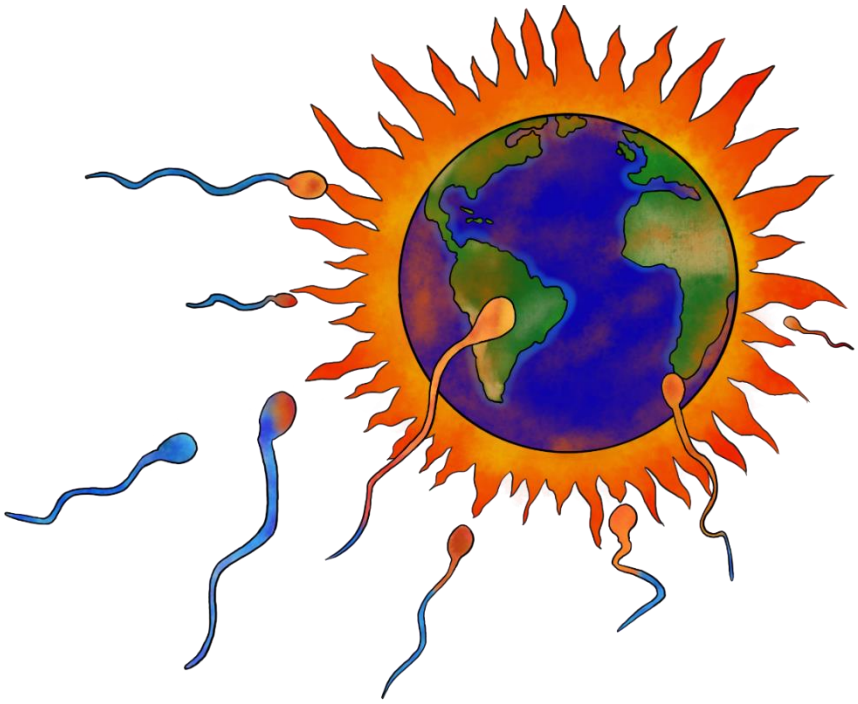
# The Impact of Climate Change on Fertility



Thesis submitted in accordance with the requirements of the University of Liverpool

for the degree of Doctor in Philosophy by Benjamin S Walsh

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In memory of Jimmy Johnson,

who always brightened the lives of others.

Forever in our hearts.



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# Preface

This project immediately stood out to me. Fertility loss at high temperatures is a phenomenon observed across taxa, but is understudied. It was abundantly clear how this emerging field could substantially affect human lives and biodiversity itself. Under every corner I looked, I found an exciting new project that could help push forward the field. In particular I found myself interested in whether organisms may be able to cope with heat-induced sterility, through plasticity or adaptation, or some other component of ecology. As I am keen to publish all of the data chapters in this thesis, each chapter is written independently of the others and structured in the style of a research paper.

**The introduction** gives an overview of how increasing temperatures are likely to affect fertility from a wide range of taxa. My aim for this review was to bring together data across different fields and examine the general impact of high temperatures on fertility of wild populations, considering possible ways populations could cope with increasing temperatures. This review was published in Trends in Ecology and Evolution (TREE) in 2019 (Walsh et al. 2019a). Graziella Iossa's comment to our review (Iossa 2019) and our response (Walsh et al. 2019b) were also published in TREE, both of which are included in Appendix 1.

**Chapter 1** explores how thermal stress during the pupal stage affects male and female fertility in *Drosophila virilis*. I demonstrate sex-specific male sterility and consider how it could affect the operational sex ratio of wild populations. These results gave me an effective jumping-off point for subsequent experiments, and allowed me to discuss a concept I find really interesting. In 2020 this chapter was published as part of a special issue in Current Zoology, examining the impact of climate change on sexual selection (Walsh et al. 2020).

**Chapter 2** investigates whether heat-induced male sterility can be 'rescued' by a high-temperature coping mechanism, called heat-hardening. In this manuscript, I demonstrate that heat-hardening can improve survival at extreme temperatures, but not fertility. This project was profoundly affected by the COVID-19 pandemic - Steve Parratt and I actually had to store experimental *D. virilis* in our homes in order to finish the experiment. In 2021 this chapter was published in Ecology and Evolution (Walsh et al. 2021).

**Chapter 3** shifts the focus from males to females. Here, I ask whether sperm is safe from high temperatures when stored in females of *D. virilis and Zaprionus indianus*. This is my favourite chapter, as I am very happy with its simple but effective experimental design. In 2022 this chapter was published in Journal of Thermal Biology (Walsh et al. 2022).



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**Chapter 4** examines whether there is a genetic basis for heat-induced sterility. I examine how temperature affects sperm production in *D. melanogaster.* I measure testes size at benign and stress temperatures in 95 different recombinant inbred lines. This allowed me to examine whether there are any genes that predict sensitivity of fertility to temperature. This project was in collaboration with Dr. Mollie Manier and constitutes the largest dataset from my thesis including over 2000 photographs, all of which were analysed individually.

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# Introduction: The impact of climate change on fertility


## Abstract

Rising global temperatures are threatening biodiversity. Studies on the impact of temperature on natural populations usually use lethal or viability thresholds, termed the 'critical thermal limit'. However, this overlooks important sub-lethal impacts of temperature that could affect species' persistence. Here, we discuss a critical but overlooked trait, fertility, which can deteriorate at temperatures less severe than an organism's lethal limit. We argue that studies examining the ecological and evolutionary impacts of climate change should consider the 'Thermal Fertility Limit' (TFL) of species; we propose that a framework for designing TFL studies across taxa be developed. Given the importance of fertility for population persistence, understanding how climate change affects TFLs is vital for assessing future biodiversity impacts.


## 1. Biodiversity under climate change

Climate change will continue to have an increasingly dramatic effect on the global thermal environment (Intergovernmental Panel on Climate Change 2014), including increases in average local temperatures and the frequency of heat waves (Buckley and Huey 2016; Kingsolver et al. 2013). These shifts present a major threat to biodiversity and are starting to have severe impacts on the distribution and abundance of natural populations and species (Hoffmann 2010; Kellermann et al. 2012). The capacity of species to respond ecologically and evolutionarily to the challenges of global thermal change will affect

227 future biodiversity. Determining key thermally-sensitive traits across species, and
228 quantifying the ability of species to buffer the effects of thermal stress on these traits, is
229 therefore a critical research priority (Moritz and Agudo 2013).

230 Understanding the long-term impacts of climate change on populations requires robust
231 predictive models that can project responses to both current global temperatures and
232 future climate change scenarios. Currently, many such models are based on empirically
233 derived 'critical thermal limit' (CTL, see Glossary) estimates, which describe the upper and
234 lower temperature bounds beyond which critical biological functions (e.g. movement or
235 respiration) fail (Geerts et al. 2015; Kellermann et al. 2012). Comparative studies have
236 shown that measures of such viability limits more robustly predict the current
237 distributions of many species than measures derived from changes in mean fitness traits
238 under thermal stress (Overgaard et al. 2014). For this reason, CTLs have also been used to
239 infer species' sensitivity to climate change (Bush et al. 2016; Kellermann et al. 2009;
240 Kellermann et al. 2012; Mitchell et al. 2011). However, using only thermal limits to
241 viability may be misleading because different measures of CTLs do not always correlate
242 within a single species or population, leading to inconsistent estimates of population
243 persistence (Blackburn et al. 2014). It has been suggested that a multi-trait approach to
244 thermal tolerance may be give more robust estimates of species responses to climate
245 change (Blackburn et al. 2014). In particular, the focus of thermal limits needs to move
246 away from the incapacitating and lethal effects of thermal stress, to investigate how sub-

247 lethal temperatures impact fitness-related traits such as reproduction, which are critical
248 for population stability and persistence.

## 249 2. Sensitivity of fertility to temperature

250 Fertility is a major component of individual fitness and is a central determinant of
251 population growth and persistence. Evidence from a wide variety of taxa suggest that the
252 germ line and associated reproductive physiology is sensitive to thermal stress,
253 particularly high temperatures (Karaca et al. 2002; Pérez-Crespo et al. 2008; Porcelli et al.
254 2016; Reinhardt et al. 2015; Vollmer et al. 2004). Evidence, mostly from pollen
255 development, suggests that meiosis is a more thermally sensitive process than mitosis
256 (reviewed in Paupière et al. 2014; Sage et al. 2015). In mammals, the descended testicle
257 has evolved to ensure that spermatogenesis occurs at cooler-than-body temperatures
258 (Moreno et al. 2012 and references therein). Indeed, temperature induced infertility
259 imposes major economic costs in tropical climates (Peña et al. 2018). However, although
260 a number of studies have examined how temperature impacts reproductive traits (Table
261 0.1), these often use vastly different methodologies and measure different aspects of
262 reproductive biology. This collection of disparate studies makes quantitative comparisons
263 of the impact of high temperature on reproduction very difficult. Possibly for this reason,
264 thermal limits to fertility have not been systematically incorporated into predictions of
265 species responses to climate change.

266 Here, we argue that the effect of temperature on fertility requires a broad analogue of
267 CTL, termed the 'Thermal Fertility Limit' (TFL). This term would capture both the upper

($TF_{MAX}$) and lower ($TF_{MIN}$) temperature boundaries at which a species loses fertility. This new term will facilitate researchers in bringing together related work on how environmental stress impacts this broadly important component of biology, and will highlight the important biological and ecological distinction between fertility and survival when assessing species' response to climate change. We suggest that a framework be developed that will allow researchers to design and conduct thermal fertility studies in a way that generates comparable datasets across taxa. A large database of TFL measures across multiple species and populations relevant to thermal stress levels encountered in nature would provide the power to answer important evolutionary and ecological questions regarding the impact of climate change on natural populations at risk (Box 0.1 and Figure 0.1). We do not propose that TFL measures would replace CTLs. Rather, we suggest that the combination of these measures, the geographic distribution of these two limits, and the extent to which they correlate within and among species, will give valuable insight into species' ability to persist and adapt to global thermal change. To do this, we need to consider how temperature is likely to affect fertility at a mechanistic level, and how researchers can design and conduct studies of TFLs in a standardised and broadly comparable way.

## 3. Towards a methodological framework for the study of TFLs

The adoption of standardised measures for CTLs (Overgaard et al. 2014; Terblanche et al. 2007), typically either a direct or proxy measure of viability, has facilitated large-scale comparative studies of species' responses to climate change (Kellermann et al. 2012). A

challenge for the study of TFLs will be to develop a similarly standardised measure for fertility. This is a non-trivial task given the inherent complexity and potential species-specificity of reproductive components that contribute to fertility (Figure 0.2). This complexity is highlighted by the diverse methodologies and metrics of fertility employed in the existing literature on the effect of temperature on fertility (Table 0.1). For maximum utility, TFL studies should be carefully designed to either produce a quantitative point estimate of temperature limits for fertility for comparative species distribution modelling, or to generate effect size estimates for fertility loss at a given thermal stress level for future meta-analyses between groups.

## Factors in designing TFL studies

Despite the diverse elements of fertility described in Figure 0.2, we argue that the most ecologically precise limit to fertility is the point at which the qualitative ability of an organism to produce viable adult offspring under controlled conditions is lost. This limit yields a precise metric that can be applied to quantitative comparisons among taxa. However, for many species, measuring offspring production directly may be impractical, for instance if generation times are extremely slow. In such instances, proxy measurements that can be empirically correlated with fertility may also serve to capture the effect of temperature. For example, in some *Drosophila*, qualitative sperm motility has been used to quantify male fertility following heat stress, as this correlates strongly with reproductive output (reviewed in David et al. 2005). In plants, the percentage of pollen grains that germinate *in vitro* correlates with fruit productivity and has been

employed as a measure of TFLs (Acar and Kakani 2010; Sage et al. 2015). It would be unrealistic to attempt to identify a trait that captures the effect of temperature on fertility across all of biology, but taxa-specific proxies like these may be sufficient to enable meaningful comparative studies.

Whichever measurement is used, assessing fertility over a range of static temperatures will allow us to generate a fertility reaction norm. From these reaction norms we can determine the temperature at which fertility drops by a given percentage compared to benign controls; a measure analogous to a 'Lethal Dosage' in toxicology and one already used for some measures of CTLs (Lutterschmidt and Hutchison 1997). The exact proportion of fertility loss that is ecologically relevant for population stability and thus represents a true thermal fertility limit, is likely to vary from species to species. With enough data on the reproductive and population biology of a given organism, these thresholds could be explicitly modelled. Or, if reaction norms are established across a broad enough range of temperatures then it should be possible to determine any threshold and to assess if these are correlated across species.

Further, unlike viability limits, fertility is not necessarily an irreversible binary trait. Evidence suggests that complete sterility at extreme temperatures is preceded by quantitative fertility loss at intermediate conditions (Chakir et al. 2002; Rukke et al. 2018). Furthermore, recovery of fertility can occur in some heat-sterilised animals if they are returned to benign conditions (Nguyen et al. 2013; Rohmer et al. 2004), although under severe thermal stress sterility can be permanent (Jørgensen et al. 2006, pers. obs.;

331 Vollmer et al. 2004). Researchers should carefully consider the time frame over which
332 qualitative fertility is assessed following heat stress, and potentially account for the
333 recovery of fertility over time; a two-day knock-down in fertility may be inconsequential
334 for long-lived species but catastrophic for organisms that exist as adults for only days. This
335 highlights an important consideration when comparing the utility of CTLs and TFLs,
336 reinforcing that TFLs have a much more complicated relationship with time than CTLs.

337 A second important practical consideration arises when selecting an ecologically relevant
338 temperature treatment. Researchers have shown that the response of organisms to
339 thermal stress is affected by both the intensity of the temperature chosen and also the
340 duration of exposure (Terblanche et al. 2007). This is further complicated when one
341 considers the effect that hardening treatments (Overgaard et al. 2012), ramping (Mitchell
342 et al. 2011), and the observed differences between static and cyclic temperature
343 treatments (Sgrò et al. 2016, and references therein) have on thermal performance in
344 many organisms. Unlike CTLs, where the effect of temperature is often immediately
345 visible, loss of fertility requires subsequent assays following exposure to heat, and so
346 ramping assays are unlikely to be useful. Instead, researchers must choose regimes of
347 static or fluctuating temperature stress that reflect current or future thermal extremes for
348 natural populations. The need to finely balance high-throughput, standardised repeatable
349 assays with ecological realism will be a major challenge for TFL research.

350 To summarise, if researchers think about the exact trait they are going to measure, the
351 thermal regime under which it will be measured, and consider that fertility may recover

over time, then they will be well on their way to having a robust framework for studying TFLs (Box 0.2). Investigating this in model species, and testing whether it predicts species distributions better than current methods, will be a key step in determining how important TFLs are in nature.

# 4. Can species maintain fertility in the face of thermal change?

Many species are predicted to have populations pushed beyond their critical thermal maxima ($CT_{MAX}$) by climate change (Kellermann et al. 2009). As thermal fertility maxima ($TF_{MAX}$) are expected to often be lower than $CT_{MAX}$, rapid climate change is likely to push many populations and species beyond their $TF_{MAX}$. Developing standardised measures of TFLs will provide tools to investigate how species might physiologically acclimate and adapt to these changing thermal environments.

## Are thermal fertility limits plastic?

Organisms could show phenotypic plasticity in TFLs within their own lifetime or through intergenerational carry-over effects. Sub-optimal temperatures experienced at early life-history stages can affect traits such as adult size (Atkinson 1994). Experiencing some level of thermal stress can increase the fitness of individuals for a similar stress later in life, a process known as acclimation. For CTLs there is significant, but very limited, scope for coping with rising temperatures through plasticity (Sørensen et al. 2016). For instance, the degree of plasticity in upper thermal tolerance appears weakly associated with species distribution ranges (Mitchell et al. 2011). However, it is not known if similar

373 plasticity exists for TFLs, and whether plasticity in TFLs is greater than that for CTLs.
374 Exposing organisms to acclimation treatments followed by TFL measurement, or
375 investigating inter-generation carry-over effects for TFLs, may shed new light on the
376 ability of organisms to buffer the effects on fitness of ecological change.

377 There is mixed evidence for the impact of acclimation on temperature-induced sterility.
378 Male *Drosophila buzzatti* regain fertility faster following a heat stress if they had previous
379 experienced a heat-shock (Jørgensen et al. 2006). However, both *Drosophila subobscura*
380 and *Tribolium castaneum* have been shown to exhibit more extreme fertility loss when
381 exposed to multiple rather than single periods of heat stress, which does not indicate an
382 acclimation response (Porcelli et al. 2016; Sales et al. 2018). Where plasticity in thermal
383 fertility traits does exist, the underlying mechanisms remain largely unknown. However,
384 individuals are likely to cope with stress in part by using heat-shock proteins, which are
385 important in mediating upper thermal limits in insect species (Krebs and Loeschcke 1994).
386 Many, including Hsp70, are up-regulated during hardening treatments, helping individuals
387 to offset the negative fitness consequences of thermal stress (Sørensen et al. 2001). Heat
388 shock proteins are a ubiquitous component in living systems: importantly, they are found
389 in gametes, including human spermatozoa (Miller et al. 1992). Exploring the scope for
390 heat-shock protein expression to buffer the deleterious effect of high temperature on
391 fertility, and the variation in this within closely related species might explain patterns of
392 variation in TFLs.

## Can thermal fertility limits evolve?

Over long periods of environmental change, selection should favour more thermally-tolerant genotypes and a rise in both CTLs and TFLs. Including the evolvability of thermally sensitive traits into models of species' response to climate change generates vastly different predictions than equivalent models parameterised with only current measures of thermal sensitivity (Kellermann et al. 2012). However, current evidence suggests there is very little standing genetic variation and evolvability for high temperature CTLs (Kellermann et al. 2012), although this is debated (reviewed in Terblanche et al. 2007). Whether TFLs can evolve rapidly is unknown. Limited evidence in *Drosophila* has shown male sterility under heat stress can be variable within species and may be under selection to be locally adapted across populations originating from different thermal regimes (Pedersen et al. 2011; Porcelli et al. 2016; Rohmer et al. 2004; Vollmer et al. 2004), suggesting that TFLs may be evolvable. Quantifying standing variation in TFLs across genotypes and populations of multiple species would be a good first approach for testing this.

Species with CTLs that are low and evolutionarily constrained are predicted to be at particular risk from climate change (Bush et al. 2016). For instance, tropical species have been shown to often lack genetic variation that would enable rapid evolution to cope with changing climatic variables such as temperature and desiccation (Deutsch et al. 2008; Kellermann et al. 2009). Establishing how these species' TFLs respond to increasing temperatures may be critical for predicting how they will be impacted by climate change.

If TFLs are substantially lower than CTLs, then these species may be more vulnerable than currently predicted. However, if TFLs are more evolvable than CTLs, this may compensate for their initially low TFLs, making CTLs more important predictors of distributions in a warming world. Until both CTLs and TFLs are examined across a variety of taxa, and the evolvability of TFLs determined, confidence in predictions about which taxa are going to be particularly vulnerable will be low (Box 0.1).

Whether populations or species can respond to thermally-induced loss of fertility, either through short-term plasticity or long-term adaptive change, is unclear. This is partly because of knowledge gaps regarding the impact of extreme temperature on fertility in animals and plants. A fundamental understanding of how extreme increases and decreases in temperature influence reproduction with negative effects on fertility is required before the ecological relevance and potential evolution of TFLs can be determined. However, it is precisely these answers that are ultimately among the most important to know, as they will improve predictions on how climate change may affect species abundance and distribution, and thereby change biodiversity across the globe.

