# Recovery from heat-induced infertility – a study of reproductive tissue responses and fitness consequences in male Drosophila melanogaster

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The predicted temperature increase caused by climate change is a threat to biodiversity. Male reproduction is particularly sensitive to elevated temperatures resulting in sterility. Here we investigate temperature induced changes in reproductive tissues and the fertility reduction in male Drosophila melanogaster. We challenged males during development and either allowed them to recover or not in early adulthood, while measuring several determinants of male reproductive success. We found significant differences in recovery rate, organ sizes, sperm production and other key reproductive traits among males from our different temperature treatments. Spermatogenesis and hence sperm maturation was impaired before reaching the upper thermal sterility threshold. While some effects were reversible, this did not compensate the earlier damage imposed. Surprisingly, developmental heat stress was damaging to accessory gland growth and female post mating responses mediated by seminal fluid proteins were impaired regardless of the possibility of recovery. We suggest that sub-lethal thermal sterility and the subsequent fertility reduction is caused by a combination of malfunctioning reproductive traits: inefficient functionality of the accessory gland and alteration of spermatogenesis.

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**Abstract**

The predicted temperature increase caused by climate change is a threat to biodiversity. Male reproduction is particularly sensitive to elevated temperatures resulting in sterility. Here we investigate temperature induced changes in reproductive tissues and the fertility reduction in male *Drosophila melanogaster*. We challenged males during development and either allowed them to recover or not in early adulthood, while measuring several determinants of male reproductive success. We found significant differences in recovery rate, organ sizes, sperm production and other key reproductive traits among males from our different temperature treatments. Spermatogenesis and hence sperm maturation was impaired before reaching the upper thermal sterility threshold. While some effects were reversible, this did not compensate the earlier damage imposed. Surprisingly, developmental heat stress was damaging to accessory gland growth and female post mating responses mediated by seminal fluid proteins were impaired regardless of the possibility of recovery. We suggest that sub-lethal thermal sterility and the subsequent fertility reduction is caused by a combination of malfunctioning reproductive traits: inefficient functionality of the accessory gland and alteration of spermatogenesis.

Temperature is a critical abiotic factor for many organisms and can turn into an environmental stressor, particularly for ectotherms. Elevated temperatures are known to affect an ectotherms physiology, behavior and, on a broader scale their overall performance (Huey and Stevenson, 1979). When critical thermal limits are exceeded, both viability and reproductive potential are harmed. Within the last years, most research assessing fitness loss under increasing temperatures have incorporated mainly measures that evaluate an organism physiological failure (viability thresholds), like death or heat coma (Walsh et al., 2019). However, often the fertility range is narrower than the viability range, such that sub-lethal temperatures already pose an important fitness loss, impeding organisms to reproduce, threatening population stability and persistence (Kellermann et al., 2012; van Heerwaarden and Sgrò, 2021; Walsh et al., 2019). Given the strong ecological implications that sterility and fertility loss exert on organisms, study the capacity of species and the mechanisms to respond ecologically and evolutionarily to the challenges of increasing temperatures has become a research priority, especially within the climate change context (Parmesan 2006). Hence, understanding the reproductive and resulting fitness consequences of exposure to elevated temperatures is key.

Sterility is one consequence when reaching thermal fertility limits at both the upper and lower ends. Most of the literature refers to males, as these have repeatedly been found to be more temperature sensitive than females (e.g. Sales et al., 2018; Zwoinska et al., 2020). However, there are also examples of female sterility due to high temperatures (e.g. in the Nile tilapia, *Oreochromis niloticus* (Byerly et al., 2005)). Based on the premise that spermatogenesis is more thermosensitive than oogenesis, the study of male fertility thresholds is of special interest, not only to understand the damage imposed on male fertility, but also to gauge the consequences for reproductive capacity and thus, fitness (Parratt et al., 2021). Even though this phenomenon has been documented for a range of taxa like insects (e.g. several Drosophila species such as *D. melanogaster* and *D. simulans* (Chakir et al., 2002)), fishes (e.g. Nile tilapia, *O. niloticus* (Byerly et al., 2005) and channel catfish, *Ictalurus punctatus* (Strüssmann et al., 1998)), reptiles (e.g. yucca night lizard, *Xantusia vigilis* (Cowles and Burleson, 1945)) and some vertebrates (e.g. Arbor Acres roosters, *Gallus gallus* (McDaniel et al., 1996), zebra finch, *Taeniopygia guttata* (Hurley et al., 2018) and rams, *Ovis aries* (Hafez, 1964)), the mechanisms underlying sterility at extreme temperatures are still unknown. The fact though that some species can recover fertility after being transferred to milder temperatures (e.g. *D. melanogaster* males (Chakir et al., 2002)) indicates that the destruction of germ cells might not explain the observed temperature induced infertility.

Previous research on *D. melanogaster* (Rohmer et al., 2004) has shown that elevated temperatures disrupt spermatogenesis causing cytological abnormalities. As a result, males have shorter cysts, show abnormalities in the shape and position of sperm nuclei, an impairment of spermatid elongation and an increase in spermatid death rate.*D. simulans* instead had shorter cysts at higher temperatures (David et al., 2005) indicating phenotypic plasticity, with temperature dependent plasticity in sperm length being adaptive in *Tribolium castaneum* (Vasudeva et al., 2019). Despite these first studies into spermatogenesis dynamics under thermal stress, little is yet known about recovery dynamics on sperm production. Furthermore, effects on the second reproductive tissue, the male accessory glands (AGs), have not been considered. The importance of the AGs

for male reproductive success has been widely studied (Chen, 1984; Gillott, 2003; Wolfner, 1997). Seminal fluid proteins (SFPs), secreted mainly by the AGs, are transferred together with sperm to the female during mating causing changes in female postmating responses (e.g. behavior and physiology (Chapman, 2001; Chen et al., 1988)). In addition, SFPs affect male sperm competitive ability, and modulate sperm storage dynamics inside the female's sperm storage organs (Avila et al., 2011) together ensuring fertility. Moreover, SFPs might have protective functions as in honeybees, *Apis mellifera* (den Boer et al., 2009) and leaf-cutter ants, *Atta colombica* (den Boer et al., 2007), where SFPs increase sperm viability. Hence, whether temperature damages either or both tissues, needs to be considered to understand the mechanisms of temperature induced sterility.

With the predicted temperature increase (at least 1.5 - 2°C for 2081-2100 (Collins et al., 2013)) and the occurrence of longer and more severe heat waves (Meehl and Tebaldi, 2004) due to global climate change, we are convinced that studying the responses of reproductive traits to stressful thermal conditions is of special interest in order to determine species persistence under possible new environmental conditions (Hoffmann, 2010; Huey and Kingsolver, 1993; Huey and Stevenson, 1979; Kellermann et al., 2012; Sinclair et al., 2016; van Heerwaarden and Sgrò, 2021; Walsh et al., 2019).

