperatures, which ensured that the eggs followed in the experiment were produced at that temperature. Females were then individually placed on bean leaf discs (2,55 cm^2). When isolated, females were left to oviposit for 24h, after which they were discarded. The number of days until the first adult male and female offspring emerged, as well as the number of offspring that reached adulthood were recorded (Figure 2.1A). Replicates with damaged mothers (alive but injured

females) were excluded from the analysis. For each temperature tested, 20 to 30 replicates were later statistically analysed.

A second experiment was performed to test how temperature affects fertility. Virgin females and males developing until the quiescent stage at the tested temperatures were placed in pairs on bean leaf discs (2,55 cm^2) to mate and oviposit. Two treatments were established: pairs where both individuals developed at 25 ºC (control; ♀C x ♂C) and pairs where only the female developed at high temperature (♀H x ♂C). Pairs were maintained for 24 hours, after which males were discarded. Females were left to lay eggs for 2 more days, after which they were also discarded, and the eggs were counted. The survival status of both individuals was recorded every day. 12 or 14 days after the establishment of the pair, male and female adult offspring were counted and removed (Figure 2.1B).

Pairs were maintained at 25°C when both individuals developed at that temperature. Otherwise, patches were kept at the tested temperature until the female was removed, being placed at 25 ºC afterwards.

For the first assay (i.e. 25, 33 and 37 ºC), we performed two blocks, which occurred in two consecutive days and included all treatments. If one of the individuals forming the mating pairs was damaged, that replicate was excluded from the analysis. For each treatment, 25 to 39 replicates were performed.

Interaction between pesticide resistance and response to temperature

Once the sublethal high temperature was chosen (36 ºC, see Results), the two previous experiments were repeated to test the effect of this temperature on developmental time, survival and fertility of both resistant and susceptible populations. These experiments followed the same procedures as in *Effect of temperature on life-history traits* (Figure 2.1), apart from the following details: an additional treatment was added (i.e., pairs were established with both individuals developed at 25 ºC (control; ♀C x ♂C), only the male or the female developed at 36 ºC (♀C x ♂H and ♀H x ♂C, respectively), and both individuals developed at 36 ºC (♀H x ♂H)) and females were given 4 days to oviposit instead of 3, to be comparable to the subsequent experiments. Additionally, pairs were maintained at 25°C when both individuals developed at that temperature. Otherwise, patches were kept at 36 ºC until the male was removed. Afterwards, they were either relocated to 25 ºC or maintained at 36 ºC, according to the developmental temperature of the female. Once the female was removed, all patches were placed at 25 ºC (see Figure 2.1B).

Replicates with damaged mothers (when testing the effect on survival and developmental time), or with individuals forming the mating pairs that were damaged (when testing the effect on fertility), were excluded from the analysis. Regarding the experiment that tested the effect on developmental time and survival, for each temperature, 20 to 30 replicates were analysed per population. For the effect on fertility, for each treatment, 13 to 40 replicates were analysed per population.

Effect of temperature on the pattern of sperm precedence

Tetranychus urticae exhibits first-male sperm precedence, that is, the fertilized offspring of a female is mostly sired by the first male (Helle, 1967; Rodrigues *et al.*, 2020). Here, we tested if the offspring paternity share is altered when the first male has reduced fertility due to developmental heat stress. This paternity share was determined by applying the pesticide Etoxazole to the offspring of a resistant female (from WuRR) that mated with both resistant and susceptible males (i.e., the first male was WuSS, and the subsequent males were WuRR). Resistance to Etoxazole can be used as a paternity marker because it is coded by a single, recessive, allele, which is fixed in our resistant population (Rodrigues *et al.*,

2020; van Leeuwen *et al.*, 2012). Because spider mites are haplodiploid and females always came from the resistant population, the pesticide application will only affect daughters (diploids), while sons (haploids), which only inherit the genetic material of their mothers, will always be resistant. Thus, when developing with pesticide, the offspring will consist of alive sons, alive daughters (sired by resistant males) and unhatched eggs (sired by susceptible males).

Four treatments were tested: pairs where both individuals developed at 25 ºC (control; ♀25 x ♂25), pairs where only the male or the female developed at 36 ºC (♀25 x ♂36 and ♀36 x ♂25, respectively) and pairs where both individuals developed at 36 ºC (♀36 x ♂36).To test the offspring paternity share, three types of mated females were created (Figure 2.2): single, double and multiple mated. First, 5 resistant virgin females and 5 susceptible virgin males were placed on a mating disc (0,5 cm^2) at 25ºC to mate for 30 minutes. Once mated, males were discarded, and females, in groups of 5, were either placed on leaf discs of 0,95 cm^2 (Single Mated females, SM) or transferred to a new mating disc (0,5 cm^2) with 5 resistant virgin males developed at 25 ºC and given 2 hours to remate (Double Mated females, DM). Again, once remated, females were placed in groups of 5 on leaf discs of 0,95 cm^2, while males were discarded after their first mating. Both matings were observed in order to verify that copulation occurred, but no data was recorded. A subset of the SM females was transferred to a new disc (0,95 cm^2) in groups of 5 females with 5 resistant virgin males developed at 25 ºC to remate multiply for 24 hours (Multiple Mated females, MM). The next day, single mated (SM), double mated (DM) and multiple mated (MM) females were individually transferred to new discs (2,55 cm^2) placed on Etoxazole-soaked cotton (diluted at 0.5g/L). Females had 4 days to oviposit, after which they were discarded, and the eggs were counted. The survival status of each female was recorded every day until removal. The number of hatched and unhatched eggs was recorded 8 days after oviposition started. Once the offspring was adult, female and male offspring were counted and removed 12 or 14 days after the onset of oviposition (Figure 2.2).

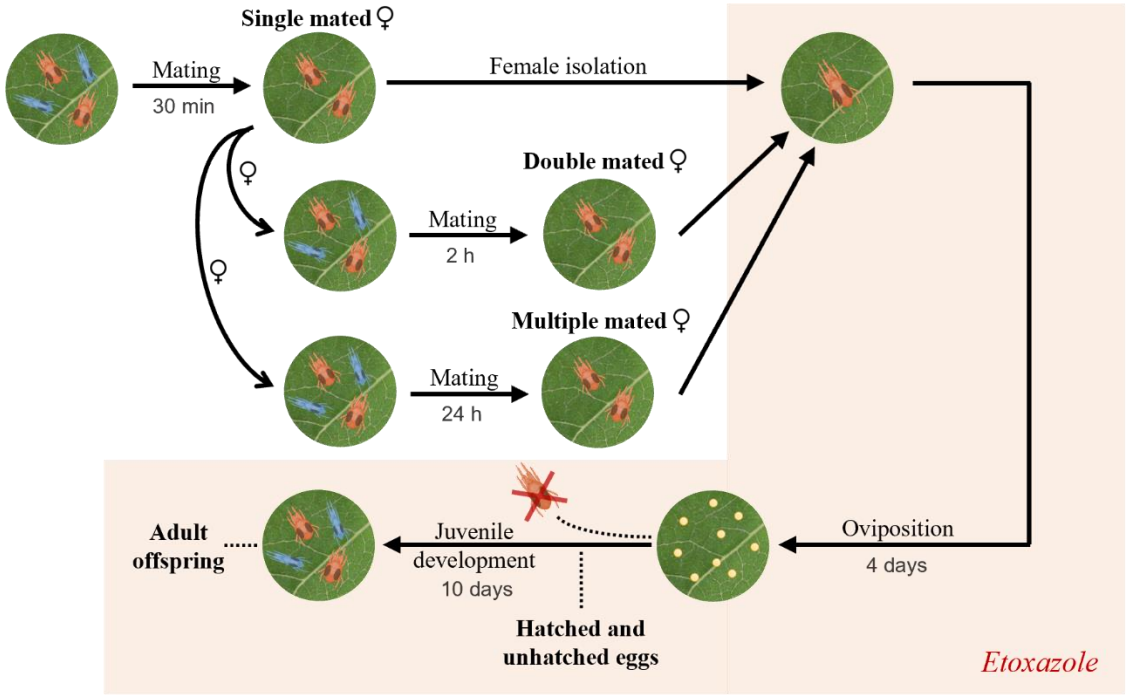

Figure 2.2 – Schematic representation of the protocol used to measure the effect of high temperatures on the pattern of sperm precedence. Steps included in the orange area take place in the presence of the pesticide Etoxazole. The orange and blue mites represent females and males, respectively.

This experiment was performed in 4 blocks, 2 per week in consecutive days, each including all treatments. SM females that did not mate and DM females that did not remate were discarded. Replicates with damaged females were excluded from the analysis. For each female mating history (SM, DM and MM), 27 to 43 replicates were analysed per treatment.

Effect of temperature on mating behavior

With this experiment, we tested if mating and female remating eagerness are affected when fertility is reduced by a high sublethal developmental temperature.

Four mating treatments, differing in the temperatures under which focal females and their first mate developed, were established: both sexes developed at 25 ºC (control; ♀25 x ♂25), males or females developed at 36 ºC (♀25 x ♂36 and ♀36 x ♂25, respectively) and both sexes developed at 36 ºC (♀36 x ♂36).

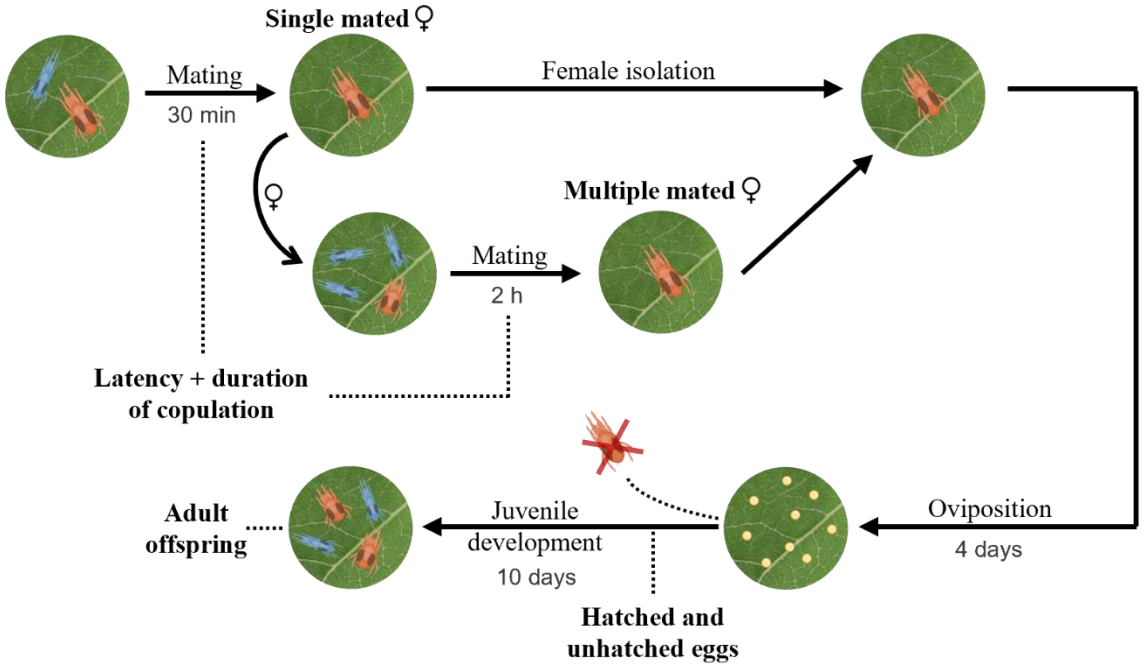

Figure 2.3 – Schematic representation of the protocol used to measure the effect of high temperatures on mating behavior. The orange and blue mites represent females and males, respectively.

Virgin females and males were paired on a disc (0,5 cm^2) and given 30 minutes to copulate. The latency to copulation and its duration were recorded using the app Stopwatch: StopNow Free. Once mated, females were either individually placed on leaf discs of 0,95 cm^2 (Single Mated females; SM) or transferred to a new disc (0,5 cm^2) to which five virgin males, that had developed at 25 ºC, were added. Females were then given 2 hours to remate multiply, with males being replaced after copulating. The latency to each copulation and their duration were also recorded. Afterwards, these females were individually placed on leaf discs of 0,95 cm^2 (Multiple Mated females; MM). The next day, both SM and MM females were individually transferred to larger leaf discs (2,55 cm^2), and its offspring was monitored as above (Figure 2.3).

This experiment was performed in 8 blocks, 2 per week in consecutive days, each including all treatments. Individuals were maintained at 25 ºC from the start of mating observations. Replicates with SM females that did not mate, MM females that did not remate or damaged females were excluded from

the analysis. For each treatment, 89 to 93 replicates were analysed for variables regarding the first mating, and 15 to 43 replicates were analysed for the remaining variables.