## Concluding remarks

Here, we have introduced and discussed the idea that measuring the thermal limit of fertility across multiple species and a broad range of taxa could be critical when assessing the impacts of global thermal change on biodiversity. While the use of critical thermal limits has proven to be informative for modelling current and future distributions of species (Kellermann et al. 2009; Kellermann et al. 2012; Mitchell et al. 2011), CTLs may

overestimate species' ability to cope with stressful temperatures**.** Research exploring TFLs (see Outstanding Questions) is needed to ascertain the extent to which they correlate with CTLs**.** To this end, we propose a general framework for TFL studies to promote large-scale cross-taxa assessments of this important but largely neglected trait**.** Focusing on TFLs with broadly standardised methodologies may improve our knowledge of how climate change will affect species' abundance, distribution, and persistence. However, the current literature on how thermal stress impacts fertility is fragmented. Stronger and more unified thermal fertility research might radically improve our predictions about the impacts of global thermal change.

**Box 0.1: Groups at risk**

**Figure 0.1** Examples of organisms that may be particularly at risk to losing fertility due to high temperatures. Clockwise from top left: broadcast spawning fish such as carp, small ectothermic insects including pollinating bees, endemic animals with limited latitudinal or elevation ranges such as the flightless cormorant, disease vectors including mosquitos, coral species that are important to highly diverse reefs, and endemic plant species including the Scottish primrose. All photos in this figure are licensed under CC BY 2.0, Credits: Joaquim Alves Gaspar, Charles Sharp, Toby Hudson & David Glass).

453 Certain groups of organisms are likely to be most vulnerable to temperature-driven
454 fertility loss. These groups may provide important case studies and primary avenues of
455 research (Fig 0.1).

456 **Ectothermic species**

457 Most plant species cannot regulate the temperature of their tissues (excluding a number
458 of species of flower (Watling et al. 2008)), forcing them to withstand ambient
459 temperatures. Likewise, ectothermic animals may also be vulnerable (Kingsolver et al.
460 2013), as they rely on behavioural rather than physiological thermoregulation to avoid
461 stressful microenvironments. Smaller ectothermic animals are even more at risk, as they
462 will reach ambient temperatures faster.

463 **Endemic species and species with small ranges**

464 Rare or endemic species with small latitudinal ranges are likely to be particularly at risk to
465 losing fertility as ambient temperatures increase because: i) they are likely to lack the
466 genetic variation and gene flow required to adapt to novel stressors (Hoffmann 2010),
467 and ii) in many cases they may be unable to shift their distribution range to track changing
468 climates. This will be particularly true for island endemics and species that live within
469 specialised elevational niches in mountains.

470 **Aquatic species**

471 Aquatic species, particularly broadcast spawners, are likely to be at risk because the
472 specific heat capacity of water will result in rapid changes in tissue temperatures. Further,

gametes in the water from spawning organisms will exposed directly to stressful temperatures, so will need to evolve robust physiological responses to high temperatures to retain form and function. This is likely to be a greater issue for freshwater and shallow water organisms, as these environments experience greater fluctuations in temperatures, exposing these organisms to acute stress events.

**Sessile species and life stages**

Sessile organisms, such as plants, corals and juvenile stages (e.g. pupal stages in holometabolous insects), in which movement to cooler areas during temperature spikes is not possible, may be particularly vulnerable. Similarly, due to their limited dispersal ability, belowground communities may be especially vulnerable to fertility loss under climate change (Berg et al. 2010).

**Box 0.2 Considerations when designing TFL experiments**

1. **Trait selection:** We suggest that wherever possible researchers measure both qualitative and quantitative offspring production in order to capture the ecological impact of high temperature on fertility. Where this is impossible, careful selection of proxy measures of fertility that can be empirically correlated with an individual's ability to produce offspring could be considered. Holistic measures such as these are most likely to generate broadly comparable data sets across taxa.

2. **Life-history stage:** Whilst reproduction occurs almost invariably during adult life-history stages, reproductive development and maturation can begin much earlier. Researchers should therefore consider which life stage(s) of their organism to expose to stress. For instance, do heat-treated juveniles mature into sterile adults whilst heated adults remain fertile?

3. **Ecologically valid thermal environment:** Careful attention should be given to selecting temperature regimes that reflect the current or future extremes that organisms are likely to face. For instance, are temperature spikes over a matter of a few hours more likely to impact a species' fertility than a rise in mean daytime temperature? A large body of work on CTLs has demonstrated that measures of thermal performance can be highly sensitive to the duration of stress (Terblanche et al. 2007), rates of temperature ramping (Mitchell et al. 2011) and the intensity and frequency of any temperature fluctuations (Davies et al. 2016). The latter point in particular may be key for thermal fertility, as some animals can recover fertility during periods of benign temperatures including night time (Zhao et al. 2014). Once researchers have selected a regime of temperature delivery they should strive, where possible, to measure thermal fertility over a range of temperature values. This will help capture the thermal fertility reaction norm of their organism.

4. **Implications for population stability:** To estimate the population-level effects of high temperature on fertility, researchers should consider what percentage loss

of fertility represents a meaningful threat to population stability. Factors such as the effective population size of the organism in a nature, the potential fecundity of individuals and their generation time could be used to estimate a specie's sensitivity to fertility loss. Researchers can then determine the degree of thermal stress required to push their study organism beyond this threshold.

5. **Critical thermal and fertility limits:** The power of TFLs to predict species' response to climate change will be related to the extent to which fertility and viability limits correlate with each other and across species. Low correlation would suggest that one metric cannot be substituted for the other. Which species have high and which species have low correlation and what impacts this relationship? Thus, researchers should determine both fertility and viability limits of their organism under relevant thermal regimes.

526 **Table 0.1:** Examples of thermal impacts on fertility

| Taxonomic group | Organism | Species | Impact of temperature on fertility | Measure | Refs |
|---|---|---|---|---|---|
| Cnidarian | Coral | *Acropora digitifera* | Increase of 2°C reduced the number of sperm bundles by almost 50%, and reduced egg size | Gamete number | (Paxton et al. 2016) |
| Insect | Bed bug | *Cimex lectularius* | Egg production and hatching success can fall to almost zero as a result of thermal stress | Fecundity | (Rukke et al. 2018) |
| | Red mason bee | *Osmia bicornis* | Changed odour profile, altering female mating preference | Mating preference | (Conrad et al. 2017) |
| | Beetle | *Callosobruchus maculatus* | Males reared at extreme high temperatures produce smaller sperm than benign controls | Sperm form and function | (Vasudeva et al. 2014) |
| | Beetle | *Tribolium castaneum* | Stressed males reduce sperm viability, competitiveness. Inseminated sperm within female storage organs less viable when female stressed. Transgenerational impact reducing longevity of offspring sired by stressed males | Sperm form and function, offspring production | (Sales et al. 2018) |
| | Dragonfly | *Micrathyria spp.* | Species within the genus that struggle to maintain optimal body temperatures are less efficient at defending perches at high temperatures, and lose out on breeding sites to larger species | Courtship behaviour | (May 1977) |
| | Fruit fly | *Bactrocera tryoni* | Reduced mating latency at cold temperatures, reduced mating frequency at cold temperatures | Mating latency, mating frequency, | (Meats and Fay 2000) |
| | Fruit fly | Family: Drosophilidae | Reduced mating success. Impairment of sperm elongation, resulting in loss of sperm motility and thus lower fertility | Offspring production, mating success, sperm motility | (Araripe et al. 2004; Batista et al. 2018; Chakir et al. 2002; David et al. 2005; Gefen and Gibbs 2009; Porcelli et al. 2016; Rohmer et al. 2004) |
| | Oriental fruit moth | *Grapholita molesta* | A 2h heat stress during pupation reduced fecundity but increased other adult fitness traits such as survival | Fecundity, gamete viability | (Zheng et al. 2017) |

| Taxonomic group | Organism | Species | Impact of temperature on fertility | Measure | Refs |
|---|---|---|---|---|---|
| | Wasp | *Aphidius avenae* | Low mating success rate due to reduced courtship behaviour. Reduced sperm count after developmental stress, with males at high stress fully sterile. Reduced fertilisation results in fewer females, secondarily altering sex ratios. Stressed females produce fewer eggs | Courtship behaviour, gamete number, fertilisation success and offspring production | (Nguyen et al. 2013; Roux et al. 2010) |
| Poales | Barley | *Hordeum vulgare* | Developing anther cells are compromised during thermal stress, while developing ovule cells are not | Gamete viability | (Oshino et al. 2007) |
| | Rice | *Oryza sativa* | High temperature during flowering increased pollen sterility, with greater sterility if $CO_2$ levels were high | Gamete viability | (Matsui et al. 1997) |
| Polemoniales | Tomato | *Solanum lycopersicum* | Under thermal stress pollen viability was reduced and anthers developed abnormalities. Thermally tolerant genotypes showed resistance | Gamete viability | (Müller et al. 2016) |
| Vertebrate | Chicken | *Gallus gallus domesticus* | An 8 week thermal stress results in increased sperm death and associated drop in fertility | Sperm concentration | (Karaca et al. 2002) |
| | Cow | *Bos taurus* | Ovulation failure and abortion rate is higher in cows inseminated during warm seasons | Fertilization | (De Rensis et al. 2017) |
| | Guppy fish | *Poecilia reticulata* | Males raised at stressful temperatures have shorter, slower sperm than individuals raised at benign temperatures | Sperm form and function | (Breckels and Neff 2013) |
| | Mouse | *Mus musculus* | Reduced sperm count for over 60 days after 30 minute heat shock | Gamete number | (Pérez-Crespo et al. 2008) |
| | Pig | *Sus sp.* | Sperm DNA damage higher and sperm concentration lower during warm wet season. | Sperm form and function | (Peña et al. 2018) |
| | Sea lion | *Otaria flavescens* | Stressed males desert females to thermoregulate, foregoing mating opportunities | Courtship and mating behaviour | (Campagna and Le Boeuf 1988) |
| | Zebra finch | *Taeniopygia guttata* | Daily heat waves reduced the proportion of sperm exhibiting normal morphology | Sperm form and function | (Hurley et al. 2018) |

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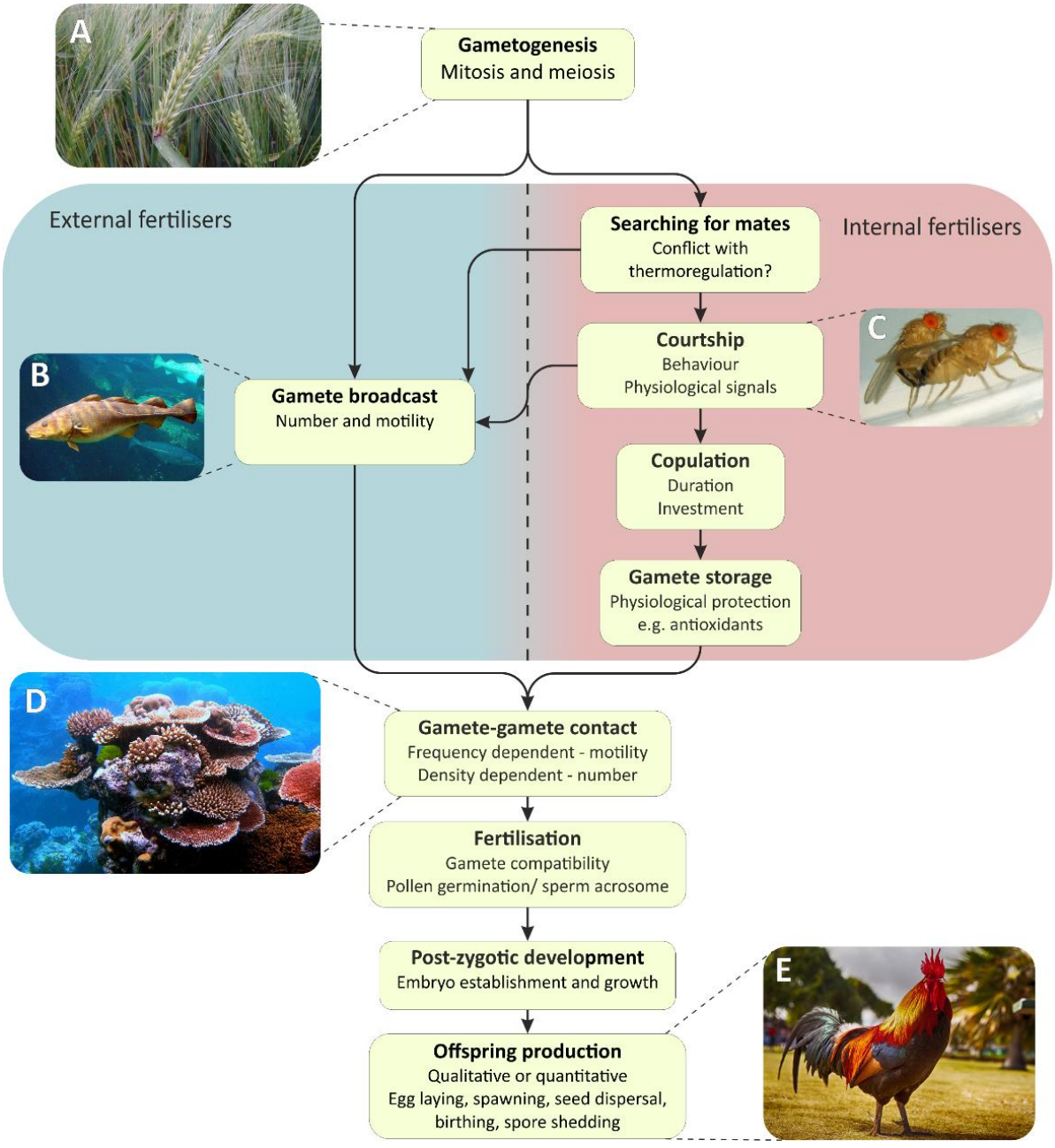


528

529 **Figure 0.2:** A generalized and simplified schematic of the stages in sexual reproduction

530 and examples of organisms for which the effect of temperature has been measured on

531 these stages (see Table 0.1)

Fertility is the emergent product of multiple physiological, developmental and behavioural processes. Not all steps are relevant to all organisms, indeed the diversity and complexity of this cascade across sexual organisms is not fully captured here. However, in all cases the 'success' of fertility begins by generating gametes and ends with the production of viable offspring. High temperature may perturbate single or multiple steps in this process but early meiotic stages can be particularly thermally sensitive (Sage et al. 2015). High temperature may affect several of these traits simultaneously within an individual, for example by both arresting gametogenesis and reducing investment in copulation behaviours. On the other hand, the effect of high temperature on a single trait, say testis development, may subsequently have cascading effects on downstream elements of reproduction such as sperm counts and motility. Photograph credits: (A) barley, Raul Dupagne; (B) cod, Hans-Petter Fjeld; (C) Drosophila mating, D. Chai; (D) coral reef, Toby Hudson; (E) rooster, Pete Linforth. All photographs licensed under CC BY 2.0.

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# Chapter 1: Sex-specific sterility caused by extreme temperatures is likely to create cryptic changes to the operational sex ratio in *Drosophila virilis*

Climate change is increasing the frequency and severity of short-term heat shocks that threaten the persistence of natural populations. However, most work addressing the evolutionary consequences of anthropogenic environmental change has focused on natural selection, with less attention paid to the impacts on sexual selection. The conditions under which sexual selection operates is a topic of debate, but a generally observed pattern is that the operational sex ratio (OSR) of a population is key to determining both the extent of competition for fertilizations and the scope for mate choice (Weir et al. 2011). Therefore, if high temperatures affect the ratio of reproductive males to females in a population this could influence sexual selection. Sub-lethal temperatures can sterilise individuals from a range of biological systems, including: plants, insects, corals, birds and mammals (reviewed in Walsh et al. 2019a). If high temperatures affect reproduction in one sex more than the other, this may create cryptic shifts in the operational sex ratio (OSR) of a population (Petry et al. 2016). However, although fertility loss at high temperatures is generally thought to be more common in males than in females (Iossa 2019), very few studies measure fertility in both sexes under identical conditions (Walsh et al. 2019b). Where sensitivity to temperature has been observed to vary between the sexes (Janowitz and Fischer 2011; Zwoinska et al. 2020),

790 the effect on population sex ratios has not been considered. Furthermore, natural
791 selection, sexual selection, and population dynamics are more likely to be affected by
792 biased sex ratios if sterility is long-lasting. However, to date patterns of sexually dimorphic
793 heat-induced sterility have not been shown over organisms' reproductive life spans. Here
794 we aim to test whether heat stress differentially affects male and female fertility in the
795 cosmopolitan fruit fly *Drosophila virilis* and if this creates cryptic bias in population sex
796 ratios over time. Specifically, we hypothesise that pupal heat-stress will significantly delay
797 adult sexual maturation and that this will be more severe in males compared to females
798 under identical conditions. To do this, we exposed pupal *D. virilis* to a sub-lethal heat
799 shock of 38°C for four hours to simulate the peak of a midday heat-wave. We chose to
800 heat pupae because they are immobile and cannot behaviourally escape heat-stress in
801 nature. We subsequently examined both complete sterility and pupal offspring
802 production over an ecologically realistic lifespan in both males and females. We combine
803 male and female time-series data to predict the effect of heat-induced sterility on the
804 OSR, and discuss its potential consequences for sexual selection.

805 We found that the rate at which newly eclosed *D. virilis* become fertile is significantly
806 influenced by the interaction between sex and temperature. While female fertility is not
807 significantly affected by heat-stress, male sexual maturation is significantly extended if
808 they are exposed to 38°C as pupae (Cox proportional hazard test interaction term: HR= -
809 1.4866, $\chi^2_{(1)} = 16.275$, $p < 0.001$; Figure 1.1a, 1.1b). Furthermore, we found that the
810 proportion of individuals that never produced offspring was predicted by a significant

811 interaction between sex and treatment, wherein males exposed to heat stress were
812 more likely than controls or females in any heat treatment to be rendered permanently
813 sterile ($\chi^2_{(1)} = 5.657$, p= 0.017; Figure S1.1). This is a relatively small effect, showing that
814 most males recovered fertility at some point during the experiment. We found that
815 control males reached sexual maturity 7 days post eclosion, in line with previous
816 observations. This results in an observed OSR for control males and females to stabilise at
817 0.5 from that point 7 days onwards (Figure 1.1c). In stressed males and females however,
818 the sterile males prevent the OSR reaching 0.5 over the 17-day duration of our
819 experiment. This results in an observed female bias in the sex ratio when flies are heated
820 as pupae (Figure 1.1c). In males, pupal heat stress significantly reduced pupal offspring
821 number by 58% (estimate= -0.870, $t_{(59,1)} = -3.925$, p< 0.001; Figure S1.2a), and variation in
822 the number of progeny from heated males was significantly lower than that in benign
823 males (F-test: $F_{(59, 1)}$= 2.837, p< 0.05). In females we find no significant effect of
824 temperature stress on pupal offspring number (estimate= -0.081, $t_{(69,1)}$= -0.928, p> 0.05,
825 Figure S1.2b), and there was no significant difference in variation of offspring number in
826 the two female treatments (F-test: $F_{(69,1)}$= 1.105, p> 0.05).

827 A small but significant proportion of males were permanently sterilised by pupal-heat
828 shock (~25%). A much larger proportion of males were rendered temporarily sterile
829 because heat-stress slowed post-eclosion sexual maturation, doubling maturation time
830 for some males. This delayed sexual maturation due to heat-stress supports findings from
831 other *Drosophila* species (Jørgensen et al. 2006). In contrast, females showed no

832 significant loss in fertility nor offspring production when stressed at sub-lethal
833 temperatures. Heat-delayed reproductive maturation in males but not females induces a
834 significant period of male sterility during which the population OSR is skewed. A major
835 question is whether our results capture what we would expect to see in natural
836 populations that experience extreme temperatures. Under benign temperature
837 conditions, male *D. virilis* eclose as sexually immature adults and become fully fertile over
838 five to seven days. We tracked fertility for up to 17 days, and almost half of heat-stressed
839 males did not become fertile until 11 days post eclosion. Best estimates suggest
840 *Drosophila* rarely survive beyond a few weeks as adults in nature (Powell 1997), so a loss
841 of fertility for even a few days could seriously impact individual fitness. This effect would
842 be particularly acute in populations and species whose life-history and phenology permit
843 limited time windows for reproduction. Further, in our study focal flies are given optimal
844 conditions and opportunity to reproduce (multiple mates, no competition, *ad libitum*
845 food, and a stable benign environment as adults). Despite these ideal conditions we still
846 see significantly higher permanent sterility in males that experience heat stress compared
847 to control males and both female treatments. These results demonstrate that sexual
848 dimorphism in sub-lethal thermal tolerance traits has the potential to shift the OSR of
849 heat-stressed populations across time. This would result in a heavily female-biased
850 populations in which the availability of fertile mates is scarce over shorter periods in
851 nature, possibly driving plastic or evolutionary changes in reproductive behaviour.
852 Whether the OSR shifts we see in our data would be sufficient to drive evolutionary
853 rather than plastic responses, and whether responses would be through sexual or natural

selection are open questions. Ultimately the selective strength of OSR biases will depend on both the short-term duration of sterilizing events and the long-term frequency of such events.