In this context, we assessed fitness loss and the ability to recover, focusing on the mechanistic basis of heat-induced sterility, of males exposed to sub-lethal developmental temperatures. Life stages undergoing fundamental changes might be particularly sensitive to environmental stressors (Lowe et al., 2021), as e.g. the pupal stage in the oriental fruit moth, *Grapholita molesta* (Zheng et al., 2017) or*Drosophila* larvae (Hoffmann et al., 2003). In addition, the lack of mobility of many species at both early and late developmental stages adds a challenge to elude thermal stress. Hence, we here exposed larvae to heat-stress and considered the resulting consequences on male fertility in early adulthood. To determine the causes of male temperature-induced sterility, we tested whether spermatogenesis is disrupted impairing mature sperm formation and secondly, measured whether a delay in AG maturation (Ruhmann et al., 2016) contributes to reduced reproductive success. With this extensive analysis of male reproductive traits, we suggest that impaired functionality of both reproductive tissues is causing temperature induced male sterility.

## Materials and methods

### Fly stocks and culturing

We used a *Drosophila melanogaster* wild type stock collected in Portugal by Prof. Élio Sucena in Azeitão, in 2007. It was established as an outbred population from 160 wild caught fertilized females with an ample degree of genetic variation within the population (Martins et al., 2014). Flies were cultured in our laboratory at standard conditions: 25°C and 60% humidity at a 12h light-dark cycle. Stocks were kept in glass bottles filled with 70mL of standard yeast-sugar (SYA) food (Bass et al., 2007). Once a week, three glass bottles with about 250 recently eclosed flies each were started, and we mixed flies across bottles regularly to maintain genetic diversity. We used a temperate population as we expect them to maintain higher phenotypic variation as they were initially adapted to exist within a broad thermal range compared with tropical populations (Hoffmann et al., 2003). Species from temperate areas are expected to maintain higher phenotypic plasticity even after adaptation to laboratory conditions and provide a more promising way to test thermal responses under a broader range of experimental temperatures and hence, give a more powerful estimation of the species ability to cope with increasing temperatures (Mathur and Schmidt, 2017). Although laboratory adaptation may alter some life-history traits, previous research has shown that some plastic responses are maintained (Trotta et al., 2006) and previous research in thermal responses still find ample variation (Parratt et al., 2021; Sales et al., 2018).

For paternity analysis in a sperm competition experiment we used flies bearing the stubble (*Sb* ) mutation as a tractable phenotypic marker. The *Sb* gene was back-crossed multiple times into the wild-type Dahomey genetic background. *Sb* is a dominant mutation that causes a short, thick bristle phenotype (Lees et al., 1945) that is visible by eye and can be easily distinguished from the wild type bristle structure. As the recessive phenotype is lethal, we used males heterozygous for this mutation in the subsequent sperm competition assay. The stock was kept under the same standard conditions as described above.

Throughout all assays, in order to obtain experimental flies, we allowed parental flies to mate for 24h and oviposit on grape-juice-agar plates [50 g agar, 600 mL red grape juice, 42.5 mL Nipagin (10% w/v solution) and 1.1 L water] with a semi-liquid baker's yeast paste distributed all around the plate to promote egg laying. We incubated plates for 24 hours and collected first instar larvae at a density of 100 larvae per vial containing 7mL of SYA food. For all experiments, flies were collected within 8 hours after eclosion as virgins on ice. Adult flies were kept in separate sex groups 20 per vial. Throughout the experiments, females were grown at 25°C, while males were exposed to different temperature treatments during development. We exposed males to two challenging temperatures, one moderate (29°C) and one severe (31°C) challenge, the latter near to the lethal threshold for *D. melanogaster* of 32°C (Petavy et al., 2001). We first tested how developmental temperature affects male fertility and whether males can recover fertility when placed at a benign temperature after eclosion. Control males were raised at the standard temperature of 25°C. We observed changes from day one to six after eclosion. As we were interested in the fitness consequences of the recovery process and the underlying mechanisms, we allowed half of the males to recover from the heat stress (denoted with an R) after eclosion, while keeping the other half at the stressful temperature. All assays described below were done under these conditions. In order to maintain temperatures precisely for our different treatments, incubators with ±0.5°C accuracy were used (INCU-Line® IL 10). Accuracy was monitored by placing a temperature logger (NOVUS®; accuracy of ±0.5°C) in each incubator throughout the course of the experiments to record temperature.

Fecundity experiment

We measured egg lying rate, fertility, fecundity, and egg to adult survival of females mated with a temperature challenged male that was either allowed to recover or not. Mating assays took place on three different days: two, four and six days after adult male eclosion to assess changes in fertility during sexual maturation. Males were paired with five-day old virgin females in all cases. New virgin males were used on each mating day and 40 pairs per treatment and day were initially set up. Pairs were allowed to mate for seven hours on day two and four, and for five hours on day six. The mating was observed, and the duration was adjusted each day in order to have an appropriate sample size for all treatments (see Supplementary table S1 for the full sample sizes). At the end of the interaction time, males were discarded. Mated females were kept at 25°C until the next day allowing them to lay eggs and were then discarded. We counted the number of eggs and the vacated vials were kept at standard conditions for 12 days allowing all the offspring to eclose. After that time, the number of offspring was counted, and we estimated the egg to adult survival. This assay was repeated independently following the same procedure and adding one mating day (one, two, four and six days after adult male eclosion). With that, we could get a better understanding of the effects of heat stress on fertility and fecundity in recently eclosed males.

Remating and sperm competition experiment

To further examine determinants of male reproductive success we assessed male post-mating competitive ability. We tested male sperm defense ability by first mating females to a temperature challenged focal male and subsequently to a Sb mutant competitor. In this assay, all males and females were five days post-eclosion when mated. Females and the Sb competitor males were grown at 25°C. For the first mating with the focal males, 50 (for the 25°C treatment) or 70 (for the remaining treatments) individual pairs were set up and given three and half hours to mate. We continuously observed pairs and noted the time when pairs were set together, started and ended mating to calculate mating latency and copulation duration. After a successful mating, males were discarded, and females were allowed to lay eggs for 48h. After this time, females were transferred to a new vial containing a virgin heterozygous Sb male. Pairs were allowed to mate for two hours (see Supplementary table S3 for the total number of mated pairs in both matings). We again recorded mating behavior as before and scored how many females remated. Females who remated were kept in the same vial for two days, allowing them to lay eggs, while Sb males were discarded. After these 48h we transferred females to new vials allowing them to lay eggs for two more days. We kept vacated vials from both the intermating interval and the four days after remating at standard conditions for 12 days allowing all the offspring to eclose. Vials were frozen and the offspring counted, whereby for the vials after remating we

determined paternity by separately counting offspring scored as presenting the Sb mutation versus the wild type phenotype. As Sb fathers were heterozygous for the mutation, we corrected the counts for paternity scores. Assuming half the offspring fathered by the Sb males present the Sb phenotype and the other half the wild type phenotype, we doubled the Sb counts and corrected the wildtype counts by subtracting the number of Sb offspring counted from the total wild type progeny number.

Maturation of the male reproductive system during recovery

In order to gain insights into the mechanistic basis of the recovery process we measured changes in male accessory gland (AG) size during the recovery process. We further checked for the presence of sperm in the seminal vesicles (SVs) and measured SV size and sperm viability to test the effects of elevated developmental temperatures on sperm production and quality. All observations were done under a microscope (ZEISS, AxioVision Software) with an Olympus SC50 5-megapixel color microscope camera; at a 50X amplification. Further measurements (measurements of the organ areas, wing length and sperm head counts) were carried out with ImageJ (Wayne Rasband).