Effect of temperature on sperm depletion and recovery of male fertility

Since developmental heat stress causes reduced fertility in males, we studied if there is sperm recovery when males are removed from heat stress and placed at 25 ºC. To test this, male fertility was tested at two time points: as soon as the male was placed at 25 ºC, and 2 days later.

Three mating treatments, that differed in the temperatures under which focal males and the females they mated with developed, were established: both sexes developed at 25 ºC (control; ♀25 x ♂25), males developed at 36 ºC (♀25 x ♂36) and both sexes developed at 36 ºC (♀36 x ♂36). One virgin focal male was placed with five virgin females on a mating disc (0,5 cm^2) at 25°C. The male was removed from that disc after 5 matings or 2 hours and placed on a disc of 2,55 cm^2. Two days later, the male was placed again on a mating disc (0,5 cm^2) for 30 minutes with a virgin female to mate, after which it was discarded. The latency to every copulation and their duration were recorded using the app Stopwatch: StopNow Free. Females were individually placed on a disc of 0,95 cm^2 immediately after copulation and their mating order was registered (1st, 2nd, 3rd, 4th, 5th and Last Mating). The following day, each female was transferred to a new leaf disc (2,55 cm^2) and its offspring was monitored as previously described (Figure 2.4).

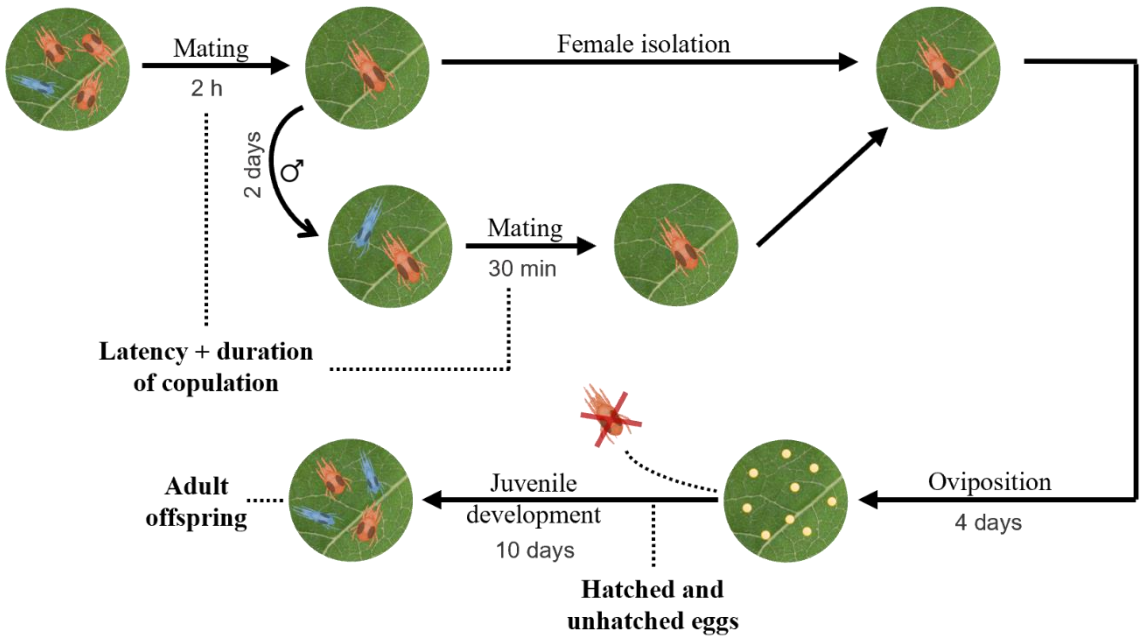

Figure 2.4 – Schematic representation of the protocol used to measure the effect of high temperatures on sperm depletion and recovery of male fertility. Orange and blue mites represent females and males, respectively.

This experiment was performed in 6 blocks, 2 per week in consecutive days, each including all treatments. Replicates with damaged females were excluded from the analysis. For each mating order, 12 to 48 replicates were analysed per treatment for variables regarding matings, and 8 to 47 replicates were analysed for the offspring sex ratio.

Statistical analyses

All statistical analyses were performed using the software R (R Core Team, 2020). We performed generalized linear models (GLM), generalized mixed-effects models (GLMM), linear models (LM) and linear mixed-effects models (LMM) implemented in *lme4* (Bates *et al.*, 2015) and *glmmTMB* (Brooks *et al*., 2017), depending on the data and error structure. Maximal models were simplified by removing non-significant terms ($p < 0.05$) from the highest- to the lowest-order interaction (Crawley, 2012). The significance of each explanatory variable was determined by chi-squared tests for discrete distributions, and Wald F tests for continuous distributions (Bolker *et al*., 2009). *A posteriori* contrasts with Bonferroni corrections were made to interpret the effect of factors with more than two levels, using *emmeans* package (Length, 2020). Graphic representations of the data were produced with the software package *ggplot2* (Wickham, 2016).

Effect of temperature on life-history traits

To determine the effects of heat stress on developmental time and survival of the offspring, we analysed two variables: days until adulthood, used as a proxy of developmental time, and number of adult offspring, indicating the offspring number that survived until adulthood.

Due to time constrains, different temperatures were applied to the mites in different blocks. Therefore, to compare the effect of different temperatures, we analysed the differences to the control temperature (i.e., trait values at control temperatures applied in the same block as the tested temperature were subtracted to the trait values measured at the temperature tested). This treated data was then used as dependent variable in the subsequent models. To check if the difference of each temperature to the control was significant, we used the function *summary* and took the values from *intercept,* which correspond to the first temperature. Then temperatures were reordered to obtain the values for each of them.

All variables were analysed using LM models with a gaussian error distribution. The data was box cox transformed (female days until adulthood: $\lambda = 1.94$; male days until adulthood: $\lambda = 0.73$; number of adult offspring: $\lambda = 0.82$; *MASS* package; Venables & Ripley, 2002) to improve the fit of the model in every variable. The developmental temperature of the offspring (33 ºC, 35 ºC or 36 ºC) was included in the models as fixed factor. Fecundity was also added as covariate in the model for the number of adult offspring to account for the variation caused by the offspring that did not reach adulthood (Table S1A). Data from 37 ºC was excluded because only 6 females survived at this temperature.

For the second experiment, that investigated the effects of developmental heat stress on fertility, we analysed the number of adult offspring, which represents female fertility. Males were not accounted for since male fertility should be more thermally sensitive than female fertility, and thus, already hindered at any temperature that affects females. Data was also treated as described above, in order to compare every tested temperature, including their difference to the control. When the experiment was done in more than one block, the mean of the control was calculated within each block, to account for that variability.

The number of adult offspring was analysed using an LM model with a Gaussian error distribution. The data was box cox transformed ($\lambda = 0.76$; *MASS* package; Venables & Ripley, 2002) to improve the fit of the model. The cross between males and females from different developmental temperatures was included in the model as fixed factor (Table S1B).

Interaction between pesticide resistance and response to temperature

To determine the effects of developmental heat stress on developmental time and survival on individuals with different resistance status, we analysed two variables: days until adulthood, used as a proxy of developmental time, and number of adult offspring, indicating the number of offspring that survived until adulthood.

All variables were analysed using GLM models with a Poisson error distribution. The developmental temperature of the offspring (25 ºC or 36 ºC), the resistance status (WuSS or WuRR) and their interaction were included in the models as fixed factors. The number of eggs laid was included as a covariate in the model for the number of adult offspring (Table S2A).

To test the effects of developmental heat stress on the fertility of both sexes, we analysed two variables: number of adult offspring, as an indicator of female fertility, and the offspring sex ratio, indicating male fertility.

The number of adult offspring was analysed using a GLM model with a quasi-Poisson error distribution. Offspring sex ratio, represented by the proportion of daughters, was computed using the function *cbind* with the number of daughters and sons as arguments. This variable was analysed with a GLM model with a beta-binomial error distribution and a parameter to account for zero inflation (ziformula ~1; package *glmmTMB*). The developmental temperature of the female and of the male (25 ºC or 36 ºC) and the resistance status (WuSS or WuRR) were included as fixed factors, as well as their interaction. Since males and females from different cohorts, with one day of interval, were used, the age of each sex was included as a random factor (Table S2B).

Effect of temperature on the pattern of sperm precedence

To test how developmental heat stress affects the pattern of sperm precedence of this species, the proportion of daughters was analysed, indicating the contribution of the subsequent males to the offspring.

The proportion of daughters was computed using the function *cbind* with the number of daughters and the number of unhatched eggs (which accounts for the contribution of the first male to the offspring) as arguments. It was analysed using a GLMM model with a binomial error distribution. The cross between the female and the first male developed at one of the temperatures (♀25 x ♂25, ♀25 x ♂36, ♀36 x ♂25 and ♀36 x ♂36), the female mating history (SM, DM or MM) and their interaction were included as fixed factors. Cross was used instead of the developmental temperatures of the female and of the first male separately to avoid convergence issues. Block and the room temperature and humidity during mating observations were included as random factors (Table S3). As expected, when females were single mated (SM), the proportion of daughters was zero, with only one replicate having a proportion of daughters above zero (in cross ♀25 x ♂36; proportion = 0.034). This lack of variance caused problems in the analysis. To deal with this, we manually added one replicate to the SM mating history of each cross: the value of these replicates were equal to the one replicate above zero in cross ♀25 x ♂36, in order to solve the variance problem while maintaining the original differences between the different crosses.

Effect of temperature on mating behavior

To analyse how mating and female remating eagerness was affected by developmental heat stress, seven variables were studied: latency to the first copulation and the duration of that copulation, indicating the

mating eagerness of both individuals; latency to the second copulation and the duration of that copulation, which indicates the eagerness of the female to mate a second time; the mating rate, designating the number of times the female remated; and the number of adult offspring and sex ratio, which gives additional information about female and male fertility, respectively.

The latencies to the first and second copulations and their durations were analysed using LMM models with a gaussian error distribution. The data was box cox transformed (latency and duration of first copulation: $\lambda = 0.39$; latency of second copulation: $\lambda = 0.11$; duration of second copulation: $\lambda = -0.05$; *MASS* package; Venables & Ripley, 2002) to improve the fit of the model. The mating rate and the number of adult offspring were analysed using GLMM models with a quasi-Poisson error distribution. Lastly, the sex ratio, represented as the proportion of daughters, was computed using the function *cbind* with the number of daughters and sons as arguments. A GLMM model was used with a beta-binomial error distribution and a parameter to account for zero inflation (ziformula ~1; package *glmmTMB*). The developmental temperatures of the female and the first male (25 ºC or 36 ºC), as well as their interaction, were added as fixed factors in all models. Additionally, the mating rate was included as a covariate in the model for the latency to the second copulation and its duration. For the models of the number of adult offspring and sex ratio, a third interaction was added with the female mating history (SM or MM). For all models, block and the room temperature and humidity were included as random factors (Table S4).

Effect of temperature on sperm depletion and recovery of male fertility

To understand how the developmental heat stress affected male sperm depletion immediately after being placed at an optimal temperature, along several matings, and after spending two days at that temperature, three variables were tested: latency to each copulation of the male and the duration of those copulations, which indicated the eagerness of each pair to copulate, and the offspring sex ratio resulting from each mating, showing the offspring of each female sired by the male. Two models were made for each variable, one to analyse the five consecutive copulations of the male and another to compare the first and last copulation (which occurred two days later; Last Mating).

The latency to the five consecutive copulations and their durations were analysed using a LM model with a gaussian error distribution. The data was box cox transformed (latency: $\lambda = 0.06$; duration: $\lambda = 0.2$; *MASS* package; Venables & Ripley, 2002) to improve the fit of the model. Moreover, the latency to the first and last copulation and their durations were analysed using LM and LMM models, respectively, with a gaussian error distribution. The data was also box cox transformed (latency: $\lambda = -0.11$; duration: $\lambda = 0.14$).

The offspring sex ratio of the five consecutive copulations, represented as the proportion of daughters, was computed using the function *cbind* with the number of daughters and sons as arguments. A GLMM model was used with a beta-binomial error distribution and a parameter to account for zero inflation (ziformula ~1; package *glmmTMB*). Lastly, the offspring sex ratio of the first and last copulation was analysed using a GLMM model with a beta-binomial error distribution.

The cross between males and females from different developmental temperatures and the identity of the male were included as fixed factors in all models. The order by which each female mated with the male (from 1st to 5th and Last Mating) was added as a covariate. Additionally, the interaction between cross and the order by which each female mated with the male was also included. In all models, block, room temperature and humidity were included as random factors (Table S5).