A key finding in our data is that shifts in the OSR happen at sub-lethal temperatures, and so are not reflected in the observable adult sex ratio. This is in contrast to observable temperature-driven sex ratio shifts in species with temperature-dependant sex determination. Therefore, cryptic sterility presents a problem for biologists trying to link observable sex-ratios in nature with evolutionary processes. Further, if cryptically sterile males behave like fertile males this could influence female mating behaviour. For example, heat sterilised *Drosophila pseudoobscura* males continue to court and mate females normally, which forces females to remate to become fertilised (Sutter et al. 2019). Increased mating rates can in turn result in female harm through direct damage, ejaculate proteins or sexually transmitted infections, all of which have been implicated in driving sexual and natural selection. Measuring how heat-induced cryptic sterility biases sex ratios and how this influences sexual selection, natural selection, and population dynamics, will inform our understanding of how climate change affects natural populations.

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## Author contributions

**Benjamin S. Walsh:** conceptualisation (lead), methodology (lead), validation (lead), formal analysis (lead), investigation (lead), data curation (lead), writing- original draft (lead), writing- review and editing (lead), visualisation (lead). **Natasha M. Mannion:** methodology (supporting), writing- review and editing (supporting). **Tom A. R. Price:** resources (lead), writing- review and editing (supporting), supervision (supporting), project administration (lead), funding acquisition (lead). **Steven R. Parratt:** conceptualization (supporting), methodology (supporting), formal analysis (supporting), supervision (lead), project administration (supporting), writing- original draft (supporting), writing- review and editing (supporting), visualisation (supporting).

## Data and materials availability

All data and analysis R code will be deposited on Dryad upon acceptance of this manuscript.

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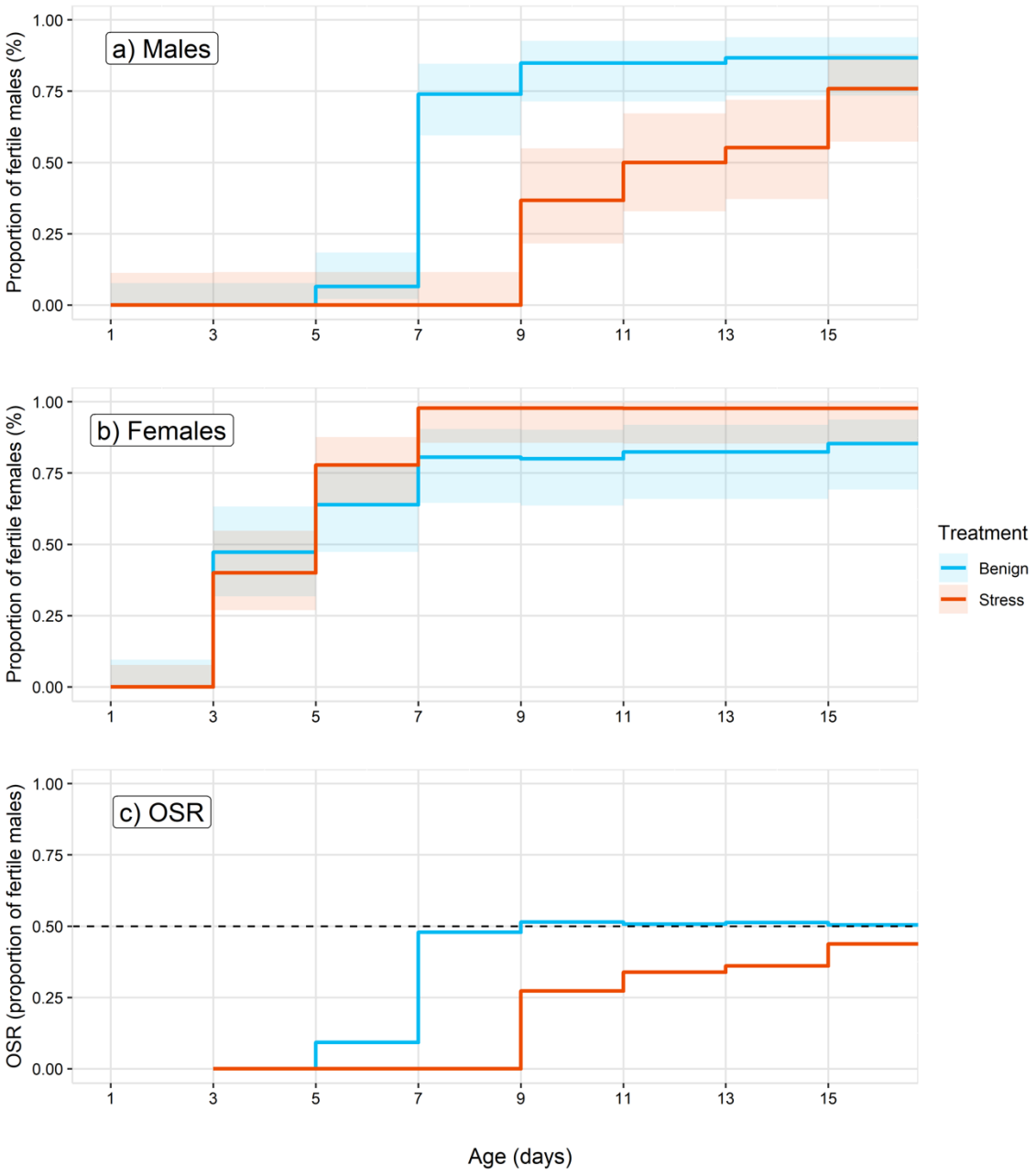


890

891 **Figure 1.1.** Cumulative proportion of a) male and b) female *D. virilis* that were

892 qualitatively fertile at each time point post-eclosion. Individuals were either kept at

893 benign temperatures (23°C) or stressed (4h at 38°C) during the pupal stage. Sample sizes:

benign males= 45, stressed males=29, benign females=35, stressed females=45. Both sexes eclose as sexually immature adults and become fertile as they sexually mature. This rate of maturation is significantly slower in males that have been exposed to 38°C heat shock as pupae. Error ribbons represent 95% confidence intervals estimated from survival model fits. c) Estimated operational sex ratio based on fertility patterns in a) and b) (OSR, proportion of fertile males as the proportion of all fertile adults). Horizontal dashed line represents a 1:1 sex ratio.

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# Supplementary information

## Materials and methods

### Animal stock maintenance

Stocks of *Drosophila virilis* (Cambridge Fly Facility StrainvS-4, isolated in 1991), were kept in a temperature-controlled room at 23°C, 12:12 L:D and ambient humidity, selected based on observations of when the laboratory populations are most stable. Stocks were maintained at moderate density (50 – 100 flies per 300ml bottle culture) on 'Propionic' medium (10g agar, 20g yeast extract, 70g cornmeal, 10g soya flour, 80g malt extract, 22g molasses, 14ml 10% nipagin, 6ml propionic acid, 1000ml $H_2O$). Ovipositing adults were tipped to new food every week to keep density relatively constant and prevent overlapping generations. Ovipositing adults were replaced with younger adult flies every 4-6 weeks.

### Assaying for sexual dimorphism in thermally induced sterility

*D. virilis* are not sexually mature when they first eclose, males and females reach maturity 6 and 9 days post-eclosion respectively (Pitnick et al. 1995). We first hypothesise that pupal heat-stress will induce a significantly longer period of complete sterility post-eclosion compared to controls. We further hypothesise that this effect will be sexually dimorphic, in that males will be rendered completely sterile for longer than females under identical pupal heat-stress conditions.

We based our assay for temperature induced sterility on Jørgensen et al. (2006), wherein the authors demonstrated that a 4-hour heat-stress of adult *D. buzzatii* suppressed male fertility. Whilst other work has used life-long stress to sterilize *Drosophila spp.* (Rohmer et al. 2004), a 4-hour shock arguably better captures ecological reality by replicating the peak of a heatwave in the middle of the day. Unlike Jørgensen et al. (2006), we applied heat stress to early-stage pupae, and tested both males and females. We use pupae to test if early-life heat stress can completely prevent reproduction in adults. Pupae are sedentary and so would be unable to behaviourally thermoregulate in nature, unlike adults.

Focal animals for our experiments were collected directly from stock bottles within 24 hours of pupation and allocated at random into groups of 30 in fresh 25 x 95mm plastic vials containing 25ml standard 'ASG' medium (10g agar, 85g sucrose, 20g yeast extract, 60g maize, 1000ml $H_2O$, 25ml, 10% Nipagin) to prevent desiccation. We did not directly control rearing density in our flies, but pupae were taken from stocks of similar age and were randomly allocated across treatments to homogenise any variation due to density during rearing. We use ASG because pilot experiments showed that the propionic acid in the 'Propionic' food reduces survival of pupae when heated. Immediately after collection, 180 pupae (3 vials containing 30 pupae per treatment) were randomly assigned to pre-heated water-baths at either a benign (23°C) or a stressful temperature (38°C) for 4 hours between 10am to 2pm. Preliminary experiments showed 38 °C to be the highest

978 temperature at which we do not see significant heat-induced mortality (Supplementary
979 Figure S1.3, Sample sizes: 23°C= 81, 37°C= 60, 38°C= 60, 39°C= 80, 40°C= 80, 41°C= 80.).

980 Following heat-stress, vials were returned to temperature-controlled rooms set at benign
981 temperature (23°C) and flies were observed daily for eclosion. In total, 35 female and 45
982 male adults eclosed from 'benign' pupae, and 45 female and 29 male adults eclosed from
983 pupae stressed at 38°C. "The sex ratio of emerging individuals did not significantly deviate
984 from the expected 1:1 at either 23°C (exact binomial test: $p = 0.38$), nor 38°C (exact
985 binomial test; $p = 0.14$). At eclosion, all flies of both sexes were isolated as virgins into
986 individual vials containing 'Propionic' food and four sexually mature virgin partners from
987 the opposite sex. We used four partners as it reduces the risk of false negative fertility
988 scores due to failures to copulate through mate-choice, or any inherent sterility in the
989 non-focal flies. Mating partners were reared from stock populations at 23°C and were 7-9
990 days post-eclosion to ensure sexual maturity (Pitnick et al. 1995). All five flies in each vial
991 (one focal male with four females, or one focal female with four males) were tipped into a
992 fresh vial of 'Propionic' food every 2 days for 15 days and all flies were discarded on day
993 17 (resulting in 8 vials of offspring per focal fly). Vials for all focal individuals from every
994 time point were kept at benign temperatures (23°C) for days 1 to 10 of the experiment,
995 and were then transported to fluctuating room temperatures (approximately 18 – 22°C,
996 UK room temperatures in early March 2020) for days 11 onwards because of a shift to
997 home-working due to the 2020 COVID-19 pandemic. This change in rearing temperature
998 was applied to all treatments equally, and given that *D. virilis* is a hardy cosmopolitan

species associated with human habitats (Mirol et al. 2008), it is unlikely this had a significant impact on individuals' ability to copulate, oviposit nor on offspring development.

We scored fertility (either completely sterile or able to sire at least one offspring) by observing the presence/absence of larvae directly in vials or by identifying the distinctive larval tracks in the food. We counted offspring production as the number of pupal cases adhered to the vial wall on the first days that adult F1 emergence was observed for that time-point. Pupal case number rather than true adult progeny counts were used due to practical limitations of home-working, but *D. virilis* lay offspring in relatively low density and almost always pupate away from their food which facilitates accurate counting.

**Statistical analyses**

All statistical analyses were completed in R (version 3.5.0), using the packages: binom (Dorai-Raj 2014), car (Fox 2011), and "ggplot2" (Wickham 2016) "survival" (Therneau 2015),.

**a) Fertility over time**

We analysed the effect of heat stress on fertility over time with inverse Cox proportional hazard survival analyses (using the "survival" package (Therneau 2015)). This allowed us to model the time in days post-eclosion until focal individuals become fertile. We fit the time point at which fertility (scored as the presence of offspring) was observed as our

response variable with sex (male or female), heat treatment (benign or stress), and their interaction as independent variables.

Some individuals never produced offspring during the experiment and so were scored as 'permanently sterile'. To determine if heat-stress increases permanent sterility, we performed a logistic regression with permanent sterility as a Bernoulli response variable and sex (male or female), heat treatment (benign or stress), and their interaction as explanatory variables. Significance of predictors and interactions was determined with Wald $Chi^2$ tests implemented in the `car` R package (Fox 2011).

**b) Offspring production**

We analysed the cumulative number of offspring produced over the 17-day mating period by fertile flies in heat stressed and non-stressed treatments. This investigates if the heat-stressed flies that maintained fertility have lower lifetime reproduction than non-stressed flies. We removed permanently sterile individuals from this dataset, because we cannot be certain that counts of 0 are generated by the same biological process as variation in integer counts. Also, any variation in the ratio between 0 counts and non-0 counts is captured in our analysis of fertility above, so including completely sterile individuals in analysis of offspring number partially re-reports this previous result (for offspring counts including 0s see Fig S1.4). Our sample sizes for offspring counts were: benign females=29, stressed females=42, benign males= 39, stressed males=22.

We tested the effect of heat treatment independently for males and females because focal males had four females to produce offspring with but focal females oviposited alone. As offspring number is typically female-driven, it is inappropriate to directly compare the two sexes – however this experimental design was necessary to maximise our detection of fertility. We used generalised linear models with quasi-Poisson distributions because of the count nature of the data and because Poisson model residuals were overdispersed.

**c) Operational sex ratio**

We combined our data on male and female complete sterility curves (see (a) above) over time to predict the OSRs for our temperature treatments. We did this by calculating the proportion of fertile males by the total proportion of fertile adults. This inherently corrects for any difference sin sample sizes in male and female treatments and allows for a potential 1:1 sex ratio. When calculated this way, the OSR can range from 0% where only females are fertile in the population, to 100% where only males are fertile in the population (Kvarnemo and Ahnesjo 1996). Because these predicted sex ratios are the product of our total observed data, we do not have variance with which to statistically test deviation from the expected 0.5. Rather, this serves as an illustration of the effect of heat on OSR.

## Results

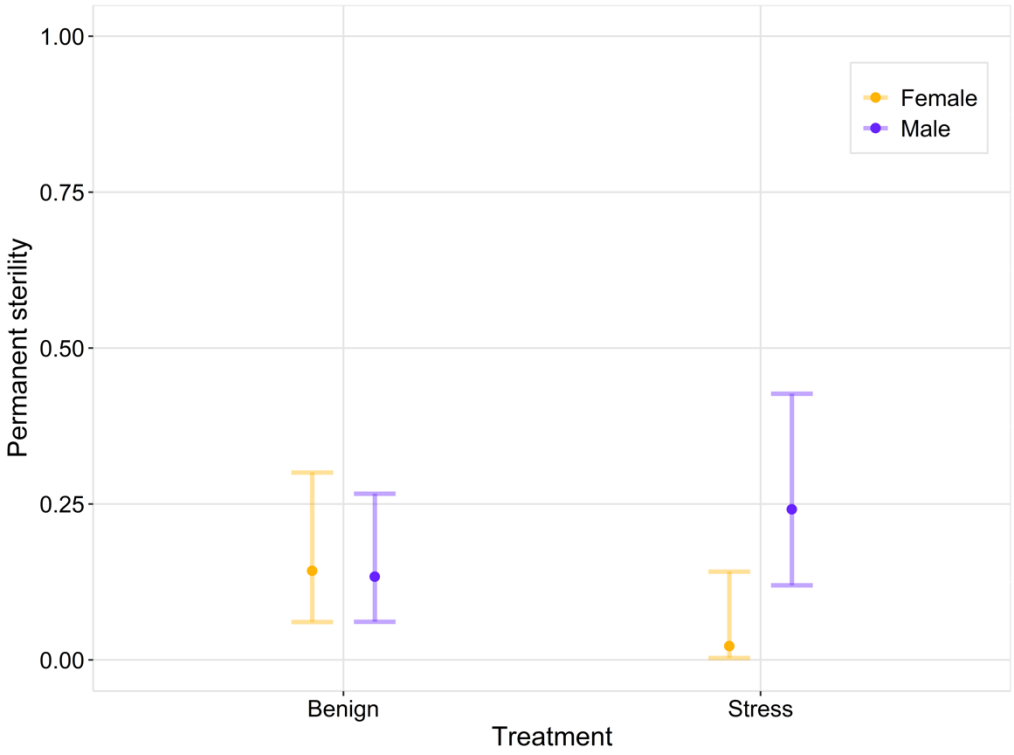



**Figure S1.1.** Proportion of male and female *D. virilis* that produced no offspring at all during the experiment (up to 17 days post-eclosion). Individuals were either kept at benign temperatures (23°C), or stressed (4h at 38°C) during the pupal stage. Error bars are 95% confidence intervals. Males exposed to heat stress were more likely than controls or females in any heat treatment to be rendered permanently sterile ($\chi^2_{(1)}$= 5.657, p= 0.017). Sample sizes: benign males= 45, stressed males=29, benign females=35, stressed females=45.