Accessory gland size changes during recovery

Measurements were done on glands dissected from virgin males, as a previous mating and the accompanying transfer of seminal fluid proteins reduces AG size (Ruhmann et al., 2016). We dissected glands from males 20-24 hours, two, four and six days old and followed procedures as carried out in (Ruhmann et al., 2016). We also measured wing length as an indicator of male body size to control for the allometric relationship between both factors (Shingleton et al., 2007). We measured both AGs and wings for each male and the mean was used for the analysis. In total, the AGs and wings of 15-26 males per treatment and day were measured.

Seminal vesicle size and sperm presence

Measurements were done on two- and six-day old virgin males. After dissecting out the reproductive tract, we isolated both seminal vesicles (SVs). We placed the SVs on a slide in a drop of phosphate buffered saline (PBS) solution and a picture of both organs was taken under the microscope immediately after dissection to subsequently measure their area. Afterwards, we checked for the presence of sperm by puncturing the central area with a thin needle. We recorded and assigned a value of 1- when sperm was present or 0- when the sperm was absent for each SV and the mean size of both SVs was used for each male in the subsequent analysis. A total of 15-20 males per treatment and day were dissected.

Sperm viability

Measurements were done only in six-day old virgin males from the control and the 29°C recovery treatment, as especially young males and males from all the other treatments had a low number of sperm in the SV preventing reliable estimates. We dissected out the male reproductive tract, the SVs were isolated and punctured following the procedure above. We stained sperm with SYBR14 ® (1:50 in DMSO) and PI (LIVE/DEAD ® Sperm Viability Kit, ThermoFisher Scientific) following the protocol explained in (Eckel et al., 2017). The temporal decrease in sperm viability of the same male was measured at three different time points: just after the staining (t0), 15 (t15) and 30 (t30) minutes later. With this procedure we can assess sperm quality and future sperm performance (Eckel et al., 2017). The time between staining and taking the first image was approximately 1 min. Pictures were taken under fluorescence (see microscope specifications above). Sperm heads were counted by eye twice once by an observer blind and the second observer non-blind to the treatment codes. As the counts were not significantly different, the mean of both counts was used for the data analysis. Green sperm were considered alive while red and red-green double stained sperm were scored as dead. A total of 21 males per temperature treatment were used.

Data analysis

All tests were done using R (version 3.6.1) and RStudio (1.2.1335). Graphs were created with the ggplot2 package in all cases (Wickham, 2016). As the data was not normally distributed, generalized linear mixed

models (GLMM) were used with the appropriate error structure for analyzing the fecundity data and sperm viability. In the first case, as the data suffered from an excess of zero counts, we used zero-inflation models as advised by Zuur and colleagues (Zuur et al., 2009). We modelled both the likelihood of sterile replicates as well as the number of offspring in one model, combining both a binomial and a count part (with a negative binomial distribution) in one model. This needed packages pscl (Jackman, 2017), lmtest (Zeileis and Hothorn, 2002) and glmmTMB (Brooks et al., 2017). The model contained the following factors: temperature, day and day$^2$, as well as the interactions between temperature and day and temperature-day$^2$. For the sperm viability analysis, we accounted for pseudoreplication as the sperm from the same male was measured in three time points. In this case, time point, and temperature were included in the model as fixed factors.

Fertility in the fecundity assay, male organ size (wing length, AG and SV size), the egg-to-adult survival as well as the behavior in the sperm competition experiment were analyzed with generalized linear models (glm) with the appropriate error structure and correction for overdispersion using the quasi-extension if necessary. Significance of factors was tested through an analysis of deviance by subtracting a factor from the full model and tested with an $F$ - or Chi-square distribution as appropriate for the error structure (Crawley, 2007). We present models with only the retained significant factors. Most of the statistical analysis were done in two different ways: in the first case, all five treatments (developmental temperature and opportunity to recover or not) were considered separately by coding them as five different treatments. In the second approach we instead included larval temperature and recovery as different factors, but this precluded us from using data from control males, allowing comparisons only among heat-challenged males. As control males both remained at their developmental temperature and were 'allowed' to recover it was not possible to assign them to either level for the factor recovery and thus precluded us from coding this as two independent treatment factors with a full-factorial design. We report always the first approach unless the contrary is specified.

A Chi-square test was applied to analyze sperm presence in the SVs and the mating and remating rates in the sperm competition experiment (Dytham, 2011). Allometry between AG size and wing length was tested by using a regression. For that, both variables were converted into the same units ($\mu m^2$) and the data was transformed to a log scale for the analysis (Shingleton et al., 2007). Day was included as a fixed factor in the model to account for the ontogenetic allometry.

Package multcomp (Hothorn et al., 2008) was used for the post-hoc comparison of wing length. Pairwise comparisons using t tests were used for analyzing differences between temperature treatments in the AG size.

**Results**

Sub-lethal temperature effects on behaviour and male reproductive output

On the basis that males of temperate *Drosophila melanogaster*strains become sterile at 30°C (Chakir et al., 2002; Petavy et al., 2001), we investigated the recovery dynamics for males of a temperate Portuguese *D. melanogaster* strain (Martins et al., 2014) developed at mid (29°C) or sub-lethal (31°C) temperatures. First, we documented viability of larvae under the different developmental temperatures and found those to be clearly challenging as only 45.5% respective 23.7% of flies eclosed when exposed to 29°C or to 31°C, in contrast to 95% of control flies eclosed successfully. Remaining at 29 or 31°C was stressful to adults as well, as males clearly did not recover fertility in contrast to males allowed to recover (Fig. 1A, C and table 1).

We next assessed the fecundity of heat-challenged males and found females mated once to a heat-challenged male laid significantly fewer eggs compared to females mated to control males (Fig. 1B, table 1). Males kept at the challenging temperature after eclosion induced lower numbers of eggs laid throughout, while a small improvement was seen for males allowed to recover. We independently repeated this experiment with the addition of sampling also day 1 after eclosion and found the pattern to be robust (Supplementary Fig. S1A, B and Supplementary table S2).

The offspring counts revealed that recovering males were initially sterile and recovered at different rates (see Fig. 1C, interaction term table 1), with offspring numbers approaching those of control males on day 6.

This is matched by a steep increase in egg-to-adult survival between day 2 and 4 (see Fig. 1D), while males remaining at the challenging conditions produced very few offspring and had a low egg to adult survival (Fig. 1D, table 1). No adult flies were produced by males grown and kept at 31°C. Males grown and kept at 29°C produced on average 82% fewer offspring on day 6, with respect to control males, while those allowed to recover after eclosion had 36% fewer offspring. The slight increase for males recovering from development at 31°C still resulted in a mean reduction of 63% in offspring produced. Hence, at this temperature males suffer a severe fitness loss even if allowed to recover, while persistent heat stress results in near complete sterility even at the moderate challenge.