Results

Effect of temperature on life-history traits

The temperature at which the offspring developed significantly affected the number of adult offspring ($F_{2,4.664} = 61.43$, $p = 0.012$; Table 3.1A). When compared to the control, the number of adult offspring at 33 ºC was higher (T = 10.304, $p = <0.001$), while at 35 and 36 ºC it was lower (35 ºC: T = 12.019, $p = <0.001$; 36 ºC: T = 8.203, $p = <0.001$). Also, there was a difference in number of adult offspring between offspring developed at 33 and 36 ºC (Table 3.1B; Figure 3.1A), indicating that at 36 ºC less offspring survives.

Developmental time in both sexes was shortened by high temperatures (females: $F_{2,12.07} = 33.52$, $p = <0.001$; males: $F_{2,6.345} = 4.16$, $p = 0.003$; Table 3.1A), with females and males having a significantly slower development at 36 ºC compared to 35 ºC (Table 3.1B; Figure 3.1B and C).

The developmental temperature also affected female fertility ($F_{7,38.812} = 6298$, $p = <0.001$; Table 3.1C). When compared to the control, the number of adult offspring of females developed at 33 ºC was higher (T = 21.025, $p = <0.001$), while at 35, 36 and 37 ºC was lower (35 ºC: T = 11.801, $p = <0.001$; 36 ºC: T = 4.577, $p = <0.001$; 37 ºC: T = 5.677, $p = <0.001$). Additionally, females developed at 33 ºC had significantly more adult offspring than females developed at 35, 36 and 37 ºC. Also, females developed at 35 ºC had higher number of adult offspring than females developed at 36 and 37 ºC (Table 3.1D; Figure 3.2). These results indicate that fertility decreases with temperature.

In sum, fertility was severely affected at 36 ºC or higher. However, at 37ºC survival was excessively reduced, hence this temperature was too high to be considered an ideal sublethal temperature. Thus, 36 ºC was chosen as the temperature to test on the following experiments.

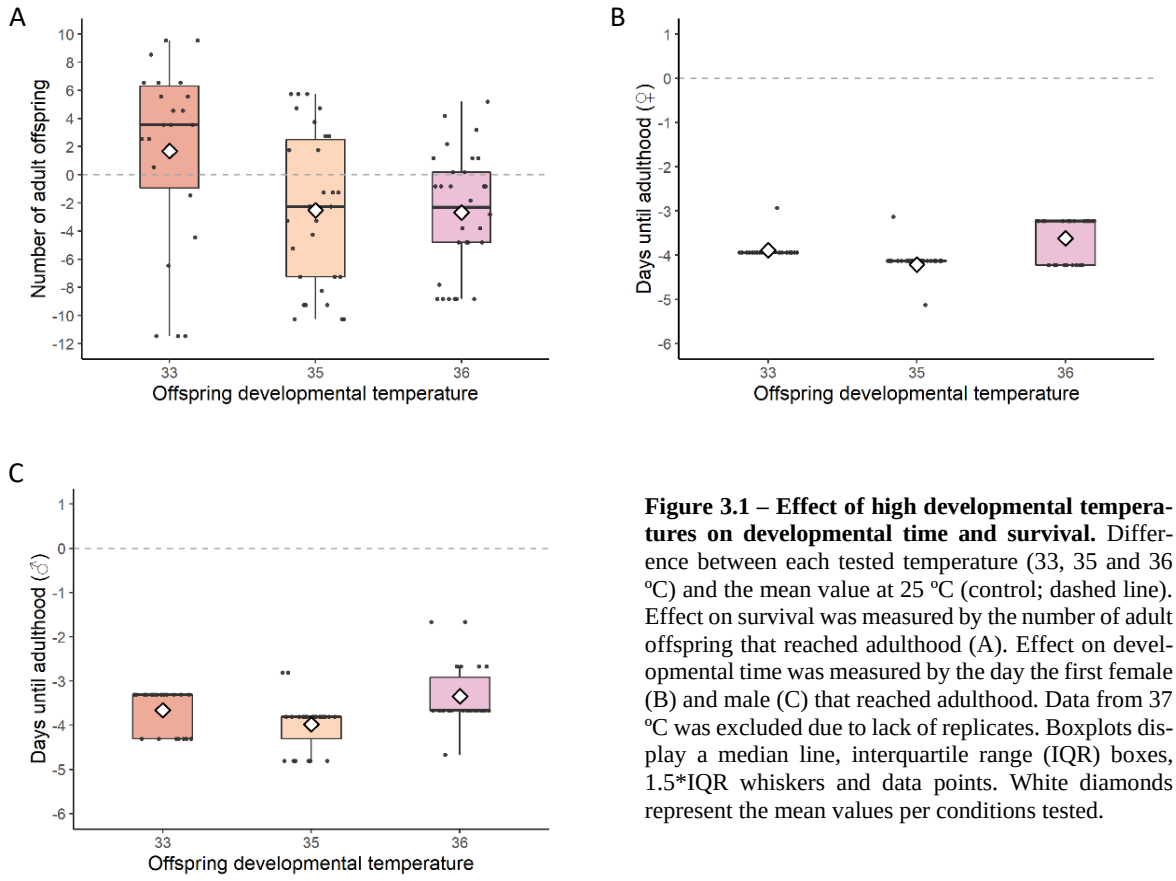

Figure 3.1 – Effect of high developmental temperatures on developmental time and survival. Difference between each tested temperature (33, 35 and 36 ºC) and the mean value at 25 ºC (control; dashed line). Effect on survival was measured by the number of adult offspring that reached adulthood (A). Effect on developmental time was measured by the day the first female (B) and male (C) that reached adulthood. Data from 37 ºC was excluded due to lack of replicates. Boxplots display a median line, interquartile range (IQR) boxes, 1.5*IQR whiskers and data points. White diamonds represent the mean values per conditions tested.

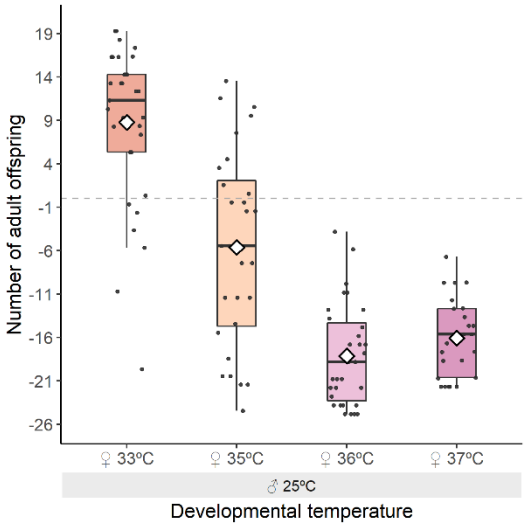

Figure 3.2 – Effect of high developmental temperatures on fertility. Difference between pairs with one individual developed at each of the tested temperatures (33, 35, 36 and 37 ºC) and the mean value of pairs developed at 25 ºC (control; dashed line). Effect on female fertility was measured by the number of adult offspring. Boxplots display a median line, interquartile range (IQR) boxes, 1.5*IQR whiskers and data points. White diamonds represent the mean values per conditions tested.

Table 3.1 – Results obtained from *Effect of temperature on life-history traits* statistical analysis and *a posteriori* contrasts of the corresponding significant explanatory variables. A and B refer to the analyses of the survival and developmental time; C and D refer to the analyses of the fertility. A) "Df": the degrees of freedom. "F": the sum of squares obtained from the F-test. "Offspring temperature": the temperature at which the females laid eggs and the offspring developed; "Fecundity": the difference between the number of eggs laid in each replicate developed at a high temperature and the mean of the number of eggs laid at the control of that high temperature. B) *A posteriori* contrasts with Bonferroni corrections were done to interpret the significant effect of the fixed factors. "T ratio": the T-test value obtained in each comparison. Comparisons: Tº - Tº (comparing different treatments, where the offspring developed at either 25, 33, 35 or 36 ºC). C) "Df": the degrees of freedom. "F": the sum of squares obtained from the F-test. "Temperature of pair": developmental temperatures of the female and male. D) *A posteriori* contrasts with Bonferroni corrections were done to interpret the significant effect of the fixed factors. "T ratio": the T-test value obtained in each comparison. Comparisons: ♀Tº x ♂Tº - ♀Tº x ♂Tº (comparing different treatments, where the female and the male developed at either 25, 33, 35, 36 or 37 ºC). Statistically significant terms are represented in bold.

A)

Var. of interest	Explanatory var.	Df (Df residuals)	F	P-value
Number of adult offspring	**Offspring temperature**	**2 (4.664)**	**61.43**	**0.012**
	Fecundity	**1 (87.045)**	**573.26**	**<0.001**
Days until adulthood (♀)	**Offspring temperature**	**2 (12.07)**	**33.52**	**<0.001**
Days until adulthood (♂)	**Offspring temperature**	**2 (6.345)**	**4.16**	**0.003**

B)

Var. of interest	Comparison	T ratio	P-value
Number of adult offspring	33 ºC – 35 ºC	0.854	1.000
	33 ºC – 36 ºC	**2.890**	**0.015**
	35 ºC – 36 ºC	2.128	0.109
Days until adulthood (♀)	33 ºC – 35 ºC	2.257	0.082
	33 ºC – 36 ºC	**-2.541**	**0.041**
	35 ºC – 36 ºC	**-4.911**	**<0.001**
Days until adulthood (♂)	33 ºC – 35 ºC	1.918	0.179
	33 ºC – 36 ºC	-1.532	0.392
	35 ºC – 36 ºC	**-3.553**	**0.002**

C)

Var. of interest	Explanatory var.	Df (Df residuals)	F	P-value
Number of adult offspring	**Temperature of pair**	**7 (38.812)**	**6298**	**<0.001**

D)

Var. of interest	Comparison	T ratio	P-value
Number of adult offspring	**♀33 x ♂25 - ♀35 x ♂25**	**5.565**	**<0.001**
	♀33 x ♂25 - ♀36 x ♂25	**11.346**	**<0.001**
	♀33 x ♂25 - ♀37 x ♂25	**9.522**	**<0.001**
	♀35 x ♂25 - ♀36 x ♂25	**5.401**	**<0.001**
	♀35 x ♂25 - ♀37 x ♂25	**3.979**	**0.001**
	♀36 x ♂25 - ♀37 x ♂25	-1.166	1.000

Interaction between pesticide resistance and response to temperature

Developmental temperature significantly affected the number of adult offspring (χ^2_1 = 122.34, p = <0.001) and the developmental time of each sex (females: χ^2_1 = 23.578, p = <0.001; males: χ^2_1 = 22.175, p = <0.001; Table 3.2A; Figure 3.3), which is consistent with the previous assay. The resistance status did not affect any of these variables.

Regarding female fertility, females developed at 36 ºC had significantly fewer adult offspring than the control (χ^2_1 = 243.924, p = <0.001; Tables 3.2B and 3.2C). This was also affected by the interaction between the developmental temperature of the male and the resistance status (χ^2_1 = 5.227, p = 0.022; Table 3.2B), with WuRR pairs having fewer adult offspring than WuSS pairs when males developed at 36 ºC (Table 3.2C; Figure 3.4A).

Male fertility, measured by the offspring sex ratio, was also affected by the developmental temperature of the female (χ^2_1 = 51.139, p = <0.001; Table 3.2B), with females developed at 36 ºC having lower proportion of fertilized eggs than the control (Table 3.2C). The interaction between the developmental temperature of the male and the resistance status of the pair also affected the offspring sex ratio (χ^2_1 = 4.895, p = 0.027; Table 3.2B). Indeed, WuRR pairs with males developed at 36 ºC showed a larger difference in proportion of daughters to pairs with males developed at 25 ºC than WuSS pairs (Table 3.2C; Figure 3.4B).