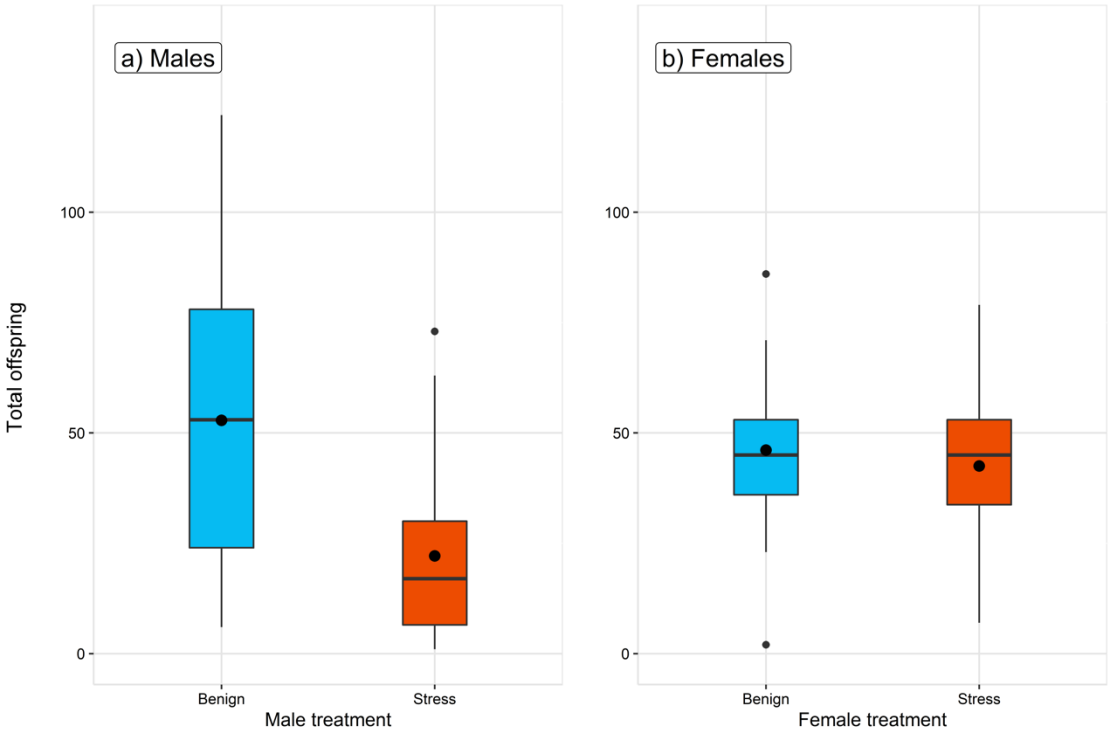
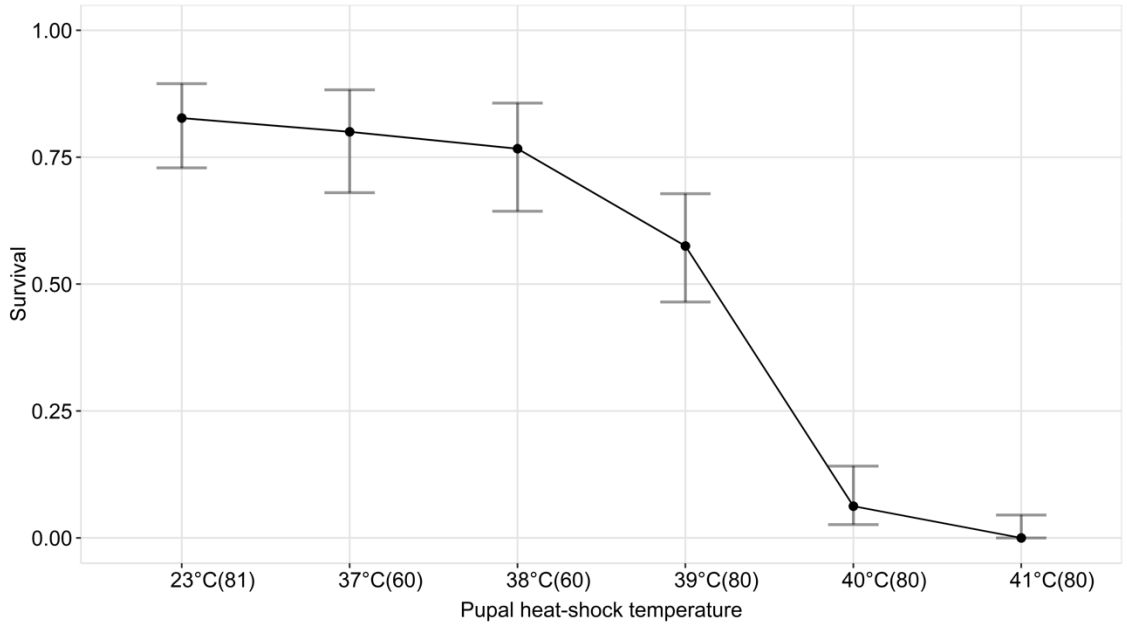
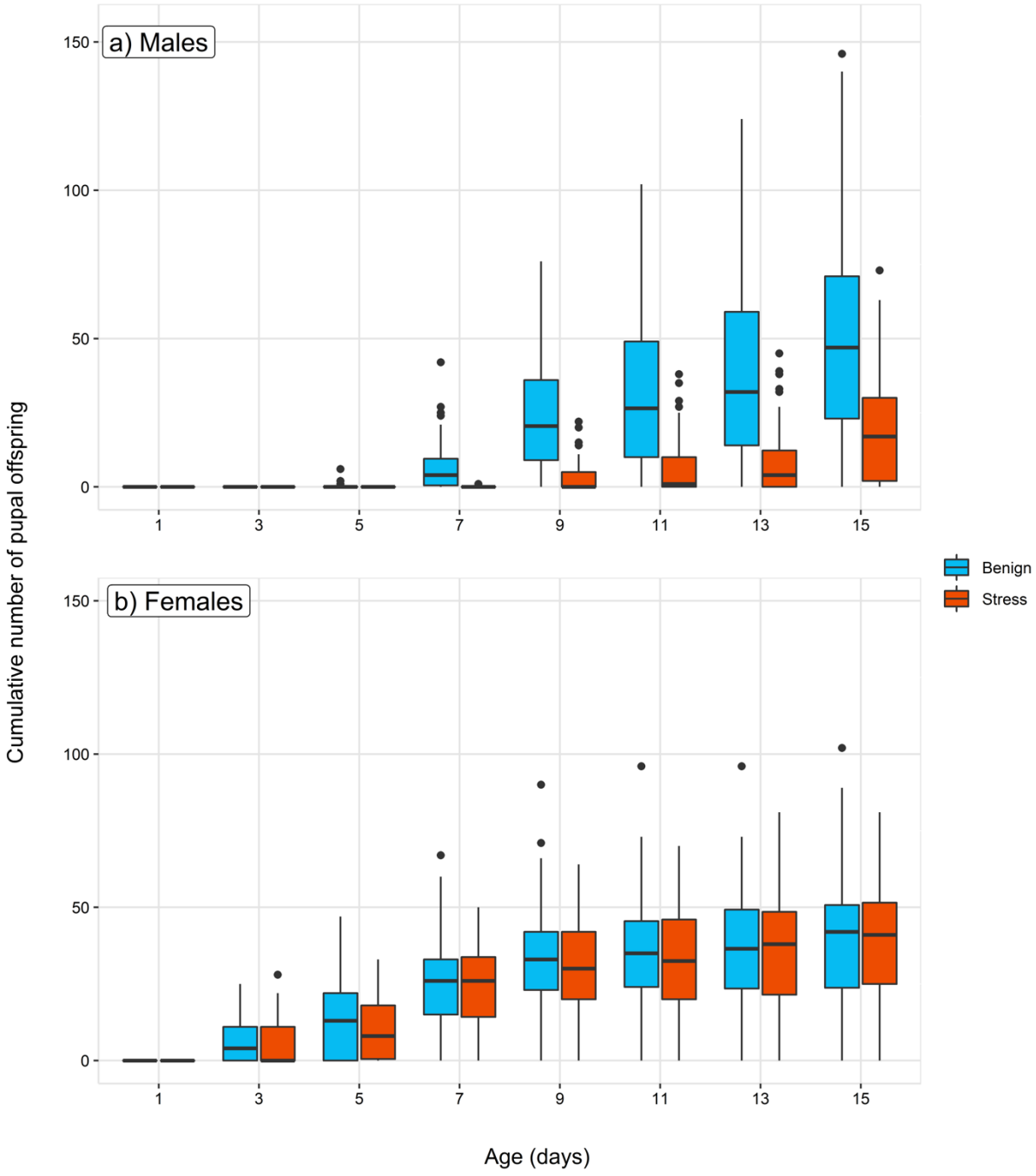



**Figure S1.2.** Total cumulative offspring number produced (laid or sired) by fertile focal individuals throughout the course of the experiment, when paired with 4 partners for 17 days. Black dots inside boxplots represent mean offspring number for each treatment. Individuals were either kept at benign temperatures (23°C) or stressed (4h at 38°C) during the pupal stage. In males, pupal heat stress significantly reduced pupal offspring number by 58% (estimate= -0.870, $t_{(59,1)}$= -3.925, p< 0.001). In females we find no significant effect of temperature stress on pupal offspring number (estimate= -0.081, $t_{(69,1)}$= -0.928, p> 0.05) Sample sizes: benign males= 39, stressed males= 22, benign females= 29, stressed females= 42.




1074

1075 **Figure S1.3**. Preliminary data demonstrating the proportion of pupae that eclose after a
1076 4h heat stress across a range of temperatures. We used these data to select 38°C as our
1077 stress temperature for the fertility assays because as it was the highest temperature that
1078 did not impose mean pupal survival below the 95% confidence intervals of survival at
1079 benign (23°C). Error bars are 95% confidence intervals. Pupal survival was assayed by
1080 heating pupae in the same way as described in the methods, but across 6 temperature
1081 treatments. Because it is difficult to record pupal death immediately following stress, we
1082 left flies to develop for 2 weeks and the number of eclosing adults were counted. Errors
1083 are 95% confidence intervals calculated with a logit link in the “binom” R package (Dorai-
1084 Raj 2014). Sample sizes are presented in brackets on the x-axis labels.




1085

1086 **Figure S1.4.** Cumulative offspring numbers produced (laid or sired) by a) male and b)

1087 female focal individuals at each measured time-point, when paired with 4 partners.

Individuals were either kept at benign temperatures (23°C) or stressed (4h at 38°C) during the pupal stage. We include counts of 0 here to illustrate how recovery of fertility happened in males but they do not recover lifetime offspring production. Sample sizes: benign males= 45, stressed males=29, benign females=35, stressed females=45.

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# Chapter 2: Plastic responses of survival and fertility following heat stress in pupal and adult *Drosophila virilis*


## Abstract

The impact of rising global temperatures on survival and reproduction is putting many species at risk of extinction. In particular, it has recently been shown that thermal effects on reproduction, especially limits to male fertility, can underpin species distributions in insects. However, the physiological factors influencing fertility at high temperatures are poorly understood. Key factors that affect somatic thermal tolerance such as hardening, the ability to phenotypically increase thermal tolerance after a mild heat shock, and the differential impact of temperature on different life stages, are largely unexplored for thermal fertility tolerance. Here, we examine the impact of high temperatures on male fertility in the cosmopolitan fruit fly *Drosophila virilis*. We first determined whether temperature stress at either the pupal or adult life-history stage impacts fertility. We then tested the capacity for heat-hardening to mitigate heat-induced sterility. We found that thermal stress reduces fertility in different ways in pupae and adults. Pupal heat stress delays sexual maturity, whereas males heated as adults can reproduce initially following heat stress, but become sterile within seven days. We also found evidence that while

heat-hardening in *D. virilis* can improve high temperature survival, there is no significant protective impact of this same hardening treatment on fertility. These results suggest that males may be unable to prevent the costs of high temperature stress on fertility through heat-hardening which limits a species' ability to quickly and effectively reduce fertility loss in the face of short-term high temperature events.

**Keywords**: sterility, plasticity, reproduction, climate change

# Introduction

Climate change is increasing the frequency of extreme temperature events (Christidis et al. 2015). A major research priority is to assess which organisms will be able to maintain fitness and cope with the changing climate. Initial efforts to explore the impact of rising temperatures on biodiversity mostly considered how thermal stress affects survival (Deutsch et al. 2008; Kellermann et al. 2012; Pinsky et al. 2019). While the impact of climate change on survival is clearly important, it has also been known for around a century that fertility is vulnerable to high temperatures in some species (Cowles 1945; Young and Plough 1926). In this paper, we use fertility to mean the ability to produce offspring; the direct opposite of sterility. We use this definition because complete sterility has the potential to be extremely important in a warming world (Parratt et al. 2021; van Heerwaarden and Sgrò 2021; Walsh et al. 2019). Heat-induced sterility occurs across diverse taxa including crops (Matsui et al. 1997) and livestock (Karaca et al. 2002), so species where fertility is lost at temperatures far below the lethal limit may represent both a major economic and conservation concern (Walsh et al. 2019) with potentially

worrying implications for humanity's resilience against climate change. Fertility loss is generally sex-specific, with males often more sensitive to fertility loss than females (Iossa 2019; Sales et al. 2018; Walsh et al. 2020; Zwoinska et al. 2020). Recent work has found that the highest temperatures *Drosophila* species are found at worldwide is strongly correlated to laboratory measurements of their lethal temperature, or the temperature at which males lose fertility, whichever is the lower (Parratt et al. 2021; van Heerwaarden and Sgrò 2021). This suggests that species distributions may often be restricted by their upper thermal limits to fertility in nature. However, we still know relatively little about the physiological factors that affect fertility loss at high temperatures.

In holometabolous insects, it is widely known that survival at high temperatures can be affected by the life-stage at which thermal stress occurs (Moghadam et al. 2019; Zhang et al. 2015). Studies on heat-induced sterility in males typically use either a single long-term stress across age-groups (Porcelli et al. 2016; Rohmer et al. 2004), or an acute stress to individuals from a single age-group (Jørgensen et al. 2006; Jørgensen et al. 2021; Sales et al. 2018; Walsh et al. 2020). However, it has recently been shown in the flour beetle *Tribolium castaneum* that the extent of male fertility loss depends on the life-stage exposed to thermal stress (Sales et al. 2021). Here, pupal and immature adults show the highest sterility after thermal stress as compared with larval and mature adults. This study reveals a critical period in the life-cycle of *T. castaneum* where fertility is particularly vulnerable to heat-stress of immature individuals. In order to uncover any general

1181 patterns in thermal sensitivity of fertility across life-stages, research should examine this
1182 across species.

1183 One way organisms can cope with thermal stress is to plastically invest resources into
1184 thermal protection after receiving a signal that the risk of extreme high temperatures has
1185 increased. For example, exposure to a short-term moderately stressful sub-lethal heat can
1186 cause organisms to make physiological changes that allow them to better survive extreme
1187 temperatures (Loeschcke and Hoffmann 2007; Moghadam et al. 2019). This response is
1188 called heat hardening, and is widespread in animals and plants (Bilyk et al. 2012;
1189 Moghadam et al. 2019; Neuner and Buchner 2012). The positive impacts of hardening in
1190 ectotherms are generally thought to occur through the upregulation of heat-shock
1191 proteins such as HSP70 (Sørensen et al. 2001). When the individual thereafter
1192 experiences extreme temperatures, the increased concentration of heat-shock proteins
1193 reduces the thermal damage. Hardening has been shown to mitigate the deleterious
1194 effects of high temperatures on a multitude of traits, including survival (Heerwaarden et
1195 al. 2016; Moghadam et al. 2019) and the ability to locate resources such as food or
1196 mating sites (Loeschcke and Hoffmann 2007). In the fruit fly *Drosophila melanogaster*,
1197 individual survival is improved at high temperatures through hardening, however the
1198 amount of protection provided changes depending on the life-stage measured
1199 (Moghadam et al. 2019). In this case, pupae show strong protection through heat-
1200 hardening, whereas adults' hardening capacity is minimal. Clearly, a full understanding of
1201 heat-hardening itself is difficult without examining multiple life-stages.

While the capacity of individuals to improve survival through heat-hardening is widespread, it remains unclear whether individuals can utilise hardening to mitigate heat-induced sterility. Some studies suggest that there is a trade-off between hardening and reproduction (Krebs and Loeschcke 1994), but other examples found hardening improves mating behaviour (Sambucetti and Norry 2015) and, in a few species, heat-hardened individuals show greater offspring production after thermal stress (Jørgensen et al. 2006; Sarup et al. 2004). Heat-induced sterility occurs at sub-lethal temperatures in many organisms (Walsh et al. 2019), including ~44% of a panel of 43 *Drosophila* species (Parratt et al. 2021). So it is likely that, in the marginal populations of particularly vulnerable species, a male's fitness could be greatly improved by maintaining fertility at sub-lethal stress temperatures. If males can plastically harden to prevent fertility loss at extreme temperatures, then populations may have the capacity to better cope with sub-lethal but stressful heat events.

Here, we explore the impact of high temperatures on male fertility in the cosmopolitan fruit fly *Drosophila virilis*, an extremely widespread model species. Critically, it has previously been demonstrated that male *D. virilis* can be sterilised by thermal stress well below their lethal temperature limit (80% of adult males sterile after four hours at 35°C, 80% of adult males dead after four hours at 38°C) (Parratt et al. 2021; Walsh et al. 2020). This sterilisation of males at survivable temperatures makes *D. virilis* an ideal species to look for heat hardening of fertility. We test the impact of temperature stress on fertility across life-history stages, heating individuals as either pupae or adults. Further, we

demonstrate the capacity for heat-hardening to improve survival at extreme temperatures and subsequently test if this hardening response can also mitigate heat-induced sterility. Importantly, we measure how fertility changes over an individual's age, to better understand the long-term fitness implications of thermal stress and hardening at different life-stages.

# Materials and Methods

In overview, we test if heat-shocks experienced during pupal and adult life-history stages result in male sterility. We also test if a brief period of heat-hardening can ameliorate these effects. In a series of experiments, adult and pupal male *D. virilis* were exposed to a 1 hour heat hardening treatment followed immediately by a 4 hour heat stress. They were then immediately assayed for survival, and their fertility was subsequently measured over 1-2 weeks to reveal temporal patterns in fertility loss and restoration. We chose a 4 hour stress because midday rises to high temperature are relatively common (Geletič et al. 2020), and we think it is ecologically reasonable that a fly in nature might be exposed to these conditions for a few hours. Moreover, it is an experimentally tractable time period, and previous work has demonstrated this method can create male sterility in many *Drosophila* species, including *D. virilis* (Parratt et al. 2021; Walsh et al. 2020).

**Animal stock maintenance**

Stocks of *Drosophila virilis* (Cambridge Fly Facility StrainvS-4, isolated in 1991), were kept in a temperature-controlled room at 23°C, 12:12 L:D and ambient humidity. Although a

long term laboratory stock, this stock was included in a recent analysis of upper thermal limits from 36 *Drosophila* species that found no significant association between time in culture and any upper thermal limit (Parratt et al. 2021), suggesting it is a reasonable model for the species. Stocks were maintained at moderate density (50 – 100 flies per 300ml bottle culture, representing a low level of larval crowding) on 'Propionic' medium (10g agar, 20g yeast extract, 70g cornmeal, 10g soya flour, 80g malt extract, 22g molasses, 14ml 10% nipagin, 6ml propionic acid, 1000ml $H_2O$). Ovipositing adults were tipped to new food every week to prevent overlapping generations and were replaced with fresh sexually mature adult flies every 4-6 weeks.

**Pupal heat-stress**

**Survival**

Pupae were collected from stock vials within 24 hours of pupation, allocated to vials of 20 pupal flies. Three vials were allocated to each treatment (giving 60 flies total per treatment, ~30 males, as sex cannot be determined in young pupae). These vials were randomly assigned to 3D-printed floating racks into pre-heated water baths (Grant TXF200) for 1 hour at either a control non-hardening temperature at 23°C ('no hardening') or a range of hardening temperatures ('hardening': 34, 35 & 36°C). These are non-lethal pupal temperatures that also do not significantly sterilise males (Walsh et al. 2020). After this hardening treatment, they were immediately moved into different pre-heated water-baths for 4-hours at either 23°C ('benign') or at a range of five sub-lethal to

lethal temperatures (37, 38, 39, 40, 41°C: 'stress'). Immediately following treatment, vials were returned to benign conditions (23°C) and emerging individuals were collected and sexed. This allowed us to assess survival of pupae at extreme temperatures, and gave us an idea of whether survival may be sex specific. However, as we were unable to determine the sex of the pupae prior to stress, we could not explicitly test for sex differences in survival thermal tolerance.

**Fertility**

Pupae were allocated to 3D-printed floating racks in pre-heated water-baths set to 23°C ('no hardening') or 36°C ('hardening') for 1h as above. Immediately following hardening, they were transferred into pre-heated water baths at 23°C ('benign') or 38°C ('stress'), chosen as the highest temperature not resulting in significant mortality from a prior study (Walsh et al. 2020). After four hours at their treatment temperature, vials were subsequently removed from the water-baths and returned to benign temperatures (23°C). Emerging males were collected and immediately moved into individual vials with 4 sexually mature virgin female partners each. These groups were moved into new vials every 2 days for 7 times, giving a total of 8 vials across 16 days where fertility was recorded. Age at reproductive maturity (ARM) was taken as the time-point (days post-pupation) of a males' first fertile vial. Estimates of *Drosophila* survival rates in nature suggest 16 days represents a substantial portion of their expected adult lifespan (Powell 1997). Males were deemed as qualitatively fertile at any given time-point if there was

evidence of larvae present in the vial (either via direct observation of larvae or observing larval tracks in the food).