When considering the mating behavior for individual pairs, we overall found little effect of a moderate heat-challenge on mating success and only males experiencing a severe heat-challenge were negatively impaired. Males raised at 31°C gained few copulations compared to males from the other treatment groups ($\chi^2 = 66.135, df = 4$, $P < 0.0001$; Fig. 2A). Males raised at 31°C had longer mating latencies (GLM with gamma distribution:$Deviance = 7.582$, $F = 3.002$, $df = 4$, $P = 0.021$; Fig. 2B) while there was no effect on copulation duration (GLM with poisson distribution: $Deviance = 8.867$, $df = 4, P = 0.065$; Supplementary Fig. S2A).

In addition to single mating productivity, we also tested male sperm competitive ability after developmental heat-exposure. We document a severe negative impact of heat on male sperm defense ability (GLM with a quasibinomial distribution: $Deviance = 5173.8$, $F = 22.177, df = 4$, $P < 0.0001$, Fig 2D), even after we allowed males to recover for 5 days.

Additionally, heat-challenged males were unable to prevent female remating, regardless of the possibility for recovery, as all females remated when the second male was present, while only 35.1% of females first mated to a control male remated ($\chi^2 = 82.624$; $df = 4$, $P < 0.0001$). This pattern was also reflected in second mating latencies (GLM with quasibinomial error distribution; $Deviance = 73.482$, $F = 181.55$, $df = 1$, $P < 0.0001$; see Fig. 2C).

Temperature effects on male reproductive tissues and mechanisms of recovery

Sperm presence in the SV and SV size

We scored the presence of mature sperm in both seminal vesicles (SV) and measured SV size in two- and six-day-old males as proxies for the amount of mature sperm available to males. We found a major impact of elevated developmental temperatures and the opportunity to recover on the presence of mature sperm in the SV ($\chi^2 = 82.01, df = 4$, $P < 0.0001$; Fig. 3A). A six-day-recovery resulted in a significant increase in mature sperm in the SV for both temperature treatments while those males not allowed to recover did not improve even after six days.

The results we observed for sperm presence were paralleled by our measure of SV size (Fig. 3B, table 2). Overall, heat-challenged males started with on average 31% smaller SVs than control males, however, by day six differences in SV size were more noticeable. Males raised at 29°C recovered to have only 15% smaller SVs compared to control males, while the other treatments retained small SV sizes resulting in a reduction of 52% for males developed at 31°C independently of ability to recover and of 43% for males raised and kept at 29°C.

We also compared sperm viability in six-day old males from the control and the 29°C recovery treatment at three timepoints after releasing sperm from SVs (Fig. 3C). This procedure allows us to determine not only sperm quality but also future sperm performance (Eckel et al., 2017). We found that, although the number of dead and alive sperm was similar at the beginning of the experiment (t0), sperm viability decreased faster over time (t15 and t30 min) when males were prior exposed to mid-challenging temperatures (GLMM with a binomial error distribution: temperature: $\chi^2 = 16.33$, $df = 1, P < 0.0001$; time point: $\chi^2 = 24.22$, $df = 2$, $P < 0.0001$; temperature * time point: $\chi^2 = 9.71$, $df = 2$, $P = 0.008$).

Accessory gland size changes during recovery

We here tested how developmental temperature affected accessory gland (AG) growth in young males. As

developmental temperature affected male body size (GLM with a gamma error distribution: $Deviance = 1.075, df = 4$, $F = 228.33$, $P < 0.0001$) and this scales with organ size, we corrected for body size in further analyses using wing length as a proxy. Overall, males were smaller on day 6 after eclosion when they developed at higher temperatures (29: 1.25mm ± 0.007; 29R: 1.23mm ± 0.007; 31: 1.21m ± 0.011; 31R: 1.17mm ± 0.009) than control males (1.37mm ± 0.007, see Supplementary table S4).

We observed a clear reduction in AG growth during the first six days after eclosion in males exposed to elevated developmental temperatures (Fig. 4A, table 2). The reduction in growth was gradual: the higher the temperature, the slower the increase in AG size, resulting in significant differences on day 6. Surprisingly, the growth trajectories did not differ for males raised at 29°C and kept at the growth temperature and those allowed to recover (see post-hoc test in Supplementary table S5). Interestingly, AG sizes of recently eclosed males (day one) were fairly similar. In a second analysis we only compared AG size of treated males and hence, excluding the control group from the analysis, using larval temperature and the possibility of recovery or not as fixed variables. This revealed that the 31°C recovery treatment resulted in males having significantly smaller AGs compared to males not allowed to recover (GLM with a gamma error distribution, recovery: $F = 18.88$, $df = 1$, $P < 0.0001$). By day six, the reduction in size was 12.8% for males of both treatments exposed to 29°C and 40.3% for those exposed to 31°C compared with the control group.

We next assessed whether the allometric relationship between the gland and body size is also altered. The regression analysis between both factors (see Supplementary table S6 and Fig. S3) indicates that in the two elevated treatments a hypometrical relationship prevails ($b<1$) highlighting that the gland is smaller than expected for the body size, while it tends to be isometrical in males grown at 25°C ($b=1$). Thus, differences in AG size can be attributed to altered growth patterns due to temperature. Overall, these results suggest that AG maturation is inhibited by elevated temperatures with males ultimately having smaller glands.

**Discussion**

We here investigated the recovery dynamics of a temperate *D. melanogaster* strain with a special focus on effects on the male reproductive tissues. Overall, sublethal temperatures severely affected a male's ability to reproduce as found in other ectotherms (Conrad et al., 2017; Nguyen et al., 2013; Parratt et al., 2020; Rodrigues et al., 2022, 2021; Sales et al., 2018; Vasudeva et al., 2019; Walsh et al., 2019; Zheng et al., 2017; Zwoinska et al., 2020). In accordance with previous findings (Chakir et al., 2002; Petavy et al., 2001), the males used here became temporarily sterile when exposed to temperatures above 29°C during development. Moreover, we found negative effects on most of our measured reproductive traits that can be explained by males transferring a sub-optimal ejaculate and despite the ability to recover, we found temperature stress to still lead to severe fitness reductions with recovery dynamics depending on the developmental temperature experienced.

The observed reduction in output could not be explained by reduced male mating rates, i.e. due to males becoming unattractive as found in male red mason bees (*Osmia bicornis,* (Conrad et al., 2017)), as we found little effect of a moderate heat-challenge of four degrees over the optimal temperature on mating behaviour. We rather suspected male ability to produce or transfer sperm to be affected. Developmental temperature can result in aberrant sperm in *D. melanogaster*(Rohmer et al., 2004) potentially explaining our reduced egg-to-adult survival. Even if males can produce sperm, they might transfer less, reducing their overall fertility (Kraaijeveld and Chapman, 2004; Seo et al., 1990; Taylor et al., 2001). Females of the parasitoid wasp*Anisopteromalus calandrae* stored 100 time less sperm when mated with a male exposed to a heatwave and even though males were transferred to the optimum temperature after a heat shock, they were not able to produce mature sperm (Nguyen et al., 2013). Constant exposure to heat stress during development resulted in reduced testes and sperm size in the bruchid beetle *Callosobruchus maculatus* (Vasudeva et al., 2014). However, while *C. maculatus* males had a lower sperm viability, no reduction in fertility in the absence of sperm competition was observed. Hence, while sperm number is important, other factors might be at play as well.