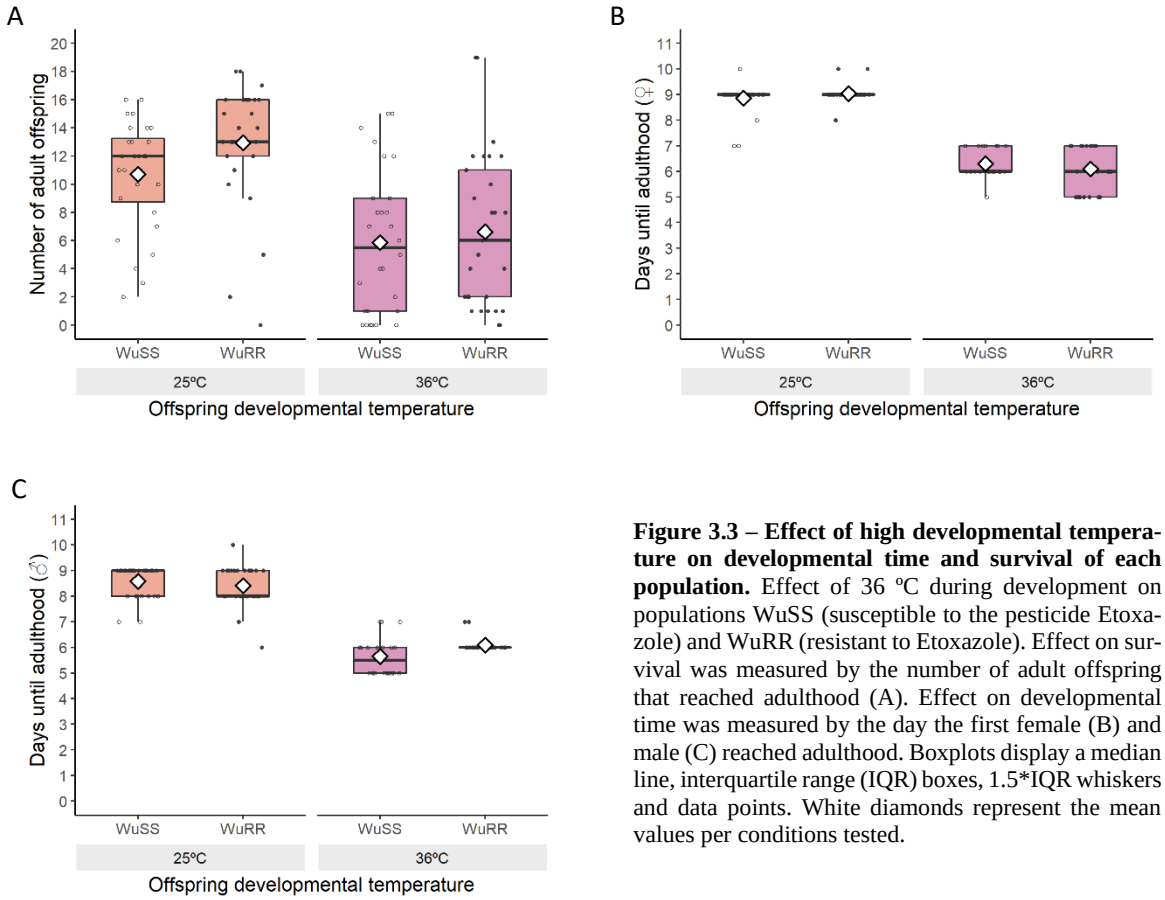

Figure 3.3 – Effect of high developmental temperature on developmental time and survival of each population. Effect of 36 ºC during development on populations WuSS (susceptible to the pesticide Etoxazole) and WuRR (resistant to Etoxazole). Effect on survival was measured by the number of adult offspring that reached adulthood (A). Effect on developmental time was measured by the day the first female (B) and male (C) reached adulthood. Boxplots display a median line, interquartile range (IQR) boxes, 1.5*IQR whiskers and data points. White diamonds represent the mean values per conditions tested.

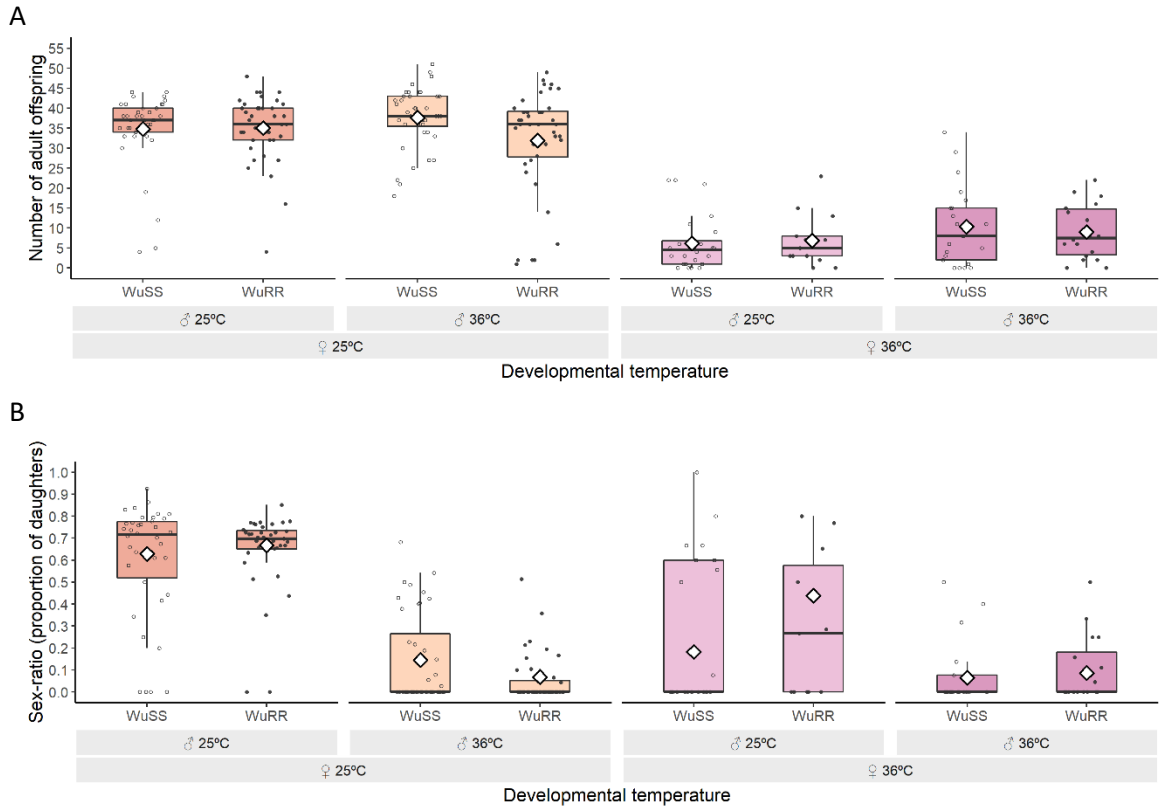

Figure 3.4 - Effect of high developmental temperature on fertility of each population. Effect of 36 ºC during development on populations WuSS (susceptible to the pesticide Etoxazole) and WuRR (resistant to Etoxazole). Effect on female fertility was measured by the number of adult offspring (A). Effect on male fertility was measured by the offspring sex ratio (proportion of daughters) (B). Boxplots display a median line, interquartile range (IQR) boxes, 1.5*IQR whiskers and data points. White diamonds represent the mean values per conditions tested.

Table 3.2 – Results obtained from *Interaction between pesticide resistance and response to temperature* statistical analysis and *a posteriori* contrasts of the corresponding significant explanatory variables. A refers to the analyses of the survival and developmental time of mites; B and C refer to the analyses of mite fertility. A) "Df": the degrees of freedom. "χ^2": the Chi-square value obtained in each analysis. "Offspring temperature": the temperature where the female laid eggs and the offspring developed; "Resistance status": resistance or susceptibility to Etoxazole. B) "Df": the degrees of freedom. "χ^2": the Chi-square value obtained in each analysis. "Resistance status": resistance or susceptibility to Etoxazole; "♀ temperature": the developmental temperatures of the females used; "♂ temperature": the developmental temperatures of the males used. C) *A posteriori* contrasts with Bonferroni corrections were done to interpret the significant effect of the fixed factors. "T ratio": the T-test value obtained in each comparison. Comparisons: (Resistance status) ♂Tº - (Resistance status) ♂Tº (comparing different treatments, where males developed at either 25 or 36 ºC and were either susceptible (WuSS) or resistant (WuRR) to Etoxazole). Statistically significant terms are represented in bold.

A)

Var. of interest	Explanatory var.	Df	χ^2	P-value
Number of adult offspring	**Offspring temperature**	**1**	**122.34**	**<0.001**
	Resistance status	1	1.977	0.160
	Fecundity	**1**	**229.46**	**<0.001**
	Offspring temperature * Resistance status	1	0.812	0.368

Var. of interest	Explanatory var.	Df	χ^2	P-value
Days until adulthood (♀)	**Offspring temperature**	**1**	**23.578**	**<0.001**
	Resistance status	1	0.001	0.977
	Offspring temperature * Resistance status	1	0.118	0.731
Days until adulthood (♂)	**Offspring temperature**	**1**	**22.175**	**<0.001**
	Resistance status	1	0.032	0.857
	Offspring temperature * Resistance status	1	0.359	0.549

B)

Var. of interest	Explanatory var.	Df	χ^2	P-value
Number of adult offspring	♀ **temperature**	**1**	**243.924**	**<0.001**
	♂ temperature	1	2.492	0.114
	Resistance status	1	0.291	0.590
	♀ temperature * ♂ temperature	1	3.170	0.075
	♀ temperature * Resistance status	1	1.471	0.225
	♂ **temperature * Resistance status**	**1**	**5.227**	**0.022**
	♀ temperature * ♂ temperature* Resistance status	1	0.207	0.649
Sex ratio	♀ **temperature**	**1**	**51.139**	**<0.001**
	♂ **temperature**	**1**	**66.215**	**<0.001**
	Resistance status	1	0.181	0.670
	♀ temperature * ♂ temperature	1	0.322	0.571
	♀ temperature * Resistance status	1	1.661	0.197

Var. of interest	Explanatory var.	Df	χ^2	P-value
Sex ratio	**♂ temperature * Resistance status**	**1**	**4.895**	**0.027**
	♀ temperature * ♂ temperature* Resistance status	1	3.062	0.080

C)

Var. of interest	Comparison	T ratio	P-value
Number of adult offspring	WuSS ♂25 – WuSS ♂36	-1.659	0.394
	WuRR ♂25 – WuRR ♂36	1.579	0.463
	WuRR ♂25 – WuSS ♂25	0.540	1.000
	WuRR ♂36 – WuSS ♂36	**-2.705**	**0.029**
Sex ratio	**WuSS ♂25 – WuSS ♂36**	**8.560**	**<0.001**
	WuRR ♂25 – WuRR ♂36	**8.137**	**<0.001**
	WuRR ♂25 – WuSS ♂25	-0.426	1.000
	WuRR ♂36 – WuSS ♂36	**-2.641**	**0.035**

Effect of temperature on the pattern of sperm precedence

The interaction between the female mating history and the developmental temperatures of the female and the first male had a significant effect on the proportion of daughters, which represents the contribution of the subsequent males on the paternity of the offspring (proportion of daughters; χ^2_6 = 22.956, p = <0.001; Table 3.3A). Indeed, double and multiple mated females (DM and MM,

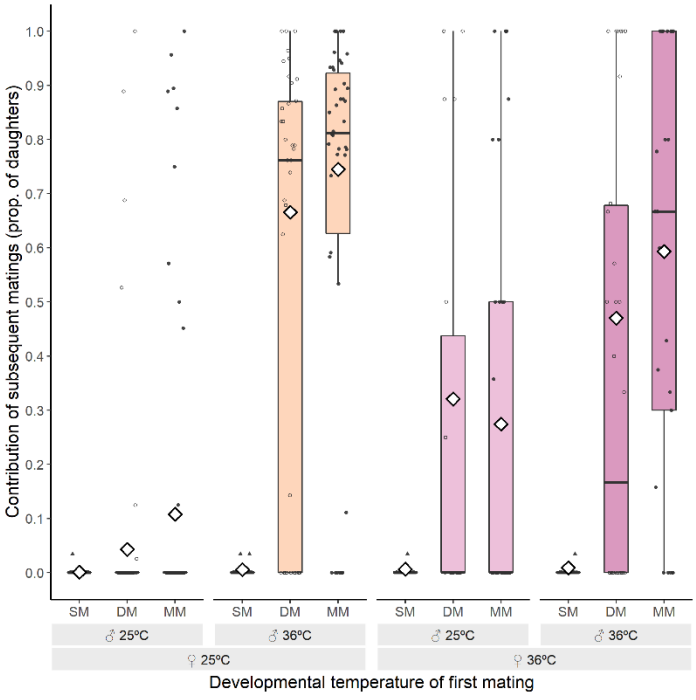

Figure 3.5 – Effect of high developmental temperature on the pattern of sperm precedence. Females were mated once (SM), twice (DM) or multiply over 24 hours (MM). The first mating involved a male susceptible to the pesticide Etoxazole and developed at the temperature described in the figure, while the subsequent matings involved males resistant to Etoxazole and developed at 25 ºC. Offspring sired by the subsequent males are represented by daughters (resist to pesticide). Boxplots display a median line, interquartile range (IQR) boxes, 1.5*IQR whiskers and data points. White diamonds represent the mean values per conditions tested. The four replicates added to the data (one per SM) are included in the figure.

respectively) developed at 36 ºC had higher proportion of daughters than females with the same mating history but that developed at 25 ºC, when the first male developed at 25 ºC (Table 3.3B). However, when the first male developed at 36 ºC, DM and MM females that developed at 36 ºC had lower proportion of daughters than females with the same mating history but that developed at 25ºC (Table 3.3B). In addition, subsequent males sired more offspring of MM females when the first male developed at 36 ºC than when the first male developed at 25 ºC. This was also true regarding DM females developed at 25 ºC (Table 3.3B). Lastly, in all crosses the proportion of daughters of SM females was significantly lower than DM and MM females, but MM females produced a higher proportion of daughters than DM females only when the female developed at 25 ºC. In particular, when the female and the first male developed at 25 ºC, despite DM and MM females having higher proportion of daughters than SM females, they were still significantly lower compared to DM and MM females from the other crosses (Table 3.3B; Figure 3.5).