**Adult heat-stress**

**Survival**

Virgin males and females (all 7 days old) were separated and allocated to vials of 10 flies per vial of their respective sex. These vials were randomly allocated to 3D-printed floating racks in pre-heated water-baths for one hour at a hardening temperature at 23°C ('no hardening') or 33°C ('hardening', determined as the highest temperature in which no sterility is observed (Parratt et al. 2021)). After this hardening treatment, vials were immediately moved into different pre-heated water-baths for four hours at either 23°C ('benign') or 38°C ('stress', determined as lowest lethal temperature from (Parratt et al. 2021)). Immediately following treatment, vials were returned to benign conditions (23°C) and left for 24 hours to ensure that any flies that were immobilised by heat but not killed could recover. After 24 hours, the number of surviving males and females from each treatment was assessed.

**Fertility**

Virgin males were allocated to vials (10 per treatment) and treated in pre-heated water-baths at 23°C ('non-hardening') or 33°C ('hardening') for 1h as above. Immediately following heat-hardening, flies were transferred into pre-heated water baths at 34°C for a

1302 further 4 hours ('stress', chosen as the lowest whole-degree Celsius temperature at which
1303 *D. virilis* are sterilised (Parratt et al. 2021)). Vials were subsequently removed from the
1304 water-baths and males were placed in new individual vials with 4 virgin female partners
1305 each. Previous experiments have shown that, when stressed as adults, male *D. virilis*
1306 initially retain fertility for several days and then become sterilised (Parratt et al. 2021).
1307 Hence, unlike our assay with pupal-stress flies, we did not passage males to new vials
1308 every 2 days immediately. Instead, we gave males an initial 7-day period in a single vial
1309 with 4 females. We then gave each male 4 new virgin females and passaged each group
1310 every 2 days for 4 times.

1311 **Statistical analyses**

1312 Measuring fertility which is a long-term adult trait when individuals are heated during
1313 different life-stages introduces significant temporal biases. We decided to measure
1314 fertility from the earliest possible time-point post-stress, and continue to measure over
1315 time. This allowed us to capture any visible loss/regain of fertility. Flies do not breed as
1316 pupae, so fertility cannot be measured immediately following heat-stress during this
1317 stage. Therefore, in order to understand how these responses change depending on life-
1318 stage, we measured fertility over a substantial period of time after stress for both pupae
1319 and adults. Due to the inherent differences this introduced, we analysed pupal and adult
1320 heat-stress separately, so comparisons of responses between stages can only be
1321 qualitative.

Data were analysed using variations on linear models. We assessed model fit by plotting patterns in residuals against fits and against predictors. All statistical analyses were completed in R (version 3.5.0), using the packages: binom (Dorai-Raj 2014), car (Fox 2011), "ggplot2" (Wickham 2016) and "survival" (Therneau 2015). We did model selection using Wald Chi-squared likelihood ratio-tests, removing non-significant interactions. We retained all main effects and reported statistics of these from type II likelihood ratio tests using the 'Anova' function from the 'car' package (Fox 2011).

**1a) Pupal survival after heat-stress**

We chose 36°C as our single experimental 'hardening' temperature since it is the highest temperature that does not reduce fertility when males experience it for 4h (Parratt et al. 2021; Walsh et al. 2020). We analysed pupal survival after heat stress using a logistic regression with survival as a Bernoulli response variable. Stress temperature, hardening treatment (non-hardened or hardened at 36°C), and their interaction were fitted as explanatory variables. To determine whether the hardening temperature altered its protective effect, we analysed pupal survival of all flies hardened at 34, 35, and 36°C prior to heat stress at the key stress temperature of 40°C where protection is observed. We performed a logistic regression with survival as a Bernoulli response variable. We used hardening temperature as the explanatory variable. Note that the 34 and 35°C hardening temperatures were not measured at 37 and 38°C temperature stress at this preliminary stage, as these temperatures are non-lethal after a 4h stress (Walsh et al. 2020).

**1b) Adult survival after heat-stress**

As every fly stressed at control temperatures (23°C) survived, we analysed adult survival at the chosen stress temperature (38°C) only, using a logistic regression with survival as a Bernoulli response variable and sex (male or female), hardening treatment (non-hardened or hardened), and their interaction as explanatory variables.

**2a) Pupal fertility over time**

We analysed the effect of heat stress on fertility over time with inverse Cox proportional hazard survival analyses (using the "survival" package (Therneau 2015)). This allowed us to model the time in days post-eclosion until focal individuals become fertile. We fit the first recorded time point at which fertility was observed (ARM) as our response variable with heat treatment (benign or stress), hardening treatment (non-hardened or hardened) and their interaction as independent variables.

**2b) Adult fertility over time**

We examined whether there was an immediate effect of heat stress on fertility, and whether hardening affects this response. We used a logistic regression with day 1 fertility as a Bernoulli response variable and stress (benign or stressed), hardening treatment (non-hardened or hardened), and their interaction as explanatory variables.

Adult fertility over time was analysed using two separate approaches due to the observed delayed sterility and how the experimental design was constructed around it. This

allowed us to pull apart different hypotheses and test them. We first tested whether heat-stress reduced fertility from day 7 onwards compared to benign temperature controls, due to delays in adult sterility. To do this we used a mixed effect logistic regression on non-hardened flies, with fertility as a Bernoulli response variable and stress, time, and their interaction as explanatory variables. Fly ID was used as a random effect to account for non-independence in the data.

We then tested whether hardening can improve fertility over time in stressed males. We used a mixed effect logistic regression on stressed flies, with fertility as a Bernoulli response variable and hardening, time, and their interaction as explanatory variables. Fly ID was used as a random effect to account for repeated measures in the data.

# Results

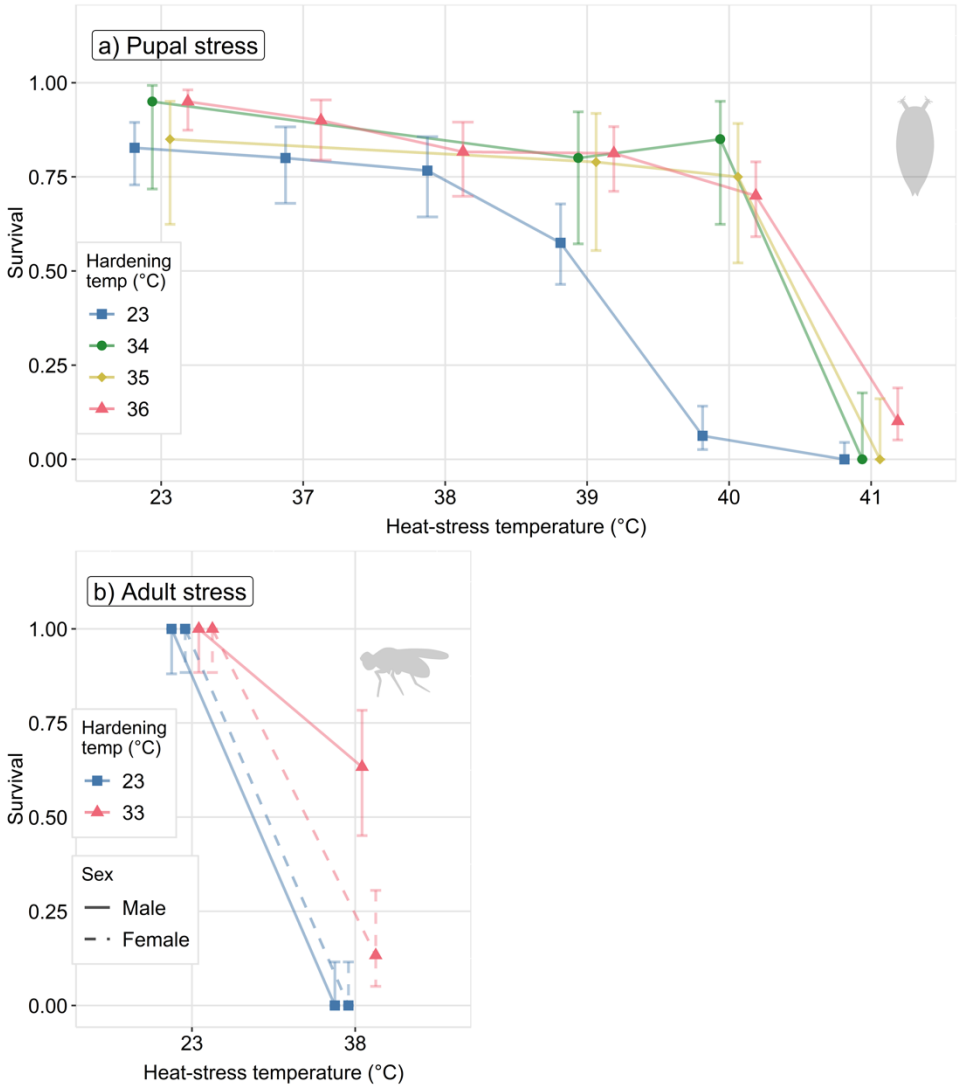


**Figure 2.1.** Proportion of surviving individuals after a 4-hour heat stress. Focal individuals were subjected to a pre-stress 'hardening' treatment for 1-hour immediately prior to temperature stress. **a)** *D. virilis* individuals of unknown sex were heated during the pupal stage and subjected to a range of stressful temperatures. A range of hardening

temperatures were also used to examine the hardening response. Note that the 34 and 35°C hardening temperatures were not measured at 37 and 38°C temperature stress. **b)** Male and female *D. virilis* were heated during the adult stage 7 days post-emergence, and subjected to two stress temperatures (23°C: benign, 38°C, stress). Error bars represent 95% confidence intervals.

**1a) Survival after pupal heat-stress**

When focusing on a single hardening temperature (36°C) compared with non-hardened controls, we found that pupal survival probability was significantly affected by the interaction between hardening and heat-stress temperature ($\chi^2_{(5)}$= 33.74, p< 0.001; Figure 2.1a). Specifically, pupae heat-hardened at 36°C showed significantly improved survival at higher stress temperatures over non-hardened pupae. Between the 3 hardening temperatures of 34, 35 and 36°C, we found no effect of hardening temperature ($\chi^2_{(2)}$= 2.040, p= 0.361; Figure 2.1a) on individual survival at the pupal stress temperature of 40°C.

**1b) Survival after adult heat-stress**

There was no interaction between hardening and sex for adult survival at 38°C ($\chi^2_{(1)}$ = 0.000, p=0.999; Figure 2.1b). However, we found a main effect of hardening on survival ($\chi^2_{(1)}$= 41.321, p< 0.001; Figure 2.1b)**.** Survival is significantly higher if adults have experienced a 1h hardening treatment at 33°C, as compared to non-hardened controls.

We also found a main effect of sex, with lower survival in females than males ($\chi^2_{(1)}$= 16.891, p< 0.001; Figure 2.1b).

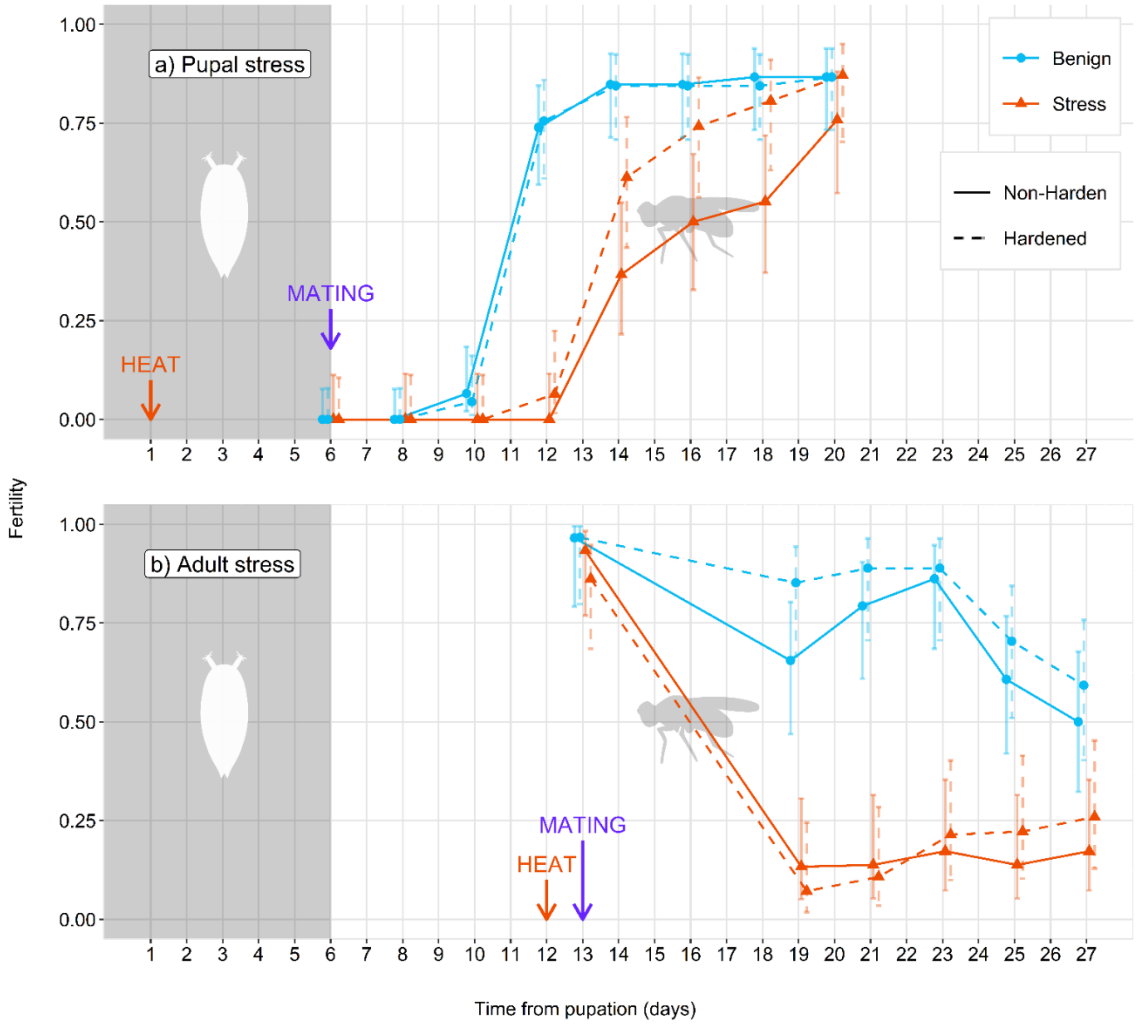


**Figure 2.2.** Cumulative fertility of male *D. virilis* over time after a 4h heat-stress. Focal individuals were subjected to a pre-stress hardening treatment for 1h immediately prior to temperature stress. The age at heat-stress is represented using an arrow, and the life-stage of the individual is represented using grey (pupal) and white (adult) background. **a)**

Individuals were heated during the pupal stage at either benign (23°C) or stressful (38°C) temperatures. Individuals were exposed to a 1h hardening treatment at 23°C ('non-hardening') or 36°C ('hardening') prior to heat-stress. Focal males were given a single group of virgin females at the first day post-eclosion. **b)** Individuals were heated during the adult stage at either benign (23°C) or stressful (35°C) temperatures. Individuals were exposed to a 1h hardening treatment at 23°C ('non-hardening') or 33°C ('hardening') prior to heat-stress. Focal males were given access to 2 groups of virgin females: one from days 1 to 6 post-heat, and another fresh set of virgin females from day 7 post-heat, to account for delayed sterility of males. Error bars represent 95% confidence intervals.

**2a) Fertility after pupal heat-stress**

There was no interaction between pupal hardening and stress temperatures on the age of reproductive maturity (ARM) (Cox proportional hazard test interaction term: HR= 0.3831, $\chi^2_{(1)}$= 1.096, p= 0.295; Figure 2.2a). However, high pupal stress temperatures increase the time after eclosion until males can produce offspring (Cox proportional hazard test interaction term: HR= -0.8862, $\chi^2_{(1)}$= 23.27, p< 0.001; Figure 2.2a). This extends the ARM, with many males eventually becoming fertile. Pupal hardening does not significantly reduce ARM at the stress temperature of 38°C (Cox proportional hazard test interaction term: HR= 0.1034, $\chi^2_{(1)}$= 0.338, p= 0.561; Figure 2.2a).

**2b) Fertility after adult heat-stress**

Adult males were given an initial group of virgin females to mate with, and there was no interaction between stress temperature and hardening treatment on immediate fertility of adult males ($\chi^2_{(1)}$= 0.244, p= 0.621; Figure 2.2b, days 13-19). We also found no effect of heat-stress on immediate fertility ($\chi^2_{(1)}$= 2.286, p= 0.130; Figure 2.2b, days 13-19), and no main effect of hardening on fertility at this initial time point ($\chi^2_{(1)}$= 0.590, p= 0.443; Figure 2.2b, days 13-19).

From 7 days post heat-stress onwards in non-hardened flies, there was no interaction between heat-stress and time ($\chi^2_{(1)}$= 3.333, p= 0.068; Figure 2.2b, days 19-27). However, we found that heat stress significantly reduced fertility through a main effect of stress ($\chi^2_{(1)}$= 28.444, p< 0.001; Figure 2.2b, days 19-27). Stressed males had lower fertility than controls after 7 days post heat-stress. We found no significant effect of time on fertility after day 7 ($\chi^2_{(1)}$= 2.413, p= 0.120; Figure 2.2b, days 19-27) meaning fertility remained low post 7 days.

There was no interaction between hardening and time on fertility at the stress temperature of 34°C when measured after day 7 ($\chi^2_{(1)}$= 2.1824, p= 0.140; Figure 2.2b, days 19-27). Hardening also did not affect fertility of heat-stressed adults ($\chi^2_{(1)}$= 0.1319, p= 0.717; Figure 2.2b, days 19-27) meaning hardening does not change the sterility pattern induced by thermal stress, even though there was a main effect of time on fertility ($\chi^2_{(1)}$= 4.265, p= 0.039; Figure 2.2b, days 19-27), where fertility increased slightly as the experiment progressed.

# Discussion

We found functionally different impacts of thermal stress at different life-history stages on fertility in *Drosophila virilis*. Pupal heat stress delays the age of reproductive maturity (ARM), whereas adult heat stress sterilises most males. Many stressed adult males are fertile immediately post-heat stress but lose fertility over a week and remain permanently sterile for the duration measured. Heat-induced sterility in *Drosophila melanogaster* has been associated with disruptions to spermatid elongation during spermatogenesis (Rohmer et al. 2004). Therefore, it is possible that mature sperm stored in the seminal vesicles of adult males are relatively unharmed and can be used by stressed males, whereas immature sperm are destroyed and the capacity to produce sperm is disrupted. However, it is unclear why pupae appear to recover fertility over the course of the experiment, whereas adults remain sterile. Benign adult males saw a drop-off in fertility over the last two time-points. Therefore, it is possible that the combination of heat-induced sterility and natural ageing prevent heated adult males from recovering fertility over the experiment. Exploring how fertility is affected by high temperature at the pupal and adult stages by looking at sperm production over an individual's lifetime may be necessary to disentangle these differences.

We found pupae were more thermally robust than adults. At 38°C, non-hardened adult *D. virilis* cannot survive, whereas pupae show high survival, and their ARM is delayed but eventually recovers. Pupae are immobile, so high physiological thermal tolerance may be particularly important for pupae as they cannot behaviourally thermoregulate to escape

heat-stress. However, the finding that pupae are more resistant to thermal stress than other life-stages contrasts with some previous studies. For example, a recent study examining flour beetles found that pupae and immature males are the most vulnerable life-stages to both fertility loss and survival at high temperatures (Sales et al. 2021). Additionally, non-hardened *D. melanogaster* pupae have very similar upper lethal limits than adults (Moghadam et al. 2019). Similarly in yellow dung flies (*Scathophaga stercoraria*), there is no simple relationship between heat-tolerance and mobility of life-stage, with early and late-stage pupae showing contrasting responses to thermal stress (Blanckenhorn et al. 2014). With no obvious pattern in how life-stage interacts with heat-induced death and sterility across species groups it is clear that studies on thermal limits should consider examining all life stages that are likely to be exposed to high temperatures in the wild.