In addition to single mating productivity, we also tested male sperm competitive ability after developmental

heat-exposure. Competitive ability is key to male reproductive success (Simmons, 2001) and was sensitive to thermal conditions in *T. castaneum* (Sales et al., 2018). We similarly document a severe negative impact of heat on male sperm defense ability, even after we allowed males to recover for 5 days. Thus, overall we also observe the previously described sensitivity of male reproductive function to elevated, but sub-lethal temperatures (Chakir et al., 2002; David et al., 2005; Sales et al., 2018; Walsh et al., 2019) in both competitive and non-competitive contexts. Reduced ability to fertilise eggs and win in sperm competition could be due to reduced sperm transfer (Kraaijeveld and Chapman, 2004; Seo et al., 1990; Taylor et al., 2001) and/ or reduced sperm storage by females (Nguyen et al., 2013). Both traits are important determinants in *D .melanogaster* sperm competition outcomes (Lüpold et al., 2013; Manier et al., 2010). Sperm storage and sperm competitive ability are mediated by receipt of SFPs (Avila et al., 2011). Thus, we continued by looking at the SVs and the AGs for possible heat damage and we will discuss those two in turn.

We observed males to recover fertility to some extent within six days, indicating that spermatogenesis was not completely damaged. Spermatogenesis in *D. melanogaster* lasts 10 days from the initial stem cell division (reviewed in (Fabian and Brill, 2012)) and starts in the early larval stages (Le Bras and Van Doren, 2006) with the reproductive system fully active during the pupal stage (Bodenstein, 1950) when most of sperm individualisation and maturation occurs (Fabian and Brill, 2012). In our assay, males started producing offspring by day 4 of the recovery process with the exception of males that had developed at 31°C, who needed much longer to recover fertility. As sperm individualisation is temperature sensitive (Ben-David et al., 2015), high developmental temperatures might disrupt proper sperm maturation. As the last step, the 64 interconnected spermatids individualize and finally the mature sperm coils into the base of the testis (Fabian and Brill, 2012; Steinhauer, 2015) and already at 29°C Ben-David and colleagues (Ben-David et al., 2015) observed the formation of fewer and more abnormal individualization complexes. Our proxy for availability of mature sperm - SV size and sperm presence in the SV- corroborated these findings with a major impact of elevated developmental temperatures and the opportunity to recover on the presence of mature sperm in the SVs, but also highlighted a delay in mature sperm formation. Although we found that sperm presence in the SV of males allowed to recover improved over time, the sperm quantity was lower than in control males. Apart from having fewer sperm, this sperm also seems more sensitive as sperm viability decreased faster in males exposed to 29°C during development within 30 mins after collection with the possibility to lead to reduced fertilisation success in the long-run. While this data shows that recovery of spermatogenesis is possible to some extent, the effects of temperature are not completely compensated during recovery. A reduction in the number of sperm ejaculated was also found in *T. castaneum*males when facing a heat shock of 5°C above the optimum temperature (Sales et al., 2018). This reduction might be the result of a significant increase in sperm cell death of exposed males (Sales et al., 2018) as we found with time. We additionally show, when elevated temperatures persist and recovery is not allowed, sperm maturation and/ or movement into the SV is not possible, as no sperm was found in the SVs of six day old males grown and kept at 31°C. A result similarly found in *D. suzukii* males raised at 30°C (Kirk Green et al., 2019) and in line with the idea that spermiogenesis is affected as found previously (Ben-David et al., 2015) halting the maturation of sperm. However, there is the potential for strong variation across genotypes in their ability to produce mature sperm as indicated by the variation in fertility at sub-lethal temperatures across isogenic lines of the Drosophila Genetic Reference Panel (Rodrigues et al., 2021).

In addition to the SVs, we also investigated the response to elevated developmental temperature on accessory gland maturation as it is the main production site of SFPs, which are important determinants of male reproductive success (Avila et al., 2010; Chapman et al., 2003). The interplay between male SFPs, sperm and the female reproductive tract is integral to ensure all stages of the reproductive cascade can proceed and culminate in the fertilization of a passing ova (Avila et al., 2010). Additionally, male SFPs can protect sperm and enhance sperm viability (den Boer et al., 2009, 2008; Holman, 2009; King et al., 2011). The growth of the accessory gland is key during sexual maturation (Ruhmann et al., 2016) and accompanied by an increase in functionality (Leiblich et al., 2012; Prince et al., 2019). We here observed a negative impact of heat-stress during development with a clear reduction in AG growth during the early stages. Surprisingly, recovery had little effect and did not aid AG maturation, which could result in reduced AG

functionality, affecting SFP properties and/or composition. This hypothesis is tentatively supported by our phenotypic data, as temperature challenged males were not able to prevent female remating, induce increased oviposition (a trait mediated by ejaculatory sex peptide (Chapman et al., 2003; Liu and Kubli, 2003) and ovulin (Rubinstein and Wolfner, 2013)) and defend their ejaculate against subsequent rivals, regardless of the possibility for recovery. These traits are determined by seminal fluid proteins like the sex peptide (Avila et al., 2010; Chapman et al., 2003; Fricke et al., 2009; Liu and Kubli, 2003) and our results point towards the possibility that heat-challenged males could not transfer functional or adequate amounts of sex peptide and potentially other SFPs. We worked under the premise that larger AG size is indicative of SFP accumulation, which is an adequate proxy for at least the first three days after eclosion (Koppik et al., 2018).

Under normal circumstances, rapid growth of the AG can be observed in the first ten days after eclosion (Box et al., 2019; Ruhmann et al., 2016) and continues at a lower rate during male adulthood (Box et al., 2019). In general, the change in AG size occurs due to changes in both its cell types – the secondary and the main cells (Box et al., 2019; Leiblich et al., 2012). Main cells increase in size due to endocycling throughout male life (Box et al., 2019). In secondary cells the vacuole like compartments (VLCs) increase in size and change nature and location (Prince et al., 2019). VLCs are vital to secondary cell functionality (Corrigan et al., 2014; Gligorov et al., 2013; Prince et al., 2019) by secreting their content into the gland lumen and communicating with neighbouring main cells (Hopkins et al., 2019). Secondary cell and proper VLC maturation is important for overall AG functionality and thus the question arises, whether development at elevated temperatures disrupts proper formation of secondary cells and/ or interferes with main cell growth. As, we did not observe an improvement for males allowed to recover compared with males kept at the growth temperature, this might indicate that processes involved in AG growth cannot be rescued and developmental temperatures might produce an irreversible damage, which could explain the inability of heat-damaged males to recover and reach the fitness of control males. However, the AG can regenerate from damage (Box et al., 2019) and possibly our chosen time span was too short to see this effect after heat-damage warranting further investigations.