Table 3.3 – Results obtained from *Effect of temperature on the pattern of sperm precedence* statistical analysis and *a posteriori* contrasts of the corresponding significant explanatory variables. A) "Df": the degrees of freedom. "$\chi 2$": the Chi-square value obtained in each analysis. "Temperature of pair": developmental temperatures of the female and male crossed in each replicate; "♀ mating history": according to the number of matings the female was involved in. B) *A posteriori* contrasts with Bonferroni corrections were done to interpret the significant effect of the fixed factors. "T ratio": the T-test value obtained in each comparison. Comparisons: (♀ mating history) ♀Tº x ♂Tº - (♀ mating history) ♀Tº x ♂Tº (comparing different treatments, where the female and the first male developed at either 25 or 36 ºC and the female mated once (SM), twice (DM) or multiply (MM)). Statistically significant terms are represented in bold.

A)

Var. of interest	Explanatory var.	Df	χ^2	P-value
Proportion of daughters	**Temperature of pair**	**3**	**516.785**	**<0.001**
	♀ mating history	**2**	**53.679**	**<0.001**
	Temperature of pair * ♀ mating history	**6**	**22.956**	**<0.001**

B)

Var. of interest	Comparison	T ratio	P-value
Proportion of daughters	**DM ♀25 x ♂25 – DM ♀25 x ♂36**	**-22.693**	**<0.001**
	DM ♀25 x ♂25 – DM ♀36 x ♂25	**-10.022**	**<0.001**
	DM ♀25 x ♂25 – DM ♀36 x ♂36	**-12.914**	**<0.001**
	DM ♀25 x ♂36 – DM ♀36 x ♂36	**4.382**	**<0.001**
	DM ♀36 x ♂25 – DM ♀36 x ♂36	-1.281	1.000
	MM ♀25 x ♂25 – MM ♀25 x ♂36	**-26.524**	**<0.001**
	MM ♀25 x ♂25 – MM ♀36 x ♂25	**-6.024**	**<0.001**
	MM ♀25 x ♂25 – MM ♀36 x ♂36	**-13.264**	**<0.001**

Var. of interest	Comparison	T ratio	P-value
Proportion of daughters	**MM ♀25 x ♂36 – MM ♀36 x ♂36**	**3.253**	**0.023**
	MM ♀36 x ♂25 – MM ♀36 x ♂36	**-5.661**	**<0.001**
	SM ♀25 x ♂25 – DM ♀25 x ♂25	**-3.918**	**0.002**
	SM ♀25 x ♂25 – MM ♀25 x ♂25	**-4.917**	**<0.001**
	DM ♀25 x ♂25 – MM ♀25 x ♂25	**-5.671**	**<0.001**
	SM ♀25 x ♂36 – DM ♀25 x ♂36	**-8.268**	**<0.001**
	SM ♀25 x ♂36 – MM ♀25 x ♂36	**-8.806**	**<0.001**
	DM ♀25 x ♂36 – MM ♀25 x ♂36	**-3.261**	**0.022**
	SM ♀36 x ♂25 – DM ♀36 x ♂25	**-4.394**	**<0.001**
	SM ♀36 x ♂25 – MM ♀36 x ♂25	**-4.014**	**0.001**
	DM ♀36 x ♂25 – MM ♀36 x ♂25	1.501	1.000
	SM ♀36 x ♂36 – DM ♀36 x ♂36	**-4.487**	**<0.001**
	SM ♀36 x ♂36 – MM ♀36 x ♂36	**-5.100**	**<0.001**
	DM ♀36 x ♂36 – MM ♀36 x ♂36	-2.514	0.239

Effect of temperature on mating behavior

The interaction between the developmental temperatures of the female and the male affected the latency to the first copulation ($F_{1,342.51} = 4.647$, $p = 0.032$; Table 3.4A), with pairs including males developed at 36 ºC taking longer to start copulation than males developed at 25 ºC by ca. 110% when the female developed at 25 ºC, and ca. 50% when the female developed at 36 ºC (Figure 3.6A). In addition, when mating with males developed at 25 ºC, females developed at 36 ºC took ca. 30% longer to mate than females developed at 25 ºC. The duration of the first copulation was only affected by the developmental temperature of the male ($F_{1,344.62} = 52.403$, $p = <0.001$; Table 3.4A), with pairs including males developed at 36 ºC having ca. 40% shorter copulations (Figure 3.6B).

The latency to the second copulation was affected by the mating rate of the female ($F_{1,126.215} = 23.842$, $p = <0.001$) and by the developmental temperature of the first male to mate with the female ($F_{1,120.670} = 17.605$, $p = <0.001$; Table 3.4A), with pairs including females first mated with males developed at 36 ºC taking ca. 55% less time to start remating than pairs where the first male developed at 25 ºC (Figure 3.6C). The duration of that copulation was affected by the interaction of the developmental temperatures of the female and of the first mate ($F_{1,119.398} = 30.959$, $p = <0.001$; Table 3.4A), with pairs including one individual developed at 36 ºC having longer copulations than the control (Table 3.4B; Figure 3.6D). The female mating rate was not affected by any factor, being similar between treatments (Table 3.4A).

The number of adult offspring was affected by the developmental temperature of the female ($\chi^2_1 = 169.33$, $p = <0.001$; Table 3.4A), with females developed at 36 ºC having ca. 65% less offspring compared to females developed at 25 ºC, independently of the female mating history (Figure 3.6E). The offspring sex ratio was affected by the interaction between the developmental temperature of the first

male and the female mating history (χ^2_1 = 75.561, p = <0.001; Table 3.4A). Indeed, single mated females that mated with males developed at 36 ºC had lower proportion of daughters than when mated with males developed at 25 ºC. In addition, females that first mated with males developed at 36 ºC had an increase of 0.52 ± 0.09 in the proportion of daughters when they remated afterwards with males developed at 25 ºC, while females that consistently mated with males developed at 25 ºC did not show a significant difference across female mating history (Table 3.4B; Figure 3.6F). The offspring sex ratio was also affected by the female developmental temperature (χ^2_1 = 22.075, p = <0.001; Table 3.4A), with females developed at 25 ºC having a higher proportion of fertilized offspring than females developed at 36 ºC (Figure 3.6F).

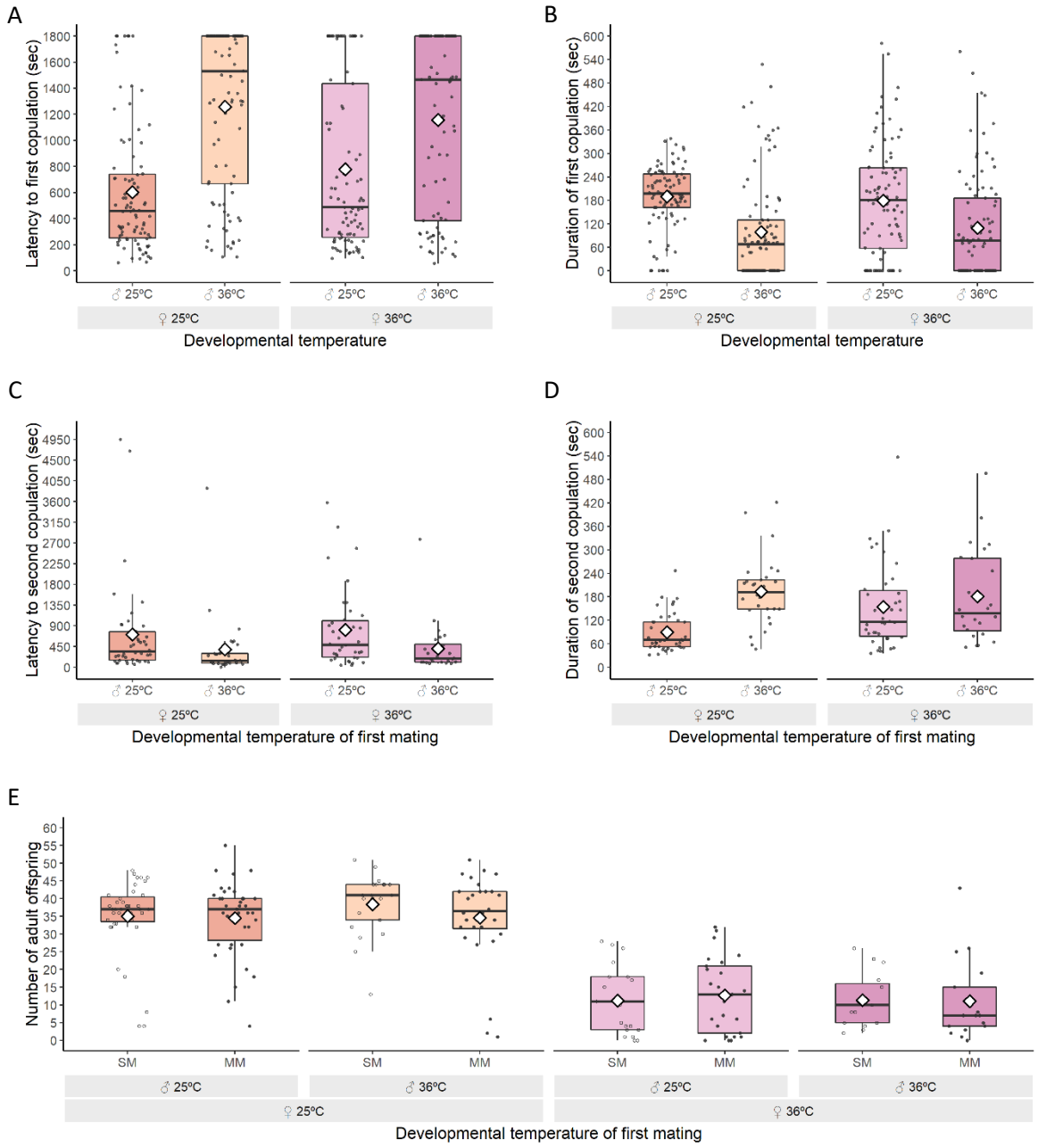

Figure 3.6 – Effect of high developmental temperature on mating behavior. Females were mated once (SM) or multiply over 2 hours (MM). The first mating involved a male developed at the temperature described in the figure, while the subsequent matings involved males developed at 25 ºC. A) Latency to mate; B) Duration of first copulation; C) Latency to remate (female's second mating); D) Duration of second copulation; E) Number of adult offspring laid by SM and MM females; F) Proportion of adult daughters laid by SM and MM females. Boxplots display a median line, interquartile range (IQR) boxes, 1.5*IQR whiskers and data points. White diamonds represent the mean values per conditions tested.

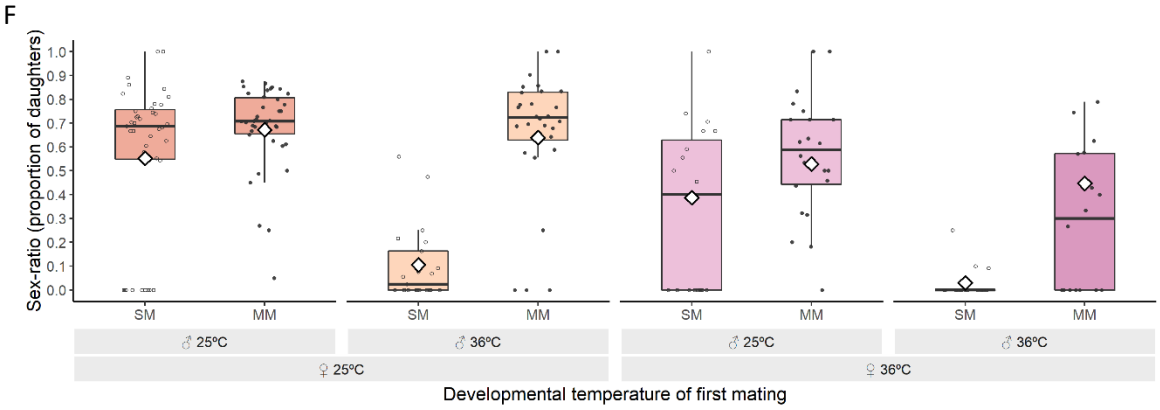

Figure 3.6 (continued)

Table 3.4 – Results obtained from *Effect of temperature on mating behavior* statistical analysis and *a posteriori* contrasts of the corresponding significant explanatory variables. A) "Df": the degrees of freedom. "χ^2": the Chi-square value obtained in each analysis; "F": the sum of squares obtained from the F-test. "♀ temperature": developmental temperature of each female used; "♂ temperature": developmental temperature of the first male; "Mating rate": number of times each MM female mated; "♀ mating history": according to the number of matings the female was involved in. B) *A posteriori* contrasts with Bonferroni corrections were done to interpret the significant effect of the fixed factors. "T ratio": the T-test value obtained in each comparison. Comparisons: ♀Tº x ♂Tº - ♀Tº x ♂Tº (comparing different treatments, where the female and the male developed at either 25 or 36 ºC); (♀ mating history) ♂Tº - (♀ mating history) ♂Tº (comparing different treatments, where the first male developed at either 25 or 36 ºC and the female mated once (SM) or multiply (MM)). Statistically significant terms are represented in bold.