As expected, we found *D. virilis* can improve high temperature survival through prior hardening at sub-lethal stress temperatures. This response occurs in both life-history stages measured. The effect is sex-specific in adults such that heat-hardened males show higher survival over heat-hardened females at lethal temperatures. A meta-analysis on sex differences in acclimation capacity, including four *Drosophila* species, found no significant differences in overall acclimation capacity between males and females (Pottier et al. 2021). However, the authors found that where differences between sexes exist, females appear to have higher acclimation capacity than males. It has previously been shown that *D. virilis* female fertility is robust to high pupal temperatures when compared

with male fertility (Walsh et al. 2020). It follows that females would be able to utilise the improved survival at high temperatures by reproducing. This makes the finding that heat-hardened males actually show higher survival than females surprising, as it is difficult to see the fitness benefit gained by permanently sterilised males surviving high temperatures.

In contrast to survival, we found no significant protective impact of this same hardening treatment on fertility at sterilising temperatures. This is true for both pupae and adults, suggesting that, although prior heat-hardening improves survival at lethal temperatures, it does not protect male fertility. Whereas previous studies found a positive impact of heat-hardening on reproduction (Jørgensen et al. 2006), here we find no measurable benefit of heat-hardening on fertility. While we demonstrated that a range of heat-hardening temperatures can protect survival, we chose a single heat-hardening treatment when testing whether heat-hardening also protects pupal and adult fertility. So we do not claim that there is no heat-hardening treatment that might protect fertility in this species. Rather, our point is that a hardening temperature that gives clear survival benefits does not appear to provide any defence for fertility. This suggests that lessons about how hardening protects survival under thermal stress cannot be directly applied to fertility.

We tested relatively short periods of hardening and stress, but longer-term acclimation to high temperatures can influence reproduction. In the flour beetle *Tribolium castaneum*, adult male development at stressful temperatures results in males producing sperm with shorter tails (Vasudeva et al. 2019). This is shown to be an adaptive morphological shift,

with shorter sperm doubling performance when males are reproducing at high temperatures. Similarly, a recent study in *D. melanogaster* found that a three-day acclimation period prior to mating increases mating success by around 70% at stressful temperatures (Stazione et al. 2019). It is known that the timing of heat-shock and heat-hardening/acclimation can drive differences in the response to temperature stress (Weldon et al. 2011; Zhang et al. 2021). Possibly, there is a delay for any physiological response to 'kick-in' before components of fertility can be protected. Many experiments demonstrating thermal plasticity of reproductive traits utilise multiple-day stress treatments (Stazione et al. 2019; Vasudeva et al. 2019), or delays between 'hardening' and thermal stress (Jørgensen et al. 2006). We did not provide our flies with such a gap, immediately moving them from hardening to stress temperatures, which might have impaired any hardening effect. Indeed, natural populations may experience more gradual transitions across sub-lethal and lethal temperatures. These may result in recovery periods between heat-hardening and stressful temperatures, or allow organisms to more gradually transition between temperatures. However, it is also possible that natural populations caught during the peak midday sun of a heatwave may not realistically have the opportunity to 'ramp-up' their physiological response. Clearly plasticity in reproductive traits is possible, however its general capacity to allow organisms to cope with climate change is still unclear (Sgrò et al. 2016). If a similar lack of strong or robust short-term heat-hardening for fertility is found across taxa, then organisms may be more vulnerable to climate change than previously thought.

There are a few notable caveats to our findings that should be taken into consideration when evaluating how species will respond to extreme temperature-stress through plasticity. A more detailed experiment in which males were provided with virgin females at shorter intervals may show some weak effects of hardening for fertility that we did not pick up with our design. In addition, our work has focused almost exclusively on high temperature stress. While this is clearly important in a warming world, climate models also suggest cold stress will also increase for many organisms, as snow cover is reduced, and winters become harsher in some areas. Studying how cold stress impacts on fertility and sterility is both urgently needed, and fortunately more developed than sublethal impacts of high temperature stress.

Superficially, it seems that improving survival of males via heat-hardening may be less beneficial to fitness than previously thought, given that males may be alive but permanently sterilised. Parratt et al. (2021) found that males from 19 of 43 Drosophila species could survive apparently permanently sterilising temperatures, suggesting there must be a biological explanation. The adaptive benefit of heat-hardening is particularly confusing if it protects survival without allowing individuals any opportunities to reproduce. However, a key finding here is that both life-stages measured still have a limited capacity to reproduce after heat-shock. Males heated as pupae are eventually sexually mature, and heated adult males can reproduce within a few days, before long-term sterility manifests. Therefore, the improved survival at extreme temperatures may provide more males with these limited opportunities to use up surviving mature sperm,

without protecting reproductive traits directly. It is also possible that if males sterilised as adults were kept long term, they may restore some fertility over time. Alternatively, male hardening could simply be a neutral by-product of selection on females for survival at high temperatures, as females are far better able to maintain fertility at near-lethal temperatures (Walsh et al. 2020).

To gain a more complete understanding of how natural populations will be affected by heat-waves, measuring the difference of survival and fertility between life-stages will be important. Our findings also suggest that research needs to consider that heat-hardening may not be a sufficient plastic rescue mechanism, although heat hardening effects on fertility in more taxa need to be tested. Importantly, studies showing the positive effects of heat-hardening should consider whether surviving individuals are fully fertile. This will allow researchers to more fully understand the adaptive benefits of these responses.

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## Conflicts of interest

None declared

## Author contributions

**Benjamin S. Walsh:** conceptualisation (lead), methodology (lead), validation (lead), formal analysis (lead), investigation (lead), data curation (lead), writing- original draft (lead), writing- review and editing (lead), visualisation (lead). **Steven R. Parratt:** conceptualization (supporting), methodology (supporting), formal analysis (supporting), writing- review and editing (supporting), visualisation (supporting). **Natasha M. Mannion:** methodology (supporting), writing- review and editing (supporting). **Rhonda R. Snook:** writing- review and editing (supporting). **Amanda Bretman:** writing- review and editing (supporting). **Tom A. R. Price:** conceptualization (supporting), resources (lead), writing- original draft (supporting), writing- review and editing (supporting), supervision (lead), project administration (lead), funding acquisition (lead).

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# Chapter 3: Female fruit flies cannot protect stored sperm from high temperature damage


## Abstract

Recently, it has been demonstrated that heat-induced male sterility is likely to shape population persistence as climate change progresses. However, an under-explored possibility is that females may be able to successfully store and preserve sperm at temperatures that sterilise males, which could ameliorate the impact of male infertility on populations. Here, we test whether females from two fruit fly species can protect stored sperm from a high temperature stress. We find that sperm carried by female *Drosophila virilis* are almost completely sterilised by high temperatures, whereas sperm carried by female *Zaprionus indianus* show only slightly reduced fertility. Heat-shocked *D. virilis* females can recover fertility when allowed to remate, suggesting that the delivered heat-shock is damaging stored sperm and not directly damaging females in this species. The temperatures required to reduce fertility of mated females are substantially lower than the temperatures required to damage mature sperm in males, suggesting that females are worse than males at protecting mature sperm. This suggests that female sperm storage is unlikely to ameliorate the impacts of high temperature fertility losses in males, and instead exacerbates fertility costs of high temperatures, representing an important determinant of population persistence during climate change.



**Keywords:** fertility, female sperm storage, heat stress, climate change

# 1. Background

Anthropogenic climate change poses a significant challenge to global biodiversity. We urgently need to understand how rising average temperatures, and an increasing number of short-term extreme temperature events (Perkins-Kirkpatrick and Lewis 2020), will affect natural populations. Understanding how high temperatures affect organisms can allow researchers to predict the vulnerability of species and inform conservation efforts, revealing which temperature-sensitive traits are particularly important for determining species persistence. Initial research focused on temperatures required to kill or incapacitate individuals, and it has been shown that species' physiological temperature limits correlate with the maximum temperatures species experience in the wild (Kellermann et al. 2012). It has been known for around a century that high temperatures can sterilise individuals (Cowles 1945; David et al. 2005; Young and Plough 1926). Recent work has found that the temperature that sterilises over 80% of males in a species, named a species' upper thermal fertility limit (TFL), correlate more strongly with maximum temperatures that species experience in the wild than lethal limits (Parratt et al. 2021; van Heerwaarden and Sgrò 2021). This indicates that upper TFLs are significant determinants of current species distributions, and are therefore likely to shape population persistence as climate change progresses.

Temperature-induced sterility occurs across a wide-variety of taxonomic groups (David et al. 2005; Hurley et al. 2018; Karaca et al. 2002; Sage et al. 2015; Walsh et al. 2019a). Sterility is used here to describe an individual that is unable to produce viable offspring,

which could be driven by one or more of the many different components of reproduction (Walsh et al. 2019a). A study of 43 *Drosophila* fruit fly species found that males from nearly half the species (19/43) are sterilised at temperatures significantly lower than temperatures required to kill them (Parratt et al. 2021). Male fertility generally seems more sensitive to high temperatures when directly compared with female fertility (Iossa 2019; Sales et al. 2018; Walsh et al. 2020), although the converse is possible (Janowitz and Fischer 2011). The relative sensitivity of male fertility in animals has been attributed to disruption of spermatogenesis or death of mature sperm as a result of thermal stress (Rohmer et al. 2004; Sales et al. 2018). Typically, the effect of temperature on fertility is measured by directly heating males, and subsequently measuring the reproductive capacity of focal males when paired with females following heat-stress (Jørgensen et al. 2006; Karaca et al. 2002; Parratt et al. 2021; Sales et al. 2018; Walsh et al. 2020; Zwoinska et al. 2020) or by measuring other traits linked to fertility (Hurley et al. 2018; Paxton et al. 2016). Likewise, studies measuring female fertility generally stress females prior to mating (Walsh et al. 2019b; Walsh et al. 2020), in order to isolate the effect of temperature on female reproductive physiology, such as oocytes. However, while it is clearly important to measure the effect of thermal stress prior to mating, the effect of high temperatures on females post-mating has been largely ignored (but see McAfee et al. 2020; Sales et al. 2018). This is important because sperm can spend a significant proportion of time within the female reproductive tract prior to fertilisation.

Sperm storage is characterised by temporal delays between insemination and fertilisation, during which sperm is maintained within a female's reproductive tract. Female sperm storage is common across taxa, including mammals, birds, reptiles, fish and insects (Holt 2011; Sever and Hamlett 2002). The time that sperm can be kept viable inside a female varies substantially. In birds and reptiles, sperm storage durations range from seven days up to seven years, in mammals for less than a day up to six months in some bat species, amphibians from four to thirty months, in fish from only days to around two years, and over a decade in some eusocial hymenoptera (Birkhead and Møller 1993; Holt and Lloyd 2010; Holt 2011; Keller 1998; Levine et al. 2021; Pamilo 1991). The method of sperm storage can also vary substantially, and phylogenetic evidence suggests long-term storage of sperm has arisen independently across taxa (Holt and Lloyd 2010). For example in birds and some reptiles, inseminated spermatozoa are stored in microscopic sperm storage tubules (SSTs) embedded in the infundibulum, which allow sperm to survive for extended periods of time (Holt 2011; Sasanami et al. 2013). Females from the majority of insects and some other arthropods store sperm in a highly chitinised specialised organ called the spermatheca. Most insects have one spermatheca, but some insects have two or three (Pascini and Martins 2017). However, while female sperm storage for extended durations is taxonomically widespread (Birkhead and Møller 1993), the impact of high temperatures on sperm stored within mated females is currently understudied. The few efforts to examine the impact of high temperatures on sperm stored within females include mated females of the red flour beetle (*Tribolium castaneum*), which show a 33% reduction in offspring production when exposed to a

heatwave treatment (Sales et al. 2018). Also, a four hour heat-stress at 42°C significantly reduces the viability of sperm stored by honey bee queens (McAfee et al. 2020), although in this study the authors do not directly test whether this reduces female offspring production. Given the urgency of understanding the consequences of rising temperatures, we need a better understanding of the thermal robustness of female sperm storage.

Fruit flies from the family Drosophilidae provide a useful model group to explore this question. Female *Drosophila* typically possess a pair of spermathecae and a seminal receptacle, the latter of which is a thin extended tubule arising from the uterus (Pitnick et al. 1999). *Drosophila* have been proposed as a model system for studying sperm-female interactions, in order to better understand fertilisation across taxa (Heifetz and Rivlin 2010). *Drosophila* are also a model taxon for studying thermal reproductive physiology, including examining how high temperatures affect fertility of both males and females prior to mating (David et al. 2005; Parratt et al. 2021; Sgrò et al. 2016; Walsh et al. 2020). However, to our knowledge there has been no substantial effort to examine how high temperatures affect the capacity of mated females to produce offspring in *Drosophila*.

Here, we explore the impact of heat stress on sperm storage in females from two Drosophilidae species. We test the tropical pest species *Zaprionus indianus*, and a more temperate species *Drosophila virilis*. Parratt et al. (2021) showed that males of both species die when exposed to ~38°C for 4 hours, and that immediate male fertility is compromised when male *D. virilis* are exposed to ~37°C for 4 hours , but male *Z. indianus* remain fertile. This indicates that mature sperm stored by *D. virilis* males are damaged by

thermal stress, but *Z. indianus* maintain fertility. In contrast, the same study found that developing sperm appear to be damaged by high temperatures in both species. Males of both species are sterile 7 days after being heated at ~35°C for 4 hours, indicating that developing sperm are damaged by increased temperatures. However, we do not know the effect of high temperatures on sperm stored within mated females.

We test three components of female fertility across time. Firstly, we test the expectation that female fertility will be more robust to high temperatures than male fertility. Secondly, we test whether sperm stored in mated females are more or less sensitive to high temperatures than sperm stored in a males seminal vesicles and developing sperm within the testes, investigated previously. Finally, we explore whether mated females that are heated to a point that sterilises them can recover fertility, after being presented with new male partners. If sterilised mated females can recover by remating, this would suggest that heat induced sterility of mated females is caused by damage to sperm and not direct damage to females.

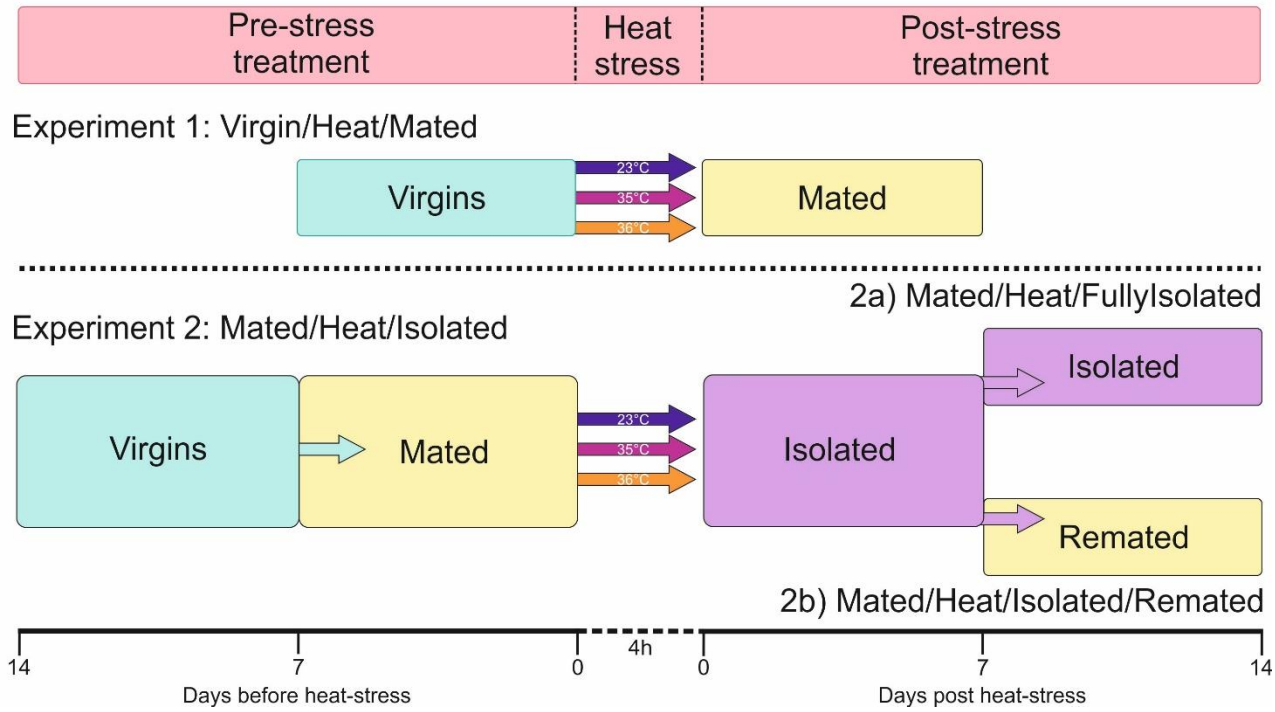
# 2. Material and Methods

## 2.1 Animal stock maintenance

Stocks of *Drosophila virilis* (Cambridge Fly Facility StrainvS-4, isolated in 1991) and *Zaprionus indianus* (DSSC Stock #: 50001-0001.05 ISOFEMALE, isolated in 2004), were kept in a temperature-controlled incubator (LMS 600NP Series 4) at 23°C, 12:12 L:D and ambient humidity. Stocks were maintained at moderate density (50 – 100 flies per 300ml bottle culture). *D. virilis* were kept on standard cornmeal-molasses-agar media, and *Z. indianus* were kept on banana medium. Ovipositing adults for both species were tipped to new food every week to prevent overlapping generations, and were replaced with fresh sexually mature adult flies every 4-6 weeks.

## 2.2 Experimental treatments

Experimental treatments are summarised in Figure 3.1. We assessed whether heat stress influences fertility of females when delivered before mating (Experiment 1). We then completed an experiment with two more treatment combinations (Experiment 2a & 2b) to address the outstanding question of whether mated females can protect stored sperm from temperature damage experienced post-mating, and isolate effects on stored sperm from changes to female egg-laying behaviour.




1862

1863 **Figure 3.1. Experimental design outlining the two experiments**. Each treatment
1864 designation combines various pre and post-stress mating treatments. **Experiment 1:**
1865 **Virgin/Heat/Mated**, where virgin females were heat-stressed and mated following heat-
1866 stress. **Experiment 2: Mated/Heat/Isolated,** where mated females are heat-stressed and
1867 kept alone for 7 days to produce offspring from previous matings. After 7 days post heat-
1868 stress, the experiment was divided into two treatments. For an additional 7 days, females
1869 were either kept in isolation **(2a: Mated/Heat/FullyIsolated)**, or given new male partners
1870 to mate with **(2b: Mated/Heat/Isolated/Remated)**. Focal females were exposed to either
1871 benign (23°C) or stress (35 & 36°C) temperatures for 4h in water baths. Day 0 in the post-

stress treatment represents the time-point when the fertility assay begins (Figure 3.2 & Figure 3.3).

We chose to mate females at 7 days old when fully sexually mature, and kept this consistent between experiments. Therefore, females from Experiment 1 are 7 days old at heat-stress, whereas females from Experiment 2 are 14 days old at heat-stress. Prior to heat stress, females from Experiment 1 were separated at emergence and kept as virgins in groups of 10 per vial for 7 days, to standardise density prior to the experiment. Females from Experiment 2 were separated as virgins and kept in groups of 10 for 7 days, then provided with sexually mature males (7 days old) at a 1:1 sex ratio for a further 7 days prior to heat-stress. This produced an 'assumed' mated treatment, where females would have many opportunities to mate with a variety of males.