## Conclusion

Under predicted climate change scenarios, an increase in temperature is expected next to an occurrence of longer and more severe heat waves (Meehl and Tebaldi, 2004). Temperatures in the range of four to six degrees above the optimum temperature as tested here are easily reachable in many areas worldwide, especially during the summer (Solomon et al., 2007). From an ecological point of view, this could lead to severe consequences for species distributions and persistence, particularly as recent research highlights a lack of genetic variability in male sub-lethal fertility limits (van Heerwaarden and Sgrò, 2021; Zwoinska et al., 2020). This lack would severely hamper a species ability to mitigate escape from this predicament through evolutionary adaptation to the novel conditions. Already now, a species thermal fertility limit is a better predictor of species ranges than the critical thermal limit across 43 Drosophila species (Parratt et al., 2020). Our findings echo this recent interest in understanding the impact of temperature on male reproduction. We here add insights on the fitness costs of heat stress and mechanisms allowing recovery. In sum, we show that sub-lethal thermal sterility and the subsequent fertility reduction could be caused by a combination of malfunctioning reproductive traits: inefficient functionality of the accessory gland and alteration of spermatogenesis. In addition, we show that the possibility of recovery after exposure, even when facing a mid-challenge, does not mitigate the damage imposed on reproduction by elevated thermal stress during development. Moreover, five days of recovery is not enough to rescue SFP functionality, and the AGs fate is mainly determined during development, which could explain the inability of heat-damaged males to recover and reach the fitness of control males. We found AG functionality more thermosensitive than spermatogenesis as SFPs induced female post-mating responses were already impaired at the mid-challenging temperature of 29°C. Mature sperm though was found in males raised at 29°C and particularly in those allowed to recover, which could explain the progressive increase in fertility observed in recovering males.

*Data Accessibilty Statement*

Raw data will be submitted to DataDryad upon acceptance of the ms.

*Author contributions*

BCD and CF designed the experiments, BCD collected and analysed the data, BCD wrote the first version of the manuscript and both authors edited the manuscript.

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*Competing interests*

We declare to have no competing interests.

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**Table 1** . Generalized mixed models following a gaussian distribution was used to analyze the number of eggs, and a quasibinomial distribution for the egg-to-adult survival. The offspring number produced was analyzed using a Zero-inflated negative binomial model. The model included a logit part that analysed whether an offspring was produced or not, while the count part analysed the number of offspring produced following a negative binomial distribution. Males previously developed at 25, 29 or 31°C and were kept at the growth temperature or moved to the control temperature to recover. Pairs were allowed to mate on day 2, 4, or 6 after eclosion. In all cases, male developmental temperature and opportunity to recover were analysed as the factor temperature with five levels, with day of measurement coded as a factor with three levels.

| **Factor** | Deviance | F | df | P |
|---|---|---|---|---|
| *Number of eggs* | *Number of eggs* | *Number of eggs* | *Number of eggs* | *Number of eggs* |
| **Temperature** | 66149 | 33.532 | 4 | $< 0.0001$ |
| **Day** | 4804.2 | 4.871 | 2 | 0.008 |
| **Temperature * Day** | 11029 | 2.935 | 8 | 0.004 |
| *Egg-to adult survival* | *Egg-to adult survival* | *Egg-to adult survival* | *Egg-to adult survival* | *Egg-to adult survival* |
| **Temperature** | 8607.6 | 56.138 | 4 | $< 0.0001$ |
| **Day** | 1172.3 | 15.291 | 2 | $< 0.0001$ |
| **Temperature * Day** | 1371 | 6.863 | 8 | $< 0.0001$ |
| *Offspring number* | *Offspring number* | *Offspring number* | *Offspring number* | *Offspring number* |

**Count part logit part**

**Factor** df $\chi^2$ $\Pi$ df

$\chi^2$ $\Pi$

**Temperature** 4 26.31 <0.0001 12 162.81 <0.0001

**Day** 2 0.23 0.89 10 67.82 <0.0001

**Temperature * Day** 8 44.75 <0.0001

**Table 2** . Results of a generalized linear models with a Gamma error distribution for male accessory gland and seminal vesicle size. Both traits were measured in two- and six-day old adult males. Males previously developed at 25, 29 or 31°C and were kept at the growth temperature or moved to the control temperature to recover.

**AG size**

| **Factor** | Deviance | F/$\chi^2$ | df | P |
|---|---|---|---|---|
| Temperature | 7.233 | 75.474 | 4 | $< 0.0001$ |
| Day | 8.595 | 358.720 | 1 | $< 0.0001$ |

| | | | | |
|---|---|---|---|---|
| $Day^2$ | 4.736 | 197.670 | 1 | $< 0.0001$ |
| Wing length | 0.531 | 22.144 | 1 | $< 0.0001$ |
| **SV size** | **SV size** | **SV size** | **SV size** | **SV size** |
| Temperature | 8.286 | 91.652 | 4 | $< 0.0001$ |
| Day | 6.709 | 296.830 | 1 | $< 0.0001$ |
| Temperature * Day | 0.804 | 11.437 | 4 | $< 0.0001$ |

**Figure legends**

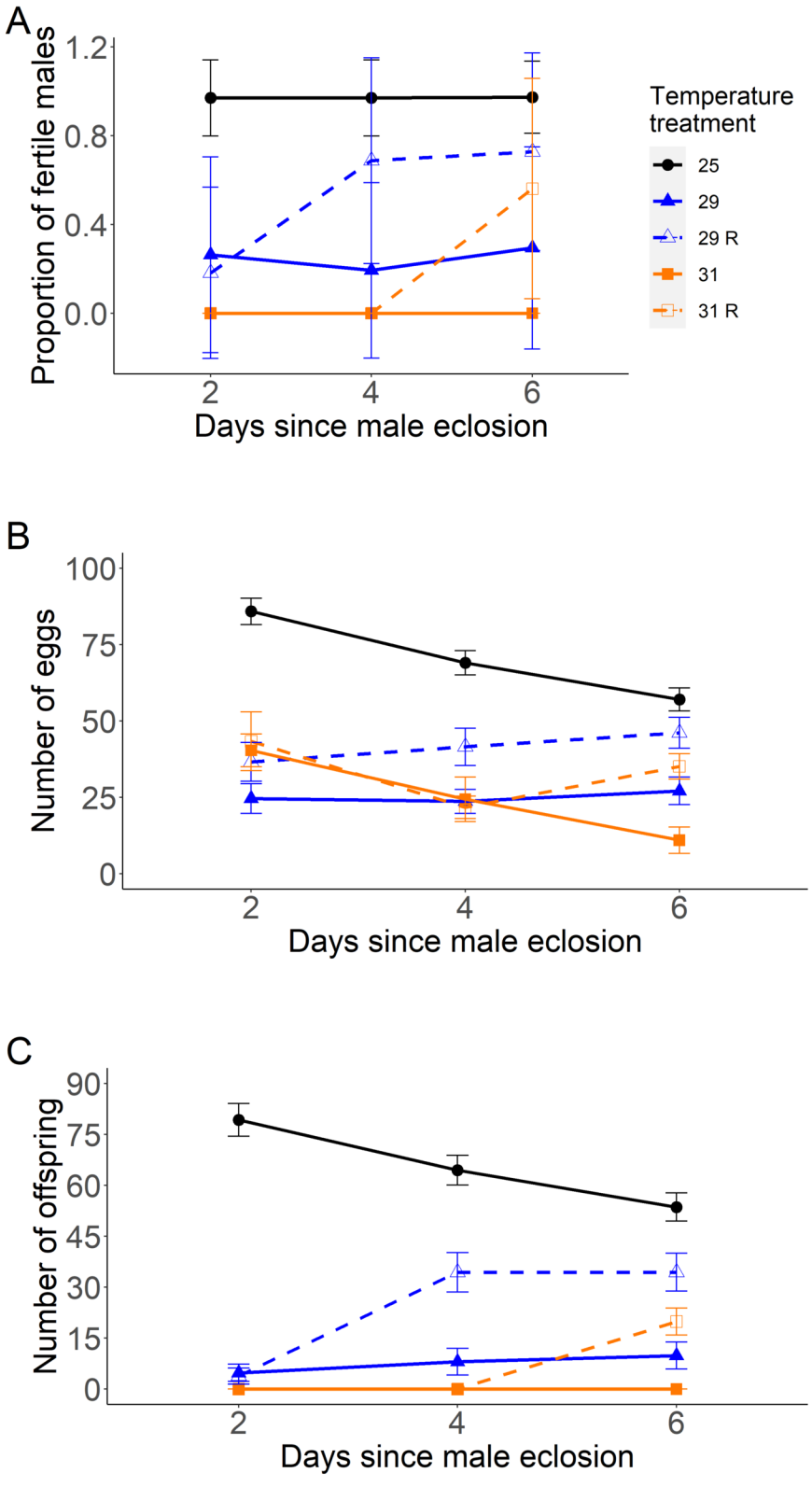
**Fig. 1 Comparison of reproductive output for heat-challenged males and those allowed to recover (Mean ± SE).** Proportion of fertile males (A) measured as the fraction of males at least producing one offspring, the number of eggs laid after a single mating (B), the offspring number (C) and egg to adult survival (D). Males were allowed a single mating two-, four- or six- days after eclosion after having developed at (°C): 25 (circle symbol), black; 29 (triangle symbol), blue; or 31 (square symbol), orange. Males grown and kept after eclosion at the developmental temperature are shown as a solid line, while males allowed to recover (R) at 25°C after eclosion, are shown as a dashed line.