A)

Var. of interest	Explanatory var.	Df (Df residuals)	χ^2/F	P-value
Latency to mate	♀ temperature	1 (343.81)	2.961	0.086
	♂ temperature	**1 (342.42)**	**49.096**	**<0.001**
	♀ temperature * ♂ temperature	**1 (342.51)**	**4.647**	**0.032**
Duration of mating copulation	♀ temperature	1 (345.42)	1.179	0.278
	♂ temperature	**1 (344.62)**	**52.403**	**<0.001**
	♀ temperature * ♂ temperature	1 (342.63)	2.859	0.092
Latency to remate	♀ temperature	1 (116.544)	1.854	0.176
	♂ temperature	**1 (120.670)**	**17.605**	**<0.001**
	Mating rate	**1 (126.215)**	**23.842**	**<0.001**

Var. of interest	Explanatory var.	Df (Df residuals)	χ^2/F	P-value
Latency to remate	♀ temperature * ♂ temperature	1 (116.151)	0.202	0.654
Duration of remating copulation	**♀ temperature**	**1 (117.555)**	**11.862**	**<0.001**
	♂ temperature	**1 (121.028)**	**30.959**	**<0.001**
	Mating rate	1 (110.23)	0.000	0.998
	♀ temperature * ♂ temperature	**1 (119.398)**	**8.548**	**0.004**
Mating rate	♀ temperature	1	0.786	0.375
	♂ temperature	1	1.022	0.312
	♀ temperature * ♂ temperature	1	0.031	0.861
Number of adult offspring	**♀ temperature**	**1**	**169.33**	**<0.001**
	♂ temperature	1	0.229	0.632
	♀ mating history	1	0.983	0.322
	♀ temperature * ♂ temperature	1	0.009	0.926
	♀ temperature * ♀ mating history	1	0.008	0.931
	♂ temperature * ♀ mating history	1	1.513	0.219
	♀ temperature * ♂ temperature * ♀ mating history	1	0.004	0.948
Sex ratio	**♀ temperature**	**1**	**22.075**	**<0.001**
	♂ temperature	1	0.370	0.543
	♀ mating history	1	1.023	0.312
	♀ temperature * ♂ temperature	1	1.176	0.278

Var. of interest	Explanatory var.	Df (Df residuals)	χ^2/F	P-value
Sex ratio	♀ temperature * ♀ mating history	1	0.103	0.749
	♂ temperature * ♀ mating history	**1**	**75.561**	**<0.001**
	♀ temperature * ♂ temperature * ♀ mating history	1	0.079	0.779

B)

Var. of interest	Comparison	T ratio	P-value
Latency to mate	♀25 x ♂25 – ♀36 x ♂25	-1.721	0.345
	♀25 x ♂36 – ♀36 x ♂36	1.324	0.745
	♀25 x ♂25 – ♀25 x ♂36	**-7.007**	**<0.001**
	♀36 x ♂25 – ♀36 x ♂36	**-3.883**	**<0.001**
Duration of remating copulation	**♀25 x ♂25 – ♀36 x ♂25**	**-3.444**	**0.003**
	♀25 x ♂36 – ♀36 x ♂36	0.981	1.000
	♀25 x ♂25 – ♀25 x ♂36	**-5.564**	**<0.001**
	♀36 x ♂25 – ♀36 x ♂36	-1.177	0.966
Sex ratio	MM ♂25 – MM ♂36	-0.608	1.000
	SM ♂25 – SM ♂36	**9.318**	**<0.001**
	MM ♂25 – SM ♂25	-1.011	1.000
	MM ♂36 – SM ♂36	**9.076**	**<0.001**

Effect of temperature on sperm depletion and recovery of male fertility

The latency to each of the five consecutive matings was affected by the interaction between the developmental temperature of the pair and the order by which each female mated with the male ($F_{2,18.547}$ = 269.3, p = <0.001; Table 3.5A), with pairs where males developed at 36 ºC taking longer to start copulation and having a significantly different variation between values across the male mating order compared to the control (♀25 x ♂25 - ♀25 x ♂36: T = -3.251, p = 0.001; ♀25 x ♂25 - ♀36 x ♂36: T = -2.216, p = 0.027; Figure 3.7A). The duration of these matings was also affected by the interaction between the developmental temperature of the pair and the mating history of the male ($F_{2,10.098}$ = 622.7, p = <0.001; Table 3.5A), with ♀25 x ♂36 pairs having a different variation between values across the male mating order compared to the other pairs (♀25 x ♂25: T = -4.478, p = <0.001; ♀36 x ♂36: T = -2.679, p = 0.008; Figure 3.7C).

Regarding the first and last mating of the male, which occurred two days later, their latencies were affected by the developmental temperature of the pair ($F_{2,83.364} = 47.962$, $p = <0.001$; Table 3.5A), with pairs where males developed at 36 ºC taking longer to start copulation than the control (Table 5 B). ♀25 x ♂36 pairs also took significantly longer to copulate than ♀36 x ♂36 pairs (Table 5 B). These latencies were also affected by the mating history of the male ($F_{1,43.115} = 12.403$, $p = <0.001$; Table 3.5A), indicating that the latency to the last mating was significantly different from the latency to the first mating of the male (Figure 3.7B). The duration of these matings was affected by the interaction between the developmental temperature of the pair and the mating history of the male ($F_{2,3.428} = 689.3$, $p = 0.034$; Table 3.5A), with pairs where males developed at 36 ºC having a different variation between values across the male mating order compared to the control (♀25 x ♂25 - ♀25 x ♂36: T = -2.437, $p = 0.016$; ♀25 x ♂25 - ♀36 x ♂36: T = -1.995, $p = 0.047$; Figure 3.7D).

Lastly, the offspring sex ratio of the five consecutive mated females was affected by the interaction between the developmental temperature of the pair and the mating history of the male ($\chi^2_2 = 6.066$, $p = 0.048$; Table 3.5A), with ♀25 x ♂36 pairs having a different variation between values across the male mating order compared to the control (T = -2.389, $p = 0.017$; Figure 3.7E). Moreover, the offspring sex ratio of the first and last female to mate with the male was affected by the developmental temperature of the pair ($\chi^2_2 = 141.249$, $p = <0.001$; Table 3.5A), with pairs where the male developed at 36 ºC having less proportion of daughters than the control (Table 3.5B; Figure 3.7F).

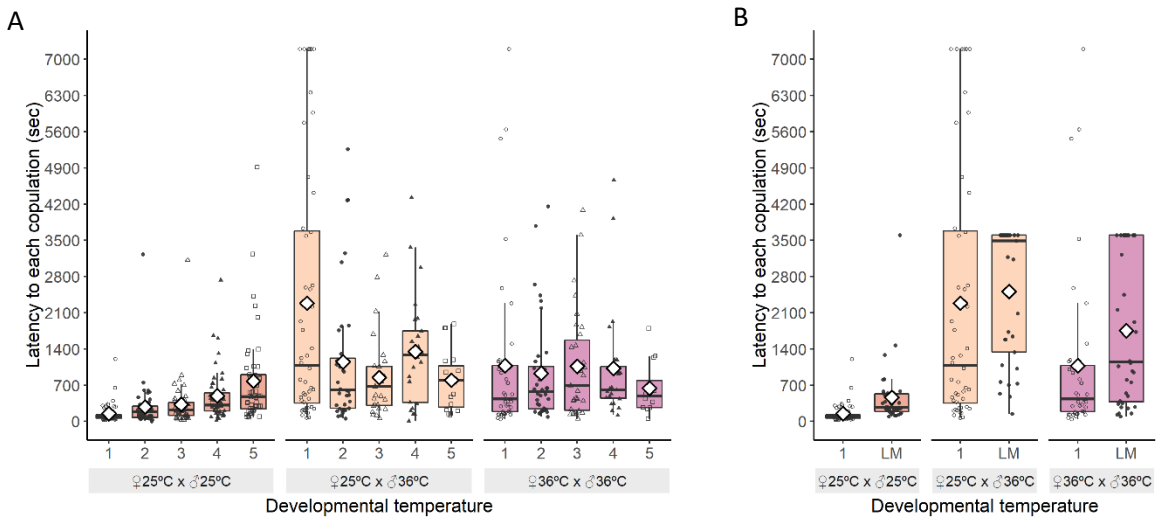

Figure 3.7 – Effect of high developmental temperature on sperm depletion and recovery of male fertility. Males could mate up to 5 times with different females for 2 hours. Each mating is represented on each graph by the number corresponding to the order of the mating. Two days later, males were mated with one female, which is represented as LM (Last Mating). All females mated with one male developed at the same temperature (25 or 36 ºC). A) Latency to the first mating and between matings; B) Duration of each mating; C) Number of adult daughters of each mating; D) Proportion of daughters of each mating. Boxplots display a median line, interquartile range (IQR) boxes, 1.5*IQR whiskers and data points. White diamonds represent the mean values per conditions tested.

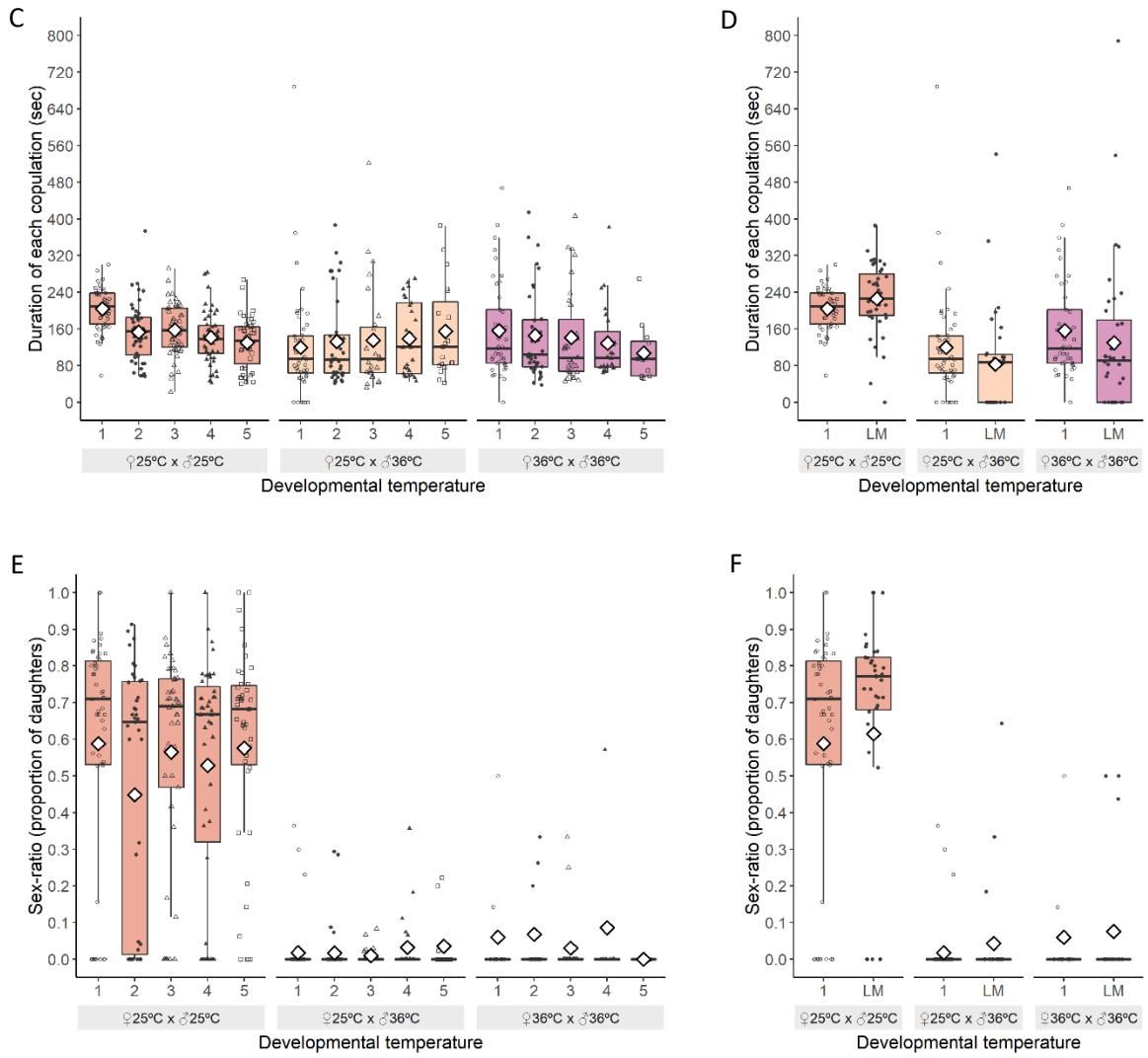

Figure 3.7 (continued)

Table 3.5 – Results obtained from *Effect of temperature on sperm depletion and recovery of male fertility* statistical analysis and *a posteriori* contrasts of the corresponding significant explanatory variables. A) "Df": the degrees of freedom. "χ^2": the Chi-square value obtained in each analysis; "F": the sum of squares obtained from the F-test. "Temperature of pair": developmental temperatures of the female and male crossed in each replicate; "♀ number": females ordered by their mating with the male, from 1st to 5th and Last Mating; "♂ identity": replicate number of each male. B) *A posteriori* contrasts with Bonferroni corrections were done to interpret the significant effect of the fixed factors. "T ratio": the T-test value obtained in each comparison. Comparisons: ♀Tº x ♂Tº - ♀Tº x ♂Tº (comparing different treatments, where the female and the male developed at either 25 or 36 ºC). Statistically significant terms are represented in bold.