Immediately following heat stress, females were transferred to individual fresh food vials**.** In Experiment 1, virgin females were immediately placed with four 7 day old virgin males. This mating group was moved to fresh vials twice, creating three 'time-points' where fertility was recorded. Females in Experiment 2 were isolated and transferred to fresh vials giving three time-points over 7 days. Experiment 2 was then split into two treatments. Females from Experiment 2a were kept in isolation for an additional 7 days, producing three more time-points where females were isolated. Females from Experiment 2b were placed with 4 males following the first 7 days of isolation. This mating group was transferred onto new vials twice more, giving three time-points where the females were isolated, followed by three recorded time-points where females were

paired with males. Females were deemed as qualitatively fertile at a given time-point if there was evidence of larvae present in their vial (1/0), measured by directly observing larvae or their distinctive tracks in the food. We use a binary fertile/infertile measure rather than counting pupae or adults because our methods were likely to result in many sterile vials, producing a dataset of offspring counts with many zeros. Quantitative models typically have difficulty with such data.

## 2.3 Heat-stress

Groups of 10 females were transferred to fresh 25 x 95mm plastic vials, containing 25ml of 'ASG' medium (10g agar, 85g sucrose, 20g yeast extract, 60g maize, 1000ml $H_2O$, 25ml, 10% Nipagin) to prevent desiccation and keep humidity consistent. These vials were randomly assigned to pre-heated water-baths (Grant TXF200) for four hours at either control: 23°C, or two stress temperatures: 35°C & 36°C. The chosen temperatures do not affect survival or immediately sterilise mature adult males of either species, but result in substantial delayed sterility of males, likely due to the destruction of developing sperm (Parratt et al. 2021). Immediately following temperature-treatment, flies were returned to benign temperatures for the remainder of the experiment (23°C). Sample sizes are given in table 3.1.

**Table 3.1.** Sample sizes of experimental treatments as summarised in Figure 3.1.

| Experiment number | *Drosophila virilis* | | | *Zaprionus indianus* | | |
|---|---|---|---|---|---|---|
| | 23°C | 35°C | 36°C | 23°C | 35°C | 36°C |
| Experiment 1 | 29 | 29 | 30 | 25 | 25 | 25 |
| Experiment 2 | 27 | 26 | 18 | 23 | 23 | 24 |
| Experiment 2b | 27 | 26 | 18 | 22 | 23 | 23 |

## 2.4 Statistical analyses

Species and experiments were analysed separately due to inherent differences in methodological design as summarised in Figure 3.1. Treatment of females in Experiment 2 are identical from the start of the experiment until the experiment is split after 7 days into the post stress treatment. Therefore, data from Experiment 2 over the first three time-points were analysed together. The final three time-points of Experiment 2a after splitting were not statistically analysed, as all flies of both species in these final three time-points were completely sterile with only one exception, making these data uninformative. Experiment 2b was analysed after the treatments were split and females were presented with new males, in order to assess differences in fertility recovery across temperature treatments.

To assess the effect of temperature on fertility we used generalised linear mixed models with Bernoulli error distributions. We fitted fertility as a binary response variable,

temperature and time-point and their interaction as fixed effects, and focal fly ID as a random effect to account for repeated measures. We did model selection using Wald Chi-squared likelihood ratio-tests, removing non-significant interactions. We retained all main effects and reported statistics of these from type II likelihood ratio tests using the 'Anova' function from the 'car' package, in the statistical software 'R' (version 3.5.0). We then reported any pairwise comparisons in which $p<0.05$ by using the Wald statistic and p-value from the model summary(). To do this we ran the model multiple times, setting each level in turn as the baseline compared with the other levels.

# 3. Results

## 3.1 Experiment 1: Virgin/Heat/Mated

Experiment 1 exposed virgin females to benign or stressful temperatures and subsequently mated them. There was no significant interaction between temperature and time on fertility of *D. virilis* from Experiment 1 ($\chi^2_{(2)}= 3.977$, $p= 0.137$; Figure 3.2). There was no main effect of temperature ($\chi^2_{(2)}= 0.093$, $p= 0.954$; Figure 3.2), or time ($\chi^2_{(1)}= 0.301$, $p= 0.583$; Figure 3.2) on fertility of *D. virilis*. Fertility was initially high, and remained so for the three time-points measured.

There was also no significant interaction between temperature and time on fertility of *Z. indianus* from Experiment 1 ($\chi^2_{(2)}= 3.946$, $p= 0.139$; Figure 3.2). While the absolute proportion of fertile females heated at 36°C was consistently lower than controls, there was no overall main effect of temperature on fertility of *Z. indianus* ($\chi^2_{(2)}= 4.469$, $p= 0.107$; Figure 3.2). However, there was a significant effect of time on fertility ($\chi^2_{(1)}= 10.911$, $p<$

0.001; Figure 3.2), where flies from all temperatures show increased fertility rates over time.

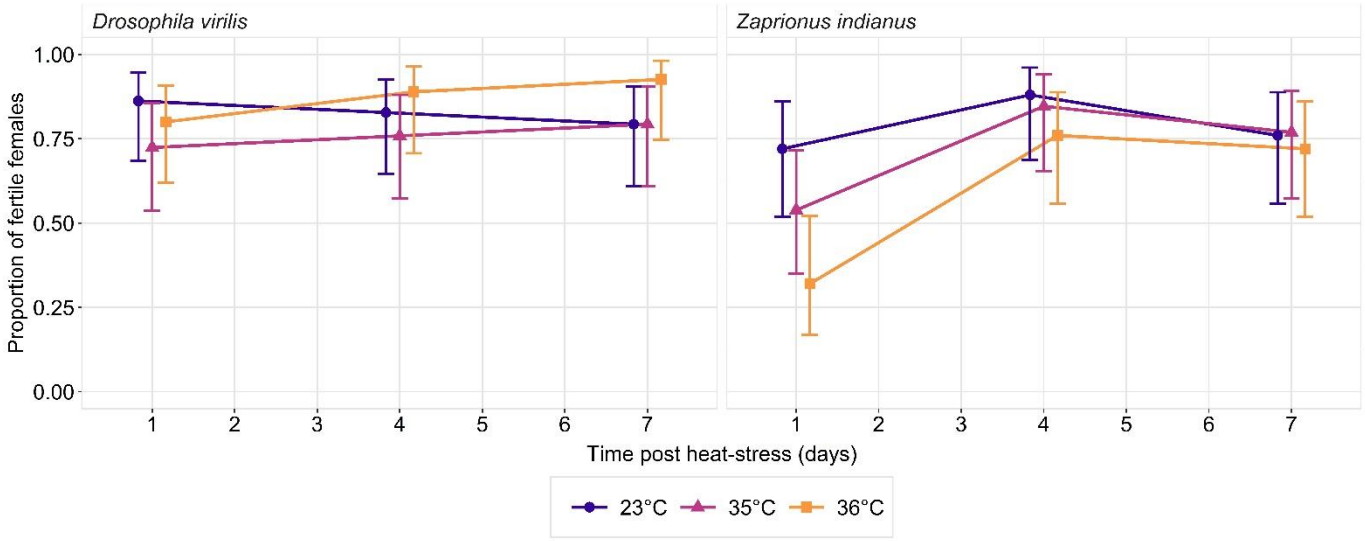



**Figure 3.2. Proportion of fertile *D. virilis* and *Z. indianus* females over time in Experiment 1: Virgin/Heat/Mated**. Virgin females were heat-shocked at either benign (23°) or two stress temperatures (35 & 36°C) for 4 hours, and paired with 4 male partners immediately following heat-stress. This mating group was given three days to lay eggs, then tipped onto fresh vials twice, giving three recorded time-points where fertility was measured. Error bars represent 95% confidence intervals. Sample sizes for each species are given in Table 3.1.

## 3.2 Experiment 2: Mated/Heat/Isolated

Experiment 2 exposed mated females to benign or stressful temperatures, then isolated individuals immediately following heat-stress. Not all females from the pre-stress 'mating'

treatment produced offspring, with controls producing a baseline fertility of around 70% in *D. virilis* and around 80% in *Z. indianus* (Figure 3.3).

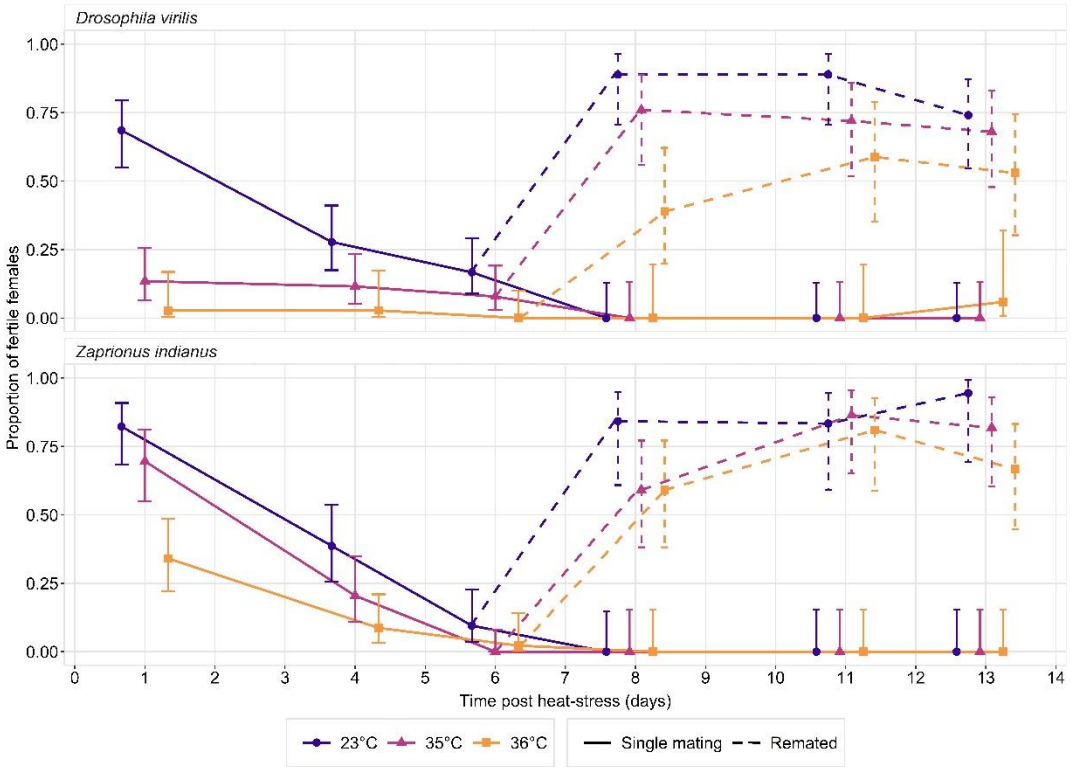



**Figure 3.3. Proportion of fertile *D. virilis and Z. indianus* females over time in Experiment 2: Mated/Heat/Isolated**. Mated females were heat-shocked at either benign (23°) or two stress temperatures (35 & 36°C) for 4 hours. Following heat stress, all females were isolated and allowed to lay eggs in fresh vials three times. After 6 days, the experiment was split into two treatments. **2a Mated/Heat/FullyIsolated:** females remained isolated and moved onto three fresh vials to lay any remaining eggs. **2b Mated/Heat/Isolated/Remated:** focal females were paired with new male partners, and

the mating group were given three fresh vials to produce offspring. Error bars represent 95% confidence intervals. Sample sizes for each species are given in Table 3.1.

There was a significant interaction between temperature and time on fertility of *D. virilis* in Experiment 2 prior to treatment splitting ($\chi^2_{(2)}$= 9.943, p< 0.007; Figure 3.3). Fertility of controls started high immediately following heat treatment and fell over time, whereas fertility at stress temperatures started low and remained low for the duration. There was a main effect of temperature ($\chi^2_{(2)}$= 21.146, p< 0.001; Figure 3.3) and time ($\chi^2_{(1)}$= 17.352, p< 0.001; Figure 3.3) on fertility of *D. virilis* in Experiment 2. Both stress temperatures showed lower fertility than controls, and all treatments showed a decline in fertility over time.

There was no significant interaction between temperature and time on fertility of *Z. indianus* from Experiment 2 ($\chi^2_{(2)}$= 1.777, p= 0.411; Figure 3.3). However, there was a significant overall effect of temperature ($\chi^2_{(2)}$= 80.161, p< 0.001; Figure 3.3) and time ($\chi^2_{(1)}$= 99.756, p< 0.001; Figure 3.3) on fertility of *Z. indianus*. In this species the highest temperature of 36°C results in significantly lower fertility than both controls (p< 0.001) and the stress temperature of 35°C (p< 0.001). All temperatures result in a loss of fertility over time.

## 3.3 Experiment 2b: Mated/Heat/Isolated/Remated

A subsection of females from experiment 2 were given the chance to remate 1 week following heat-stress. There was no significant interaction between temperature and time

on fertility of *D. virilis* after females were given the chance to remate in Experiment 2b ($\chi^2_{(2)}$= 3.549 , p= 0.170; Figure 3.3). However, we found a significant effect of temperature on fertility in *D. virilis* ($\chi^2_{(2)}$= 9.520, p= 0.009; Figure 3.3). Specifically, fertility of females exposed to the stress temperature of 36°C was significantly lower than fertility from the control 23°C (p= 0.002) and stress temperature of 35°C (p= 0.046). There was no significant effect of time on fertility ($\chi^2_{(1)}$= 0.515, p= 0.473; Figure 3.3).

There was no significant interaction between temperature and time on fertility of *Z. indianus* when females were given the opportunity to remate in Experiment 2b ($\chi^2_{(2)}$= 1.049, p= 0.592; Figure 3.3). There was also no main effect of temperature on fertility ($\chi^2_{(2)}$= 4.250, p= 0.119; Figure 3.3). However, there was a significant effect of time on fertility ($\chi^2_{(1)}$= 4.775, p= 0.029; Figure 3.3), where fertility slightly increases over time.

# 4. Discussion

We found little evidence that virgin females are susceptible to fertility loss at high temperatures. Heat-stress did not influence fertility of virgin *D. virilis* or *Z. indianus* females that were then mated after heat-stress, although it should be noted that females were not heated up to their lethal limit. Fertility of *Z. indianus* females was initially lower at the first time-point measured post heat-stress, and increased over the duration of the experiment. Conversely, fertility of *D. virilis* females was consistently high over the duration, suggesting that *Z. indianus* females were slower to mate and produce offspring with their paired males than *D. virilis*.

Mated females given no opportunity to remate used up their viable sperm reserves within the first week of laying. However, we found that heat-stress reduced the number of fertile females of both species, likely through destruction of stored mature sperm. This is curious because a previous study found that mature sperm in males of both species from the same experimental lines appear to be largely unaffected by the same temperature treatments (Parratt et al. 2021). We find that mated females are sterilised at temperatures around 2°C lower than those required to completely sterilise 80% of males from our study species (Parratt et al. 2021). Hence our results suggest that females of both species are worse at protecting mature sperm from high temperatures than males. However, as we did not directly observe sperm death within females, it is also possible that there is an alternative explanation for female sterility, such as embryonic death.

We found that the temperatures required to sterilise mated females differ between the two species. Four hours at either 35°C or 36°C almost completely sterilise *D. virilis* females (~90% of females produce no offspring), whereas mated *Z. indianus* females are mostly fertile when stressed at 35°C and only a small majority are sterilised when exposed to 36°C for four hours (~60% of females produce no offspring). The finding that mature sperm from *Z. indianus* is likely more resilient than sperm from *D. virilis* is consistent with our previous study that heated adult males of each species, although it should be noted that these experiments were not conducted together. Males of *D. virilis* require temperatures of no less than 37°C for 4h to immediately sterilise the majority of males, whereas males of *Z. indianus* are fertile up to their lethal temperature of ~38°C (Parratt et

2029 al. 2021). While the absolute temperatures required to sterilise males and mated females
2030 appear to be different, these results combine to suggest that mature sperm from *Z.*
2031 *indianus* are generally more thermally robust than those from *D. virilis*. It is unclear
2032 exactly why this may be the case, however *Z. indianus* tend to live in slightly warmer
2033 areas than *D. virilis*. The temperature experienced by individuals at the upper edge of
2034 their thermal range in the hottest month of the year (Tmax+1sd: WorldClim.org BIO05) is
2035 36.1°C for *Z. indianus*, whereas it is 32.6°C in *D. virilis* (Parratt et al. 2021). Therefore, *Z.*
2036 *indianus* sperm may better adapted to high temperatures than *D. virilis*, although this is
2037 beyond the scope of this study.

2038 To unpick effects of high temperatures on stored sperm from direct effects on females,
2039 we then gave a chance for mated females to 'recover' fertility after they had used up their
2040 viable stored sperm. We found that while the majority of females exposed to all
2041 temperatures were able to produce offspring when paired with new males, females
2042 heated at 36°C performed worse than controls in *D. virilis*. Therefore, it is likely that 36°C
2043 thermal stress results in some permanent damage to females of this species, possibly due
2044 to elevated ROS due to thermal stress. There could also be a trade-off between the cost
2045 of additional mating and any increased fecundity, if females are in a worsened condition
2046 as a result of heat-stress. Measuring additional reproductive traits, such as the number of
2047 emerging offspring, could reveal more subtle changes in reproduction that could begin to
2048 address these questions. However, the almost complete sterilisation of sperm stored in
2049 female *D. virilis* paired with a general capacity to 'recover' fertility suggests that initial

sterilisation in this species is likely due to the destruction of stored sperm by high temperatures and not direct effects on females. Mated *Z. indianus* females were equally able to recover fertility when paired with new males, regardless of the heat-stress temperature experienced. While the temperatures required to reduce fertility of mated females were higher in this species, there was no long-term effects of temperature on female recovery when females were presented with new males, suggesting that this initial reduction of fertility in *Z. indianus* is also driven by effects on stored sperm.

Sterilisation of mated females could be particularly devastating to species with low remating rates. However, females can use facultative polyandry to improve offspring production when mating with sub-fertile males (Sutter et al. 2019; Vasudeva et al. 2021). For example, heat-shocked males of the flour beetle *Tribolium castaneum* have low numbers of viable sperm after heat-stress (Vasudeva et al. 2021). Here, females increase their remating rate when mated with a heat-shocked male, rescuing fertility to normal levels. However, whether increased polyandry is observed when sperm within the female is sterilised by high temperatures remains an open question. Also, there may be species where facultative polyandry is impossible, for example in seasonally reproducing animals with discrete mating opportunities. Those particularly at risk include species that store sperm for long periods of time, such as hymenopteran insects that have been observed to store sperm for up to 10 years (Keller 1998; Pamilo 1991). In these cases, sterilisation of mated females may actually be worse for population persistence than sterilisation of males.

Understanding how high temperatures affect male fertility has improved our ability to predict the consequences of climate change on species (Parratt et al. 2021; van Heerwaarden and Sgrò 2021; Walsh et al. 2019a). When these severe long-term effects on male fertility are combined with the immediate sterilisation of mated females like we have demonstrated, the impact of rising temperatures on wild populations may be exacerbated. Further, we find here that the temperatures required to sterilise mated females are not always consistent with the temperatures required to sterilise males. It will be important to determine whether this is true across species and taxa to help forecast vulnerability climate warming effects. Species where sperm in both males and mated females cannot be protected may be particularly vulnerable, whereas species where females can protect sperm effectively may be more resilient to an increasing incidence and severity of heat-waves.

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## CRediT authorship contribution statement

**Benjamin S. Walsh**: conceptualization, methodology, validation, formal analysis, investigation, data curation, writing- original draft, writing- review and editing, visualisation. **Steven R. Parratt:** conceptualization, methodology, formal analysis, writing- review and editing, visualisation. **Rhonda R. Snook:** writing- review and editing **Amanda Bretman:** writing- review and editing **David Atkinson:** writing- review and editing. **Tom A. R. Price:** conceptualization, resources, writing- original draft, writing- review and editing, supervision, project administration, funding acquisition.