**Fig. 2 Male mating behaviour and competitive success (Mean ± SE):** proportion of males gaining a copulation (A), and their mating latencies (B). Mating latency of females remating with a competitor male (C), and proportion of offspring obtained by treatment males when defending their ejaculate against a second male (P1) (D). The results are shown according to the developmental temperature of first mating males. Males allowed to recover at 25°C after eclosion are represented with an "R"; otherwise, males were kept at the growth temperature after eclosion.

**Fig. 3 Assessment of mature sperm presence in heat-challenged males (Mean ± SE):** seminal vesicle (SV) size (A), and sperm presence in the vesicles (B) for two- and six- day old heat-challenged males. Between 14 to 16 males were measured for each day and temperature treatment; the mean of both SVs was used for each male for representing the SV size and sperm presence. Colored lines indicate the developmental temperature: 25 (circle symbol), black; 29 (triangle symbol), blue; or 31 (square symbol), orange. Males grown and kept after eclosion at the growth temperature are shown with a solid line while males allowed to recover (R) at 25°C after eclosion, are shown with a dashed line. **Sperm viability in heat-challenged males** (C)**:** Percentage of alive sperm for six-day old control and 29°C recovery males. Portrayed is the temporal decrease in sperm viability, measured at three different time points: just after the staining (t0) as well as 15 (t15) and 30 (t30) minutes later. 21 males from each temperature treatment were used.

**Fig. 4 Sexual maturation in accessory glands for heat-challenged males (Mean ± SE) (A)** : Accessory gland size measures for one-, two-, four- and six-day old heat-challenged males. Between 15 to 26 males were measured for each day and temperature treatment. The mean of both accessory glands was used for each male. Colored lines indicate the developmental temperature (°C): 25 (circle symbol), black; 29 (triangle symbol), blue; or 31 (square symbol), orange. Males grown and kept after eclosion at the growth temperature are shown with a solid line while males allowed to recover (R) at 25°C, are shown with a dashed line.

**Fig. 1**



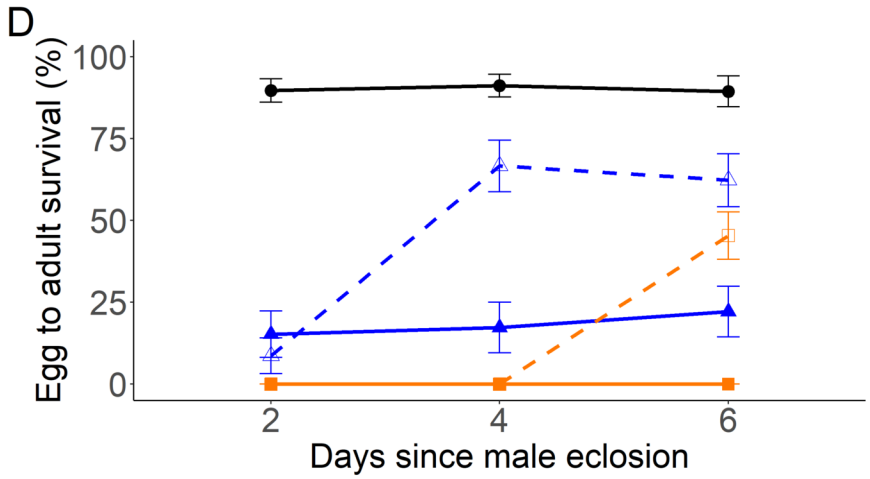
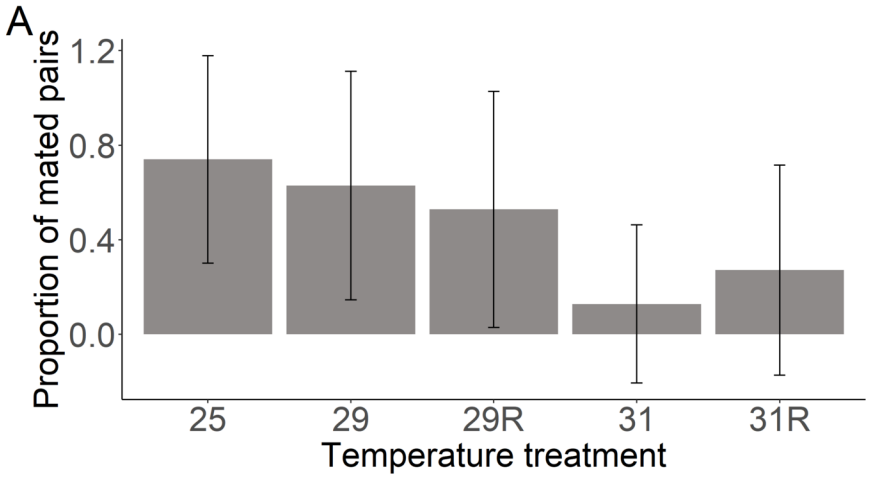
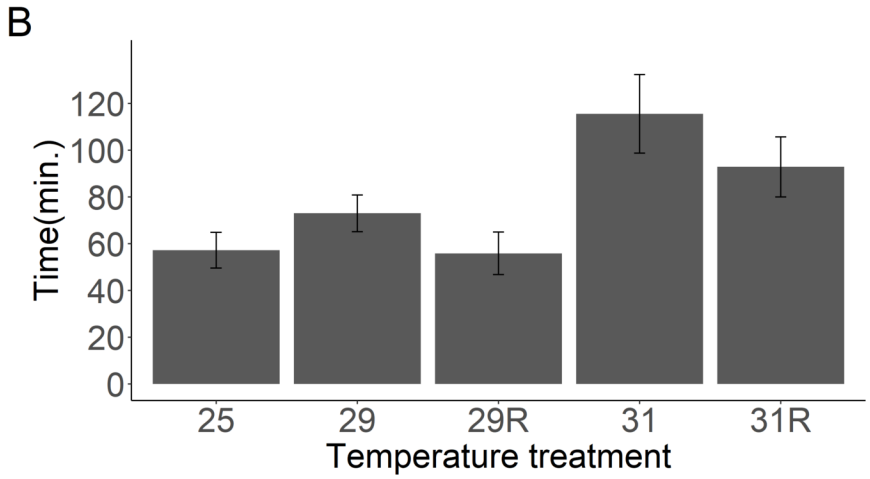