A)

Var. of interest	Explanatory var.	Df (Df residuals)	χ^2/F	P-value
Latency to 5 copulations	**Temperature of pair**	**2 (18.547)**	**269.3**	**<0.001**
	♀ number	**1 (18.260)**	**132.6**	**<0.001**

Var. of interest	Explanatory var.	Df (Df residuals)	χ^2/F	P-value
Latency to 5 copulations	♂ identity	47 (0.891)	303.9	0.680
	Temperature of pair * ♀ number	**2 (5.945)**	**86.3**	**0.003**
Latency to 1st and last copulation	**Temperature of pair**	**2 (83.364)**	**47.962**	**<0.001**
	♀ number	**1 (43.115)**	**12.403**	**<0.001**
	♂ identity	47 (0.959)	12.961	0.554
	Temperature of pair * ♀ number	2 (2.403)	1.363	0.093
Duration of 5 copulations	**Temperature of pair**	**2 (20.441)**	**1260.4**	**<0.001**
	♀ number	**1 (18.064)**	**556.9**	**<0.001**
	♂ identity	47 (1.232)	1785.1	0.147
	Temperature of pair * ♀ number	**2 (10.098)**	**622.7**	**<0.001**
Duration of 1st and last copulation	**Temperature of pair**	**2 (4.837)**	**972.7**	**0.009**
	♀ number	1 (0.025)	2.5	0.875
	♂ identity	47 (1.283)	6061.7	0.125
	Temperature of pair * ♀ number	**2 (3.428)**	**689.3**	**0.034**
Sex ratio of 5 copulations	**Temperature of pair**	**2**	**109.263**	**<0.001**
	♀ number	**1**	**4.075**	**0.044**
	♂ identity	47	58.965	0.113
	Temperature of pair * ♀ number	**2**	**6.066**	**0.048**
Sex ratio of 1st and last copulation	**Temperature of pair**	**2**	**141.249**	**<0.001**
	♀ number	1	0.521	0.471

Var. of interest	Explanatory var.	Df (Df residuals)	χ^2/F	P-value
Sex ratio of 1st and last copulation	♂ identity	47	60.910	0.084
	Temperature of pair * ♀ number	2	2.438	0.296

B)

Var. of interest	Comparison	T ratio	P-value
Latency to 1st and last copulation	**♀25 x ♂25 – ♀25 x ♂36**	**-12.694**	**<0.001**
	♀25 x ♂25 – ♀36 x ♂36	**-7.993**	**<0.001**
	♀25 x ♂36 – ♀36 x ♂36	**4.228**	**<0.001**
Sex ratio of 1st and last copulation	**♀25 x ♂25 – ♀25 x ♂36**	**10.412**	**<0.001**
	♀25 x ♂25 – ♀36 x ♂36	**6.851**	**<0.001**
	♀25 x ♂36 – ♀36 x ♂36	-2.149	0.101

Discussion

This project aimed at testing the impact of a high sub-lethal temperature on several reproductive aspects of the spider mite *Tetranychus urticae*. We showed that at a temperature where both male and female fertility are negatively affected, the resistance status of the individuals regarding the pesticide Etoxazole can further hinder male fertility. Moreover, thermally stressed males did not show a recovery of fertility across five consecutive matings or after two days at an optimal temperature. However, the pattern of sperm precedence was disrupted at this temperature, enabling subsequent males to sire offspring. In this way, the proportion of fertilized (i.e., female) offspring produced by females first mated with sterile males, can be rescued to values similar to that of females mated with fertile males. Also, matings involving males developed at high temperatures took longer to occur and were shorter than matings with fertile males. Females involved in those mating were also more eager to remate once mated with thermally stressed males.

Exposing individuals to high sublethal temperatures during their development resulted in changes throughout the three life-history traits tested. Developmental time shortened with high temperatures, which confirms earlier studies done in *Tetranychus* (Zou *et al*., 2018; Farazmand, 2020). Moreover, offspring survival and female fecundity increased from 25 to 33 ºC, decreasing afterwards with rising temperatures. This result differs from that of Kasap (2004), which shows a monotonical decrease in survival rate with rising temperatures. However, the increase in female fertility seen in our results at 33 ºC can explain why offspring survival at that temperature surpasses survival at 25 ºC, as we tested survival in absolute values while Kasap (2004) tested it as a proportion. The effect of temperature on fertility has already been seen in earlier studies regarding *T. urticae*, despite the majority of studies only testing temperatures as high as 35 ºC (Kasap, 2004; Praslička & Huszár, 2004). The same is seen in other species (Zhang *et al*., 2013; Zhao *et al*., 2014; Zheng *et al*., 2017). However, our results do not indicate that female fertility is affected at lower temperatures than survival, as seen in several other studies (Walsh *et al.*, 2019; Ma *et al.*, 2020).

At 36 ºC, male fertility (i.e. the proportion of daughters produced) was also negatively affected, as expected, since earlier studies in other species show that male fertility is often reduced at lower temperatures than female fertility (David *et al*., 2005; Sales *et al*., 2018; Iossa, 2019). Moreover, our results show a decrease in the proportion of daughters when females developed at that temperature, independently of the developmental temperature of the male, which suggests that females cannot store all sperm transferred, hence fewer eggs are fertilized as compared to when females developed at 25 ºC. Another hypothesis is that mothers produce fewer fertilized eggs due to reduced resource investment in producing eggs of large size. Indeed, female eggs are larger than male eggs and egg size determines fertilization probability, with females producing larger eggs having a higher proportion of daughters (Macke *et al*. 2011). Thus, if females produce smaller eggs as a consequence of being exposed to high temperatures, they will have less fertilized offspring.

Addressing the effects of high temperatures on spider mites does not usually include male fertility tests (Farazmand, 2020; Praslička & Huszár, 2004; Riahi *et al*., 2013). Moreover, studies addressing recovery of male fertility after exposure to high temperatures in the *Tetranychus* genus are lacking. Our results show that thermally stressed males did not recover their fertility, either throughout five matings or after two days at an optimal temperature. Several studies on other species have shown that the recovery time of male fertility is proportional to the sterilizing temperature (Chakir *et al*., 2002; Araripe *et al*., 2004; Vollmer *et al.*, 2004), and that this sterility is caused by perturbations at spermatogenesis (Rohmer *et al.*, 2004). Results from *Drosophila buzzatii* also indicate that the proportion of permanent sterility in

heat-induced males increases with the sterilizing temperature (Jørgensen *et al.*, 2006). From our results, we cannot conclude whether male sterility is permanent or temporary. Still, males clearly cannot recover fertility after a few matings or two days after being exposed to heat stress. Given that *T. urticae* has a short life span, such a period of sterility may be sufficient to significantly affect male reproductive success. However, testing this would involve recording male fertility throughout its entire adult life. In contrast, we show that males developed at 25 ºC inseminated all females during five consecutive matings after being placed at an optimal temperature, and in one mating two days later. This is consistent with results from Krainacker & Carey (1989), where males from this species can inseminate on average 15 females before becoming sperm depleted.

Although both resistant and susceptible populations to the pesticide Etoxazole had increased developmental time and decreased survival and fertility when individuals developed at 36 ºC vs 25 ºC, male fertility loss was more pronounced in the former. Costs of pesticide resistance are known to increase at high temperatures, affecting several traits. Resistance costs were seen for instance in the diamondback moth, *Plutella xylostella*, where resistance led to lower larval survival, longer developmental time and a lower fertility curve at high temperatures (Li *et al*., 2007), or in the brown planthopper, *Nilaparvata lugens*, where rising temperatures resulted in lower copulation rate and fecundity of resistant individuals (Yang *et al*., 2018). The negative effects of pesticide resistance on the fertility of male spider mites at high temperatures should decrease the selective advantage of pest resistance and thus be an important factor for crop management and control. However, pest resistance should evolve within shorter periods at high temperatures, with the decrease in developmental time, which should interfere with crop pest management.

This species presents a nearly complete pattern of first-male sperm precedence (Helle, 1967; Rodrigues *et al*., 2020). However, there are no studies on *T. urticae* addressing the effect of high temperatures on this pattern. The expected pattern was observed in our results of crosses involving males and females developed at 25 ºC, with the mean contribution of subsequent matings to the paternity of the offspring not surpassing ca. 10%, despite increasing with the mating history of the female. However, whenever at least one of the individuals developed at 36 ºC, the paternity share of subsequent mates increased significantly, with the mean proportion of daughters ranging from ca. 27% (MM females in cross ♀36 x ♂25) to 75% (MM females in cross ♀25 x ♂36). In crosses involving females that mated first with a male developed at 36 ºC then with a male developed at 25 ºC, the contribution of the second male may be explained by sperm depletion or incomplete sperm transfer by the first male. Evidence for sperm transfer being compromised due to high temperatures has been reported in other species (Nguyen *et al*., 2013; Vasudeva *et al*., 2014; Sales *et al*., 2018). In earlier studies, second matings were successful when the sperm supply from the first mating was insufficient for the female to fertilize the offspring due to sexual exhaustion or interrupted mating (Helle, 1967; Morita *et al*., 2020). These results further support our hypothesis that incomplete sperm transfer occurred in the first mating. Moreover, females developed at 25 ºC that first mated with a male developed at 36 ºC had a higher proportion of offspring fertilized by the subsequent matings when they mated with several males, as compared to females that only mated with two males. This suggests that further sperm transfer continues to be stored by the female with subsequent matings. The sperm precedence pattern was also disrupted when only the female developed at 36 ºC, which suggests that the sperm transferred to these females by the first fertile male was not fully stored. In this case, subsequent males may contribute to the paternity of the offspring. Overall, these results indicate that the disruption of the pattern of sperm precedence can occur if either the male or the female is exposed to high temperatures.

Results regarding the proportion of daughters of single vs multiple mated females from the experiment on the effect of temperature on mating behavior further support the disruption of sperm precedence previously discussed. Females that mated with a single male developed at 36 ºC produced fewer

daughters than females that mated with a male developed at 25 ºC. This result further confirms the reduction of male fertility observed in our previous experiment. Moreover, when these females remated with several males developed at 25 ºC, the proportion of daughters significantly increased, which suggests, in accordance with our results from the sperm precedence experiment, that the sperm transferred by the subsequent matings was used to fertilize the offspring. Similar results were also observed in other species (Sutter *et al*., 2019; Vasudeva *et al*., 2021). In contrast, females that mated consistently with males developed at 25 ºC did not change their proportion of fertilized offspring by multiple mating. The number of adult offspring was not affected by the condition of the male involved in the first mating or by the female mating history, which indicates that female fertility was not hindered by multiple mating. Results from Rodrigues *et al*. (2020) show that female fertility can be affected by multiple mating when these matings occur 24h after the first copula. However, when there was no interval between the first and the following matings, no effect was observed, as confirmed by our results.

As sublethal temperatures affect the pattern of sperm precedence, which often influences the mating behavior of the species, we tested if this behavior would also be affected, as studies addressing this topic on *T. urticae* are lacking. Regarding the first mating, our results showed that latency to copulation increased while mating duration decreased in females mated with thermally stressed males, when compared to mating with males developed at 25 ºC. This suggests that females can identify and resist mating with low-fertility males. Another non-mutually exclusive explanation is that exposure to developmental high temperatures affects male mobility, or their perception of the presence of a female. The mating duration seen when the first mating involved males developed at 25 ºC is in agreement with earlier studies (Overmeer, 1972; Oku, 2014). Moreover, Satoh *et al*. (2001) show that copulations lasting more than 40 seconds ensure fertilization of the offspring. As mating duration involving thermally stressed males were longer than that, this may not fully explain the reduction of male fertility observed in these males.