## Data and materials availability

All data and analysis R code is available at

https://datadryad.org/stash/share/7wn67Q4UVZBXStL1OKTk87xJ9CzXh-GrQ1H2ZoxC7TA

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# Chapter 4: The genetic basis of reduced sperm production at high temperatures in *Drosophila melanogaster*


## Abstract

As ambient temperatures continue to rise, we need to know whether species will be able to cope. However, the capacity of species to evolve upper physiological limits is still unclear. Here, we examine genetic variation in spermatogenesis disruption at high temperatures in *Drosophila melanogaster*. We first demonstrate that high temperatures reduce the proportion of fertile males in a mixed population and then link this to reductions in seminal vesicle size, where males store mature sperm. We subsequently use this proxy to examine variation in seminal vesicle size loss due to high temperatures in 95 lines of the Drosophila Synthetic Population Resource, a panel of recombinant inbred lines (RILs) created from genotypes representing an extremely broad range of different climates. We measured seminal vesicle size of benign males and stressed males for each RIL, and then calculated the size 'loss' due to high temperatures. Of the 95 RILs, 75 showed significant reductions in seminal vesicle area due to high temperatures. All responses varied considerably across lines and showed high broad-sense heritability. However we did not find any genetic variants associated with seminal vesicle size at benign temperatures, or its sensitivity to high temperatures. Our results agree with previous studies, although the power of our analyses may be reduced by the relatively

small number of lines assayed. Our results reaffirm that genetic variation in physiological responses to high temperatures is possible. However, local standing variation in the sensitivity of sperm production at high temperatures may not be enough to prepare species for increasing ambient temperatures. Therefore, increasing upper fertility limits through evolution may not allow populations to cope with rising temperatures.

# Background

Climate change is increasing average ambient temperatures, as well as causing a rising frequency of heatwaves. Understanding how these high temperatures will affect biodiversity is a major research priority. High temperatures can compromise many different biological processes, so research typically focuses on severe effects of thermal stress such as death, and more recently, loss of fertility (Hurley et al. 2018; Parratt et al. 2021; Sales et al. 2018; van Heerwaarden and Sgrò 2021; Walsh et al. 2019). Upper lethal temperatures have previously been linked to species' distributions in insects, suggesting that upper limits determine range boundaries and species persistence (Kellermann et al. 2012). However, it has recently been shown that sub-lethal sterilising temperatures of *Drosophila* males may be even better predictors than lethal temperatures (Parratt et al. 2021; van Heerwaarden and Sgrò 2021). If true across other species, then many species' ranges will shrink due to limited ability to breed at the increasing high temperatures associated with continued climate warming.

One possible avenue species could use to mitigate the effect of rising temperatures on survival and fertility is through evolving higher thermal limits. Unfortunately, current data

2264 suggest that increased upper thermal tolerance is difficult to evolve, particularly for
2265 surviving high temperatures (Hoffmann 2010). There are three main approaches that
2266 have been used to understand evolvability of upper thermal limits. First, natural
2267 populations can be examined for local adaptation to high temperatures. Second,
2268 laboratory populations can be selected for higher thermal tolerance. Third, genotypes can
2269 be assayed for variation in thermal tolerance. Research has found that in some *Drosophila*
2270 species, populations in higher temperature locations sometimes have higher thermal
2271 limits than populations in low temperature locations, both for survival (Hoffmann et al.
2272 2002) and fertility (Porcelli et al. 2016; Rohmer et al. 2004). However, upper lethal limits
2273 generally show very limited genetic variability and evolvability (Blackburn et al. 2014;
2274 Kellermann and van Heerwaarden 2019; Rezende et al. 2011; van Heerwaarden and Sgrò
2275 2021). The majority of these studies focus on *Drosophila* survival*,* however male fertility
2276 across *Drosophila* is typically a critical reproductive trait which can be compromised by
2277 sub-lethal temperatures (Parratt et al. 2021; van Heerwaarden and Sgrò 2021). A recent
2278 study selecting for higher upper fertility limits in laboratory populations of five species of
2279 *Drosophila* found little evidence of evolution of higher thermal tolerances (van
2280 Heerwaarden and Sgrò 2021). Furthermore, a recent study by Zwoinska et al. (2020)
2281 found phenotypic variation of male fertility loss at high temperatures in *Drosophila*
2282 *melanogaster*, but little evidence of additive genetic variance. This suggests that there is
2283 high broad-sense heritability in fertility limits in the species, but did not provide any
2284 evidence that the *D. melanogaster* used in this study can improve upper limits to
2285 reproduction through evolution.

2286 However, the Zwoinska et al. (2020) study measured within-population variation in
2287 thermal fertility loss, using the Drosophila Genetic Reference Panel (DGRP). The DGRP are
2288 a set of inbred lines derived from a single natural population of *D. melanogaster* from
2289 Raleigh, USA. It is possible that inter-population variation in thermal tolerance is greater
2290 than intra-population variation. If this were true, then long distance gene flow between
2291 populations, or even human directed genetic rescue, might allow populations at risk of
2292 thermal extinction to evolve increased thermal tolerance. Key evidence for this comes
2293 from a study by Porcelli et al. (2016), which found that populations of *D. subobscura* from
2294 Spain maintain fertility at high temperatures better than their conspecifics from Northern
2295 Europe. If local adaptation for upper limits to fertility is possible, utilising genotypes from
2296 a variety of different locations may help to reveal whether evolution can improve fertility
2297 loss at high temperatures. Here, we use a worldwide sample of *D. melanogaster* from the
2298 Drosophila Synthetic Population Resource (DSPR). The DSPR is a panel of recombinant
2299 inbred lines (RILs) created using founder lines from 6 continents, representing an
2300 extremely broad range of different climates (King et al. 2012). There are two synthetic
2301 populations (A & B) constructed from 8 founder lines each which share only one line,
2302 making 15 lines total between the populations. For each population, the 8 founder lines
2303 were crossed to bring all alleles together. These large populations were maintained
2304 through random mating for 50 generations, at which point a set of 750 RILs were created
2305 from each population by 25 generations of full-sibling mating. This created a panel of RILs
2306 that represent a fine-scale mosaic of genomic segments from the 8 founder lines. The
2307 genomic structure of each RIL was determined using a set of SNP markers and a Hidden

Markov Model with a high degree of confidence. By phenotyping a panel of RILs from either population of the DSPR, researchers are better able to understand the genetics of complex traits in *Drosophila*. Given that the original populations were taken from six continents, from locations experiencing a broad range of temperatures, if genetic variation for thermal tolerance exists within the species, then the DSPR should have an excellent chance of revealing it.

Previous studies examining evolvability and variation in fertility limits have measured the capacity of males to produce offspring after thermal stress (van Heerwaarden and Sgrò 2021; Zwoinska et al. 2020). Recording the capacity to produce offspring as a binary response (where a male producing at least one offspring is classed as fully fertile) can be very useful, particularly when we expect to observe large differences in the proportion of sterile individuals, such as in interspecies comparisons (Parratt et al. 2021; van Heerwaarden and Sgrò 2021). However, in some cases less severe reductions of fertility are observed (Sales et al. 2018; Vasudeva et al. 2021), where individuals are typically able to produce some offspring, but the numbers are reduced. While a critical measure of fertility, offspring production is a complex composite trait that requires both males and females (Walsh et al. 2019), introducing some capacity for noise when attempting to isolate the effect of high temperatures on male fertility.

An alternative is to use a continuously distributed measure which does not depend on interactions between males and females may more precisely measure individual differences in how individuals' fertility is affected by high temperatures. Fertility loss is

often associated with disruptions to sperm production in males (Hurley et al. 2018; Karaca et al. 2002; Rohmer et al. 2004; Sales et al. 2018), so examining quantities of mature sperm in males after heat stress can provide a useful proxy for heat-induced sterility. In *Drosophila*, sperm are produced by stem cells in the testes and transferred to the seminal vesicles after sperm elongation and maturation (Demarco et al. 2014). The seminal vesicles increase in size as they fill with mature sperm. A reduction in the size of a male's seminal vesicle suggests fewer mature sperm are present and can be used as an indicator of sterility (Mishra and Singh 2005; Naveira and Fontdevila 1991; Zeng and Singh 1993). Measuring the loss in seminal vesicle size due to heat stress could thereby provide a proxy for spermatogenesis disruption, and help elucidate the mechanism behind any observed losses of fertility due to high temperatures. Note as well that male fertility is affected differently depending on the life-stage that experiences thermal stress (Porcelli et al. 2016; Sales et al. 2021; Walsh et al. 2021; Zhang et al. 2015). For example, pupal thermal stress can result in an increase to the age of reproductive maturity (ARM) in *Drosophila virilis* (Walsh et al. 2020; Walsh et al. 2021). Pupae are immobile, so they are unable to behaviourally thermoregulate to avoid heat-stress. Therefore, a pupa trapped in stressful temperatures has no option but to physiologically cope with high temperatures. Here, we explore the effect of high pupal temperatures on offspring production of male *D. melanogaster* fruit flies, identifying a 'sub-fertile' temperature stress at which around half of males are fully sterile.

We then examine the potential to use seminal vesicle surface area as a proxy for fertility loss. Using this proxy, we examine the variation in seminal vesicle size loss due to high temperatures in 95 RILs from the DSPR. This allows us to determine the extent of phenotypic genetic variation in spermatogenesis disruption at high temperatures. Finally, we examine the genetic architecture of sperm loss, using a genome-wide association study (GWAS) to identify trait-associated genetic variants.

# Methods

## Experimental stocks

Experiment 1 used an outbred laboratory strain of *Drosophila melanogaster* named 'Dahomey', collected in West Africa in 1970. Experiment 2 used experimental stocks created from *Drosophila melanogaster* recombinant Inbred Lines (RILs) from the DSPR population B, kindly sent by Stuart MacDonald. Dahomey and DSPR stocks were kept in a temperature-controlled room at 25°C, 12:12 L:D and ambient humidity. Stocks were maintained at moderate density (20 flies per *Drosophila* vial) on standard 'agar sugar yeast (ASG)' medium and laying adults were replaced new generations of with adult flies every 4 weeks.

## Temperature treatment

Focal individuals for our experiments were collected directly from stock bottles within 24 hours of pupation and allocated in groups of 30 to fresh 25 x 95 cm plastic vials containing 25 ml ASG medium to prevent desiccation. Immediately after collection, these vials of 30

pupae were randomly assigned to pre-heated water-baths (Grant TXF200) at either a benign (25 °C) or stressful temperature (30-35 °C, depending on experiment) for 48 hours.

Following heat-stress, vials were returned to temperature-controlled rooms set at benign temperature (25 °C) and flies were observed daily for eclosion. All emerging individuals from each experimental vial were collected, and males were transferred to fresh ASG vials and kept at 25 °C until experimental treatment. All females used in the experiment were raised and kept at 25°C and can be considered 'benign'.

## Experiment 1: Establishing a proxy for male fertility loss using *D. melanogaster* (Dahomey)

### *1A: Effect of thermal stress on fertility*

Pupae were collected and heated as above, at either benign (25 °C), or one of 6 stress temperatures during the pupal stage in 1°C increments ranging from 30 °C to 35 °C for 48 h before being returned to benign temperature and monitored for emerging adults. Adult males were separated as virgins within 8 hours of emerging, and stored for 3 days until sexual maturity. At this point, males were aspirated into a vial containing 2 sexually mature females and given 24 h to mate. Following this 24 h period, males were immediately removed and discarded, whereas females were given a further 48 h to oviposit before being discarded. Food vials were monitored for a week, during which time fertility was measured. Males were scored as fertile (1) or infertile (0) by directly observing presence or absence of larvae or their distinctive tracks in the food.

*1B: Effect of thermal stress on offspring number*

We also examined the effect of heat-stress on offspring number. To do this, pupae were heated to 25 °C (benign) and 31 °C (stress) as above. Emerging males were treated as in Experiment 1A, however the number of emerging adult offspring from each experimental vial were counted, providing a quantitative measure of offspring production. Vials were monitored once females were removed and offspring were counted in all vials one week after the first adult fly emerged across the experiment. This accounted for differences in egg to adult timings across vials, but prevented overlapping generations of offspring.

*1C: Effect of thermal stress on sperm production*

Pupae were heated to 25°C (benign) and 31°C (stress) as above. Emerging adult males were collected as virgins and left for 3 days before being dissected, as in previous experiments. When the males were 3 days post-eclosion, they were anaesthetised under $CO_2$ and seminal vesicles were removed under a dissection microscope. To do this, the abdomen was separated from the thorax using insect dissecting pins, and the testes were removed by lightly squeezing the contents of the abdomen into phosphate-buffered saline (PBS; 0.05 M sodium phosphate, 0.1 M sodium chloride, pH 7.8). Where necessary, the accessory glands and apical tip of the testes were removed in order to get a clear picture of the seminal vesicles. Seminal vesicles were transferred to a microscope slide containing 80 µl of PBS and a cover-slip was placed on top. The seminal vesicles were photographed under 40x, using a Nikon D5100 camera mounted to a Leica dissection microscope.

All photos were analysed using ImageJ version 1.48. The scale of the ImageJ environment was calibrated using a micrometer, which returned a scale of 0.896 pixels/µm. Seminal vesicle area was calculated using the polygon selector tool and closely tracing around the image of the seminal vesicle, giving a surface area in $\mu m^2$.

## Experiment 2: Variation and heritability of fertility loss using the DSPR

In the second experiment, we measured the benign and stressed seminal vesicle area of a panel of 95 RILs from population B of the DSPR. Two levels of heat-stress (25°C benign; 31°C stress) were used and seminal vesicle area was measured as in section 1C. Up to 6 lines were assayed for each of 22 discrete blocks across the experiment.

## Body size

In order to examine whether male body size influenced seminal vesicle area, we measured body size, using thorax length which is a standard proxy in *Drosophila* (Lack et al. 2016), in a subset of 65 lines. Prior to dissection, males were laterally positioned and the thorax was photographed under the same zoom level as seminal vesicles. Thorax size was calculated in ImageJ by drawing a straight line from the base of the anterior humeral bristle to the posterior tip of the scutellum as in Lack et al. (2016), giving a single length in µm.

## Statistical Analyses

All statistical analyses were completed in the statistical environment "R" (version 3.5.0). In generalised linear models, significance of predictor variables was calculated using Wald chi-squared tests in the "car" package.

### *1A: Fertility*

In order to work out a 'sub-fertile' response temperature where variation in fertility loss is expected, we used a dose-response model to calculate point-estimates of fertility loss and survival as in Parratt et al. (2021). To do this, we used the "drc" package to calculate 50% fertility loss ($TFL_{50}$) and 50% lethality ($LT_{50}$), the latter of which is determined by the temperature at which 50% of pupae successfully eclose as adults. We deemed $TFL_{50}$ as significantly lower than $LT_{50}$ if the 95% confidence intervals did not overlap, as in Parratt et al. (2021). We used the rounded-down closest integer to $TFL_{50}$ as our 'stress' temperature going forward.

### *1B: Offspring Number*

To assess the effect of temperature on offspring number of fertile males, we used a generalised linear model with a negative-binomial distribution (using the "MASS" package) because of the count nature of the data, and because Poisson model residuals were overdispersed. We fitted offspring number as a count response variable, and temperature as a fixed effect.

### *1C: Seminal vesicle size*

To assess the effect of temperature on seminal vesicle surface area, we used a generalised linear model with a Gaussian distribution due to the continuous nature of the data. We fitted seminal vesicle area as a continuous response variable, and temperature as a fixed effect.

*2A: The effect of temperature on DSPR SV size*

To assess the effect of temperature on DSPR sperm production, we used a generalised linear model with a Gaussian distribution. We fitted seminal vesicle surface area as a continuous response variable, and RIL number, temperature, body size, and the interaction between RIL number and temperature as predictor variables.

*2B: Heritability analyses*

Broad-sense heritability analyses were calculated using mixed effect models, with the phenotype of interest fitted as a fixed effect, and ~1 fitted as a random effect. The variance and standard deviation were then extracted from these models and the heritability was calculated as the variance divided by the sum of the variance and standard deviation.

We used six different responses in the heritability analysis. **1) ln transformed benign** and **2) ln transformed stressed SV size; 3) residual benign and 4) residual stressed SV size,** which accounted for body size effects by using residuals of a linear model with benign or stressed SV size fitted as a response variable and body size fitted as a predictor variable. **5) SV size difference**, which was calculated by randomly pairing benign and stressed SV areas and taking the difference of stressed SV size on benign SV size. We ran a linear

model with benign or stressed SV size fitted as a response variable and body size fitted as a predictor variable. We subsequently extracted the residuals of this model and used these in the heritability analysis. **6) Stress response accounting for benign SV size**. After finding that the stress response depends on benign SV size, we ran a linear model with the stress response fitted as a response variable and benign seminal vesicle size fitted as a predictor variable. We subsequently extracted the residuals of this model and used these in the final heritability analysis.

*2C: Genome-wide association studies*

We used four different measures in the GWAS. **1) Benign** and **2) Stressed SV size** were transformed using natural log (ln) before the mean SV size for each line was calculated. **3) SV size difference** was calculated by taking the difference of stressed on benign SV size. **4) Stress response accounting for benign SV size.** The residuals of the linear model of the stress response on benign SV size as in section 2A were used. This allowed us to examine the genetic basis of the stress response whilst accounting for the effect of benign SV size.

GWAS were performed using the DSPR-specific “DSPRqtl” and “DSPRqtlDataB” packages, and Manhattan plots were created using the “qqman” package. The permutation levels of significance for each analysis were calculated and LOD (- log 10 (p-value)) were tested against these significance thresholds.

# Results

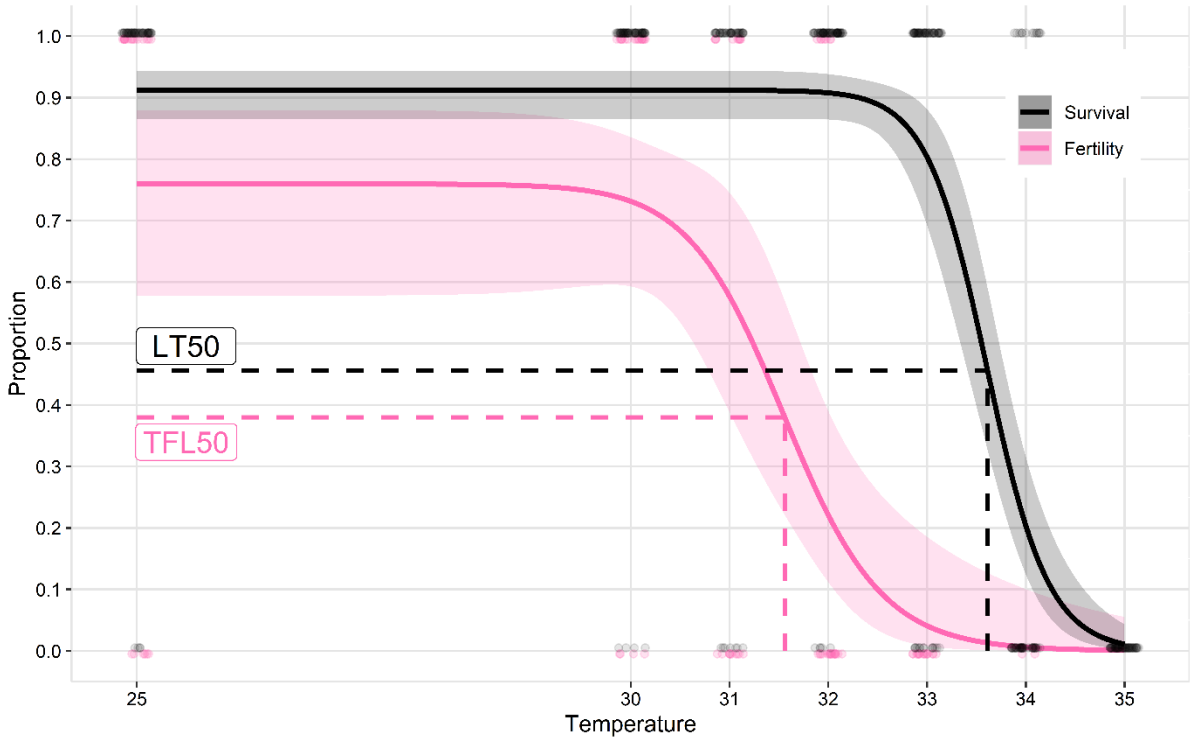