**Fig. 2**



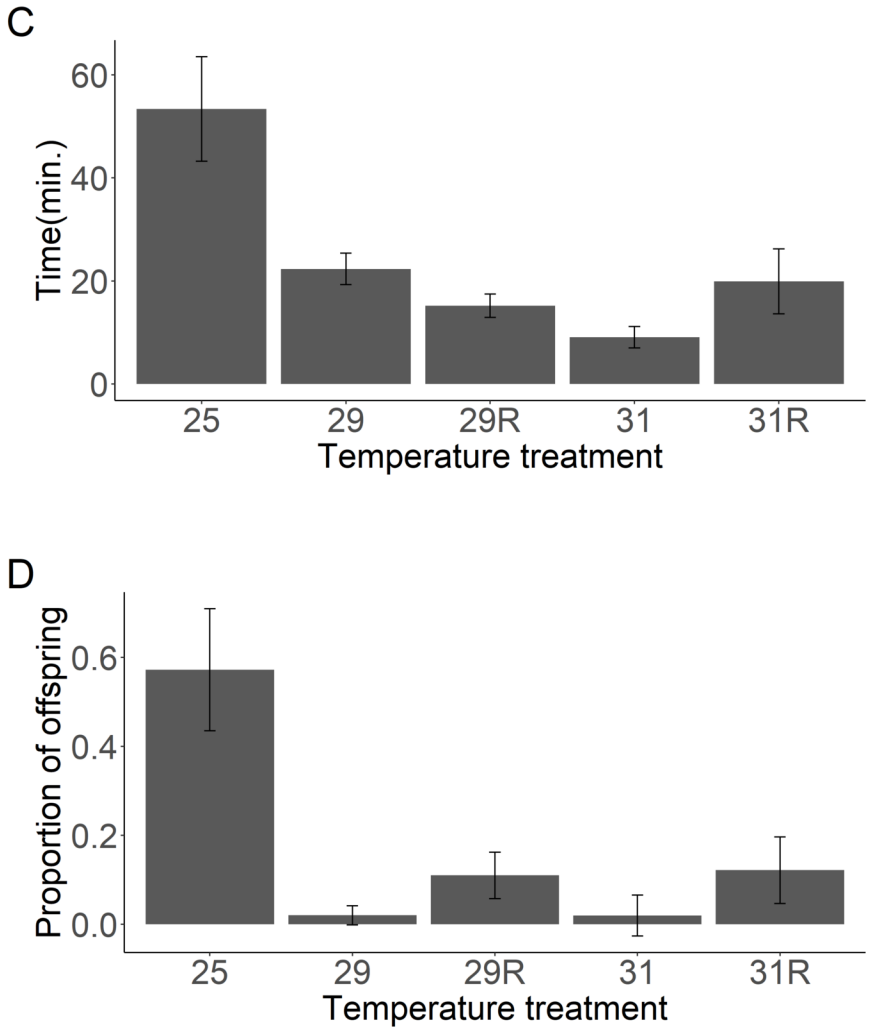



**Fig. 3**

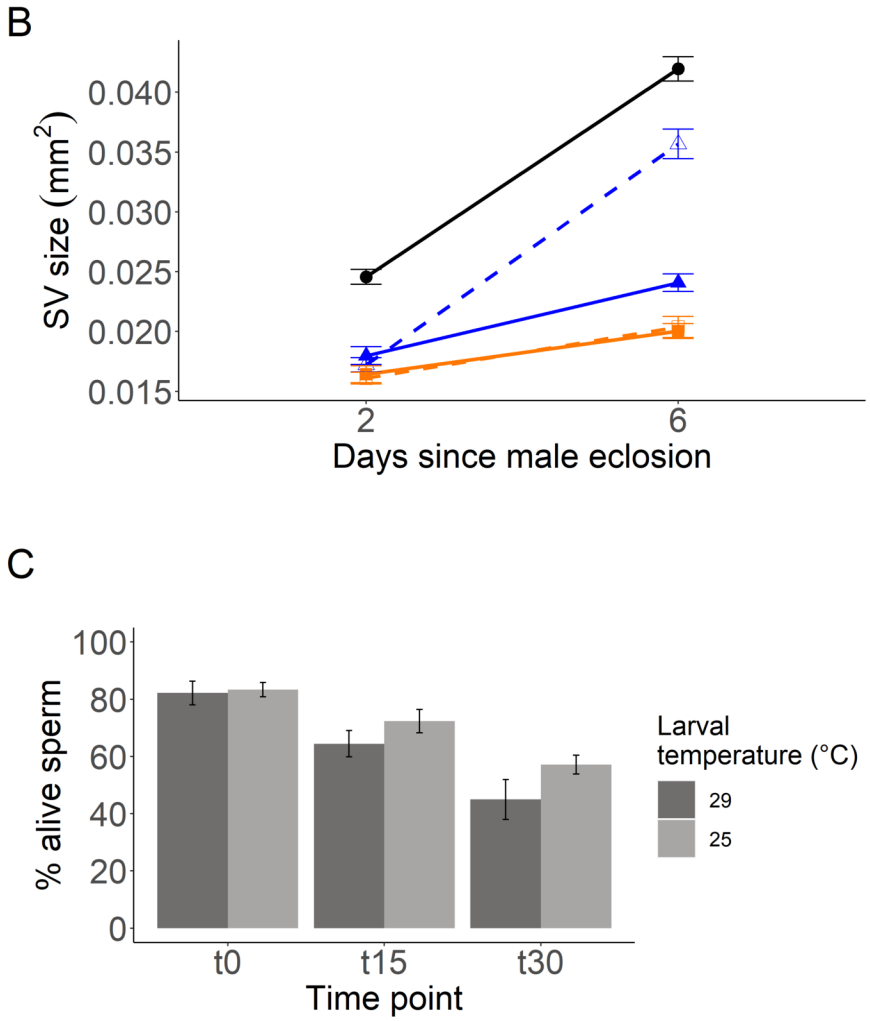



**Fig. 4**