In *T. urticae*, mating behavior is generally in agreement with patterns observed in species with first-male sperm precedence. Indeed, females are expected to seek only one mating, because multiple mating is usually costly (Macke *et al*., 2012; Rodrigues *et al*., 2020). Thus, remating in this species should take longer to occur and be shorter than the first mating of the female, as seen in Clemente *et al*. (2016), which we also observed. However, females that mated first with thermally stressed males mated faster and showed an increase in mating duration when remating with males developed at 25 ºC, compared to females that mated first with fertile males. This suggests that the proportion of offspring that the first male can fertilize affects the female eagerness to remate. This eagerness could be influenced by the quality or amount of sperm transferred to the female, or other physical or physiological male conditions that suggests low fertility. Sutter *et al.* (2019) presents similar results in a mostly monandrous fly, *Drosophila pseudoobscura*, where female remating rate increased with reduced reproductive output from the first mating, which indicates that females make dynamic remating decisions according to their current sperm storage threshold. The authors also observed that matings with heat-exposed males had longer latency and shorter duration than matings with control males, which is also in agreement with our results. When mating only with males developed at 25 ºC, females developed at 36 ºC remated for a longer period than females developed at 25 ºC. A reduction in the female ability to resist remating due to effects of thermal stress may explain this behavior. Another hypothesis is that these females benefit from remating since they did not fully store the sperm from the first mating, as discussed above.

In conclusion, the fertility loss observed at high temperatures can make *Tetranychus urticae* severely vulnerable to climate change. In particular, the reduction of male fertility in natural populations can lead to a male-biased sex ratio, which will impact the stability and persistence of these populations. However, our results show that the virtually complete pattern of first-male sperm precedence seen in *T. urticae* can be disrupted when individuals are exposed to developmental thermal stress. This may contribute to

population maintenance in the face of climate change, as it allows restoring the production of fertilized offspring, which has been compromised by earlier matings with partially infertile individuals. Still, this relies on females encountering males with normal sperm transfer patterns, which may not be guaranteed under natural conditions.

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Supplementary Material

Table S1 – Description of the statistical models used for data analysis in *Effect of temperature on life-history traits*. "Sample size": total number of replicates 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. A) Square brackets indicate the error structure used ("g": gaussian). "Offspring temperature": the temperature where the female laid eggs and the offspring developed; "Fecundity": the difference between the number of eggs laid at a high temperature and the mean of the number of eggs laid at the control of that high temperature. All analyses include replicates in which fecundity was above zero. Replicates with damaged mothers were excluded from the analysis. Due to the lack of replicates, 37 ºC was not considered in the statistical analysis. B) Square brackets indicate the error structure used ("g": gaussian). "Temperature of pair": developmental temperatures of the female and male crossed in each replicate; "Number of adult offspring": the difference between the number of adult offspring of each replicate from a treatment with a high temperature (33, 35, 36 or 37 ºC) and the mean of the number of adult offspring from the control of that high temperature. Only replicates in which fecundity was above zero were included. Replicates with individuals forming the mating pairs that were damaged were excluded from the analysis.

A)

Var. of interest	Response variable	Sample size	Maximal model	Minimal model	R subroutine [err struct.]
Number of adult offspring	Offspring at high Tº - mean (offspring at 25 ºC)	82	Offspring temperature + Fecundity	Offspring temperature + Fecundity	lm [g]
Days until adulthood (♀)	Day of 1st adult ♀ at high Tº - mean (day of 1st adult ♀ at 25 ºC)	67	Offspring temperature	Offspring temperature	lm [g]
Days until adulthood (♂)	Day of 1st adult ♂ at high Tº - mean (day of 1st adult ♂ at 25 ºC)	65	Offspring temperature	Offspring temperature	lm [g]

B)

Var. of interest	Response variable	Sample size	Maximal model	Minimal model	R subroutine [err struct.]
Number of adult offspring	Offspring (treatments) - mean (offspring; control)	262	Temperature of pair	Temperature of pair	lm [g]

Table S2 – Description of the statistical models used for data analysis in *Interaction between pesticide resistance and response to temperature*. "Sample size": total number of replicates 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. A) Square brackets indicate the error structure used ("p": Poisson). "Offspring temperature": the temperature where the female laid eggs and the offspring developed; "Resistance status": if the individuals tested came from the population resistant or susceptible to pesticide. All analyses include replicates in which fecundity was above zero. Replicates with damaged mothers were excluded from the analysis. B) Square brackets indicate the error structure used ("bbI": beta-binomial, accounting for zero inflation; "qp": quasi-Poisson). "♂": number of sons; "♀": number of daughters. "♀ temperature": developmental temperatures of the females used; "♂ temperature": the developmental temperatures of the males used; "Resistance status": if the individuals tested came from the population resistant or susceptible to pesticide; "Age of male": the day in which each male was isolated; "Age of female": the day in which each female was isolated. All analyses include replicates in which fecundity was above zero. Replicates with individuals forming the mating pairs that were damaged were excluded from the analysis.

A)

Var. of interest	Response variable	Sample size	Maximal model	Minimal model	R subroutine [err struct.]
Number of adult offspring	Number of adult offspring	117	Offspring temperature * Resistance status + Fecundity	Offspring temperature + Fecundity	glm [p]
Days until adulthood (♀)	Day of first adult female	99	Offspring temperature * Resistance status	Offspring temperature	glm [p]
Days until adulthood (♂)	Day of first adult male	96	Offspring temperature * Resistance status	Offspring temperature	glm [p]

B)

Var. of interest	Response variable	Sample size	Maximal model	Minimal model	R subroutine [err struct.]
Number of adult offspring	Number of adult offspring	233	♀ temperature * ♂ temperature * Resistance status + (1| Age of male) + (1| Age of female)	♀ temperature + ♂ temperature * Resistance status	glmmTMB [qp]
Sex ratio	cbind (♀, ♂)	233	♀ temperature * ♂ temperature * Resistance status + (1| Age of male) + (1| Age of female)	♀ temperature + ♂ temperature * Resistance status	glmmTMB [bbI]

Table S3 – Description of the statistical models used for data analysis in *Effect of temperature on the pattern of sperm precedence*. "Sample size": total number of replicates 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 (round brackets indicate that the variable was included as a random factor). Square brackets indicate the error structure used ("b": binomial). "♀": number of daughters. "Temperature of pair": developmental temperatures of the female and male crossed in each replicate ; "♀ mating history": according to the number of matings the female was involved in; "Block": the group in which each experimental unit was placed; "Room temperature": the room temperature at which observations were made; "Room humidity": the room humidity at which observations were made. This analysis only includes replicates in which fecundity was above zero. Replicates with individuals forming the mating pairs that were damaged were excluded from the analysis.

Var. of interest	Response variable	Sample size	Maximal model	Minimal model	R subroutine [err struct.]
Proportion of daughters	cbind (♀, unhatched eggs)	426	Temperature of pair * ♀ mating history + (1|Block) + (1|Room temperature) + (1|Room humidity)	Temperature of pair * ♀ mating history + (1|Block)	glmer [b]

Table S4 – Description of the statistical models used for data analysis in *Effect of temperature on mating behavior*. "Sample size": total number of replicates 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 (round brackets indicate that the variable was included as a random factor). Square brackets indicate the error structure used ("qp": quasi-Poisson; "bbI": beta-binomial, accounting for zero inflation; "g": gaussian). "♂": number of sons; "♀": number of daughters. "♀ temperature": developmental temperature of each female used; "♂ temperature": developmental temperature of the first male; "Mating rate": number of times each MM female mated; "♀ mating history": according to the number of matings the female was involved in; "Block": the group in which each experimental unit was placed; "Room temperature": the room temperature at which observations were made; "Room humidity": the room humidity at which observations were made. [a] only includes replicates in which females were multiply mated (mating rate > 1); [b] only includes replicates in which females had the chance to multiply mate (mating rate > 0; ♀ mating history = MM); [c] includes single and multiple mated females (females that were not successful in multiple mating were excluded); [d] is similar to [c] and in which fecundity was above zero. No replicates with damaged individuals were used on the analysis.

Var. of interest	Response variable	Sample size	Maximal model	Minimal model	R subroutine [err struct.]
Latency to mate	Latency to 1st mating	361	♀ temperature * ♂ temperature + (1|Block) + (1|Room temperature) + (1|Room humidity)	♀ temperature * ♂ temperature + (1|Block)	lmer [g]
Duration of first copulation	Duration of 1st mating	361	♀ temperature * ♂ temperature + (1|Block) + (1|Room temperature) + (1|Room humidity)	♂ temperature + (1|Block)	lmer [g]
Latency to remate	Latency to 2nd mating	133 [a]	♀ temperature * ♂ temperature + Mating rate + (1|Block) + (1|Room temperature) + (1|Room humidity)	♂ temperature + Mating rate + (1|Block)	lmer [g]
Duration of second copulation	Duration of 2nd mating	133 [a]	♀ temperature * ♂ temperature + Mating rate + (1|Block) + (1|Room temperature) + (1|Room humidity)	♀ temperature * ♂ temperature + (1|Block)	lmer [g]
Mating rate	Mating rate	137 [b]	♀ temperature * ♂ temperature + (1|Block) + (1|Room temperature) + (1|Room humidity)	(1|Block)	glmmTMB(qp)
Number of adult offspring	Number of adult offspring	204 [d]	♀ temperature * ♂ temperature * ♀ mating history + (1|Block) + (1|Room temperature) + (1|Room humidity)	♀ temperature + (1|Block)	glmmTMB(qp)
Sex ratio	cbind (♀, ♂)	204 [d]	♀ temperature * ♂ temperature * ♀ mating history + (1|Block) + (1|Room temperature) + (1|Room humidity)	♀ temperature + ♂ temperature * ♀ mating history + (1|Block)	glmmTMB(bbI)

Table S5 – Description of the statistical models used for data analysis in *Effect of temperature on sperm depletion and recovery of male fertility*. "Sample size": total number of replicates 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 (round brackets indicate that the variable was included as a random factor). Square brackets indicate the error structure used ("g": gaussian; "bbI": beta-binomial, accounting for zero inflation; "bb": beta-binomial). "♂": number of sons; "♀": number of daughters. "Temperature of pair": developmental temperatures of the female and male crossed in each replicate; "♀ number": females ordered by their mating with the male, from 1st to 5th and Last Mating; "♂ identity": replicate number of each male; "Block": the group in which each experimental unit was placed; "Room temperature": the room temperature at which observations were made; "Room humidity": the room humidity at which observations were made. [a] only includes data from the five consecutive copulations; [b] only includes data from the first and last copulation (occurred 2 days after; Last Mating); [c] only includes data from females of the five consecutive copulations which fecundity was above zero; [d] only includes data from females of the first and last copulation (occurred 2 days after) which fecundity was above zero. No damaged females were included on the analysis.

Var. of interest	Response variable	Sample size	Maximal model	Minimal model	R subroutine [err struct.]
Latency to 5 copulations	Latency to each mating	713 [a]	Temperature of pair * ♀ number + ♂ identity + (1|Block) + (1|Room temperature) + (1|Room humidity)	Temperature of pair * ♀ number + ♂ identity	lm [g]
Latency to 1st and last copulation	Latency to each mating	288 [b]	Temperature of pair * ♀ number + ♂ identity + (1|Block) + (1|Room temperature) + (1|Room humidity)	Temperature of pair + ♀ number + ♂ identity	lm [g]
Duration of 5 copulations	Duration of each mating copulation	713 [a]	Temperature of pair * ♀ number + ♂ identity + (1|Block) + (1|Room temperature) + (1|Room humidity)	Temperature of pair * ♀ number + ♂ identity	lm [g]
Duration of 1st and last copulation	Duration of each mating copulation	288 [b]	Temperature of pair * ♀ number + ♂ identity + (1|Block) + (1|Room temperature) + (1|Room humidity)	Temperature of pair * ♀ number + ♂ identity	lm [g]
Sex ratio of 5 copulations	cbind (♀, ♂)	432 [c]	Temperature of pair * ♀ number + ♂ identity + (1|Block) + (1|Room temperature) + (1|Room humidity)	Temperature of pair * ♀ number + ♂ identity	glmmTMB (bbI)
Sex ratio of 1st and last copulation	cbind (♀, ♂)	172 [d]	Temperature of pair * ♀ number + ♂ identity + (1|Block) + (1|Room temperature) + (1|Room humidity)	Temperature of pair + ♂ identity	glmmTMB (bb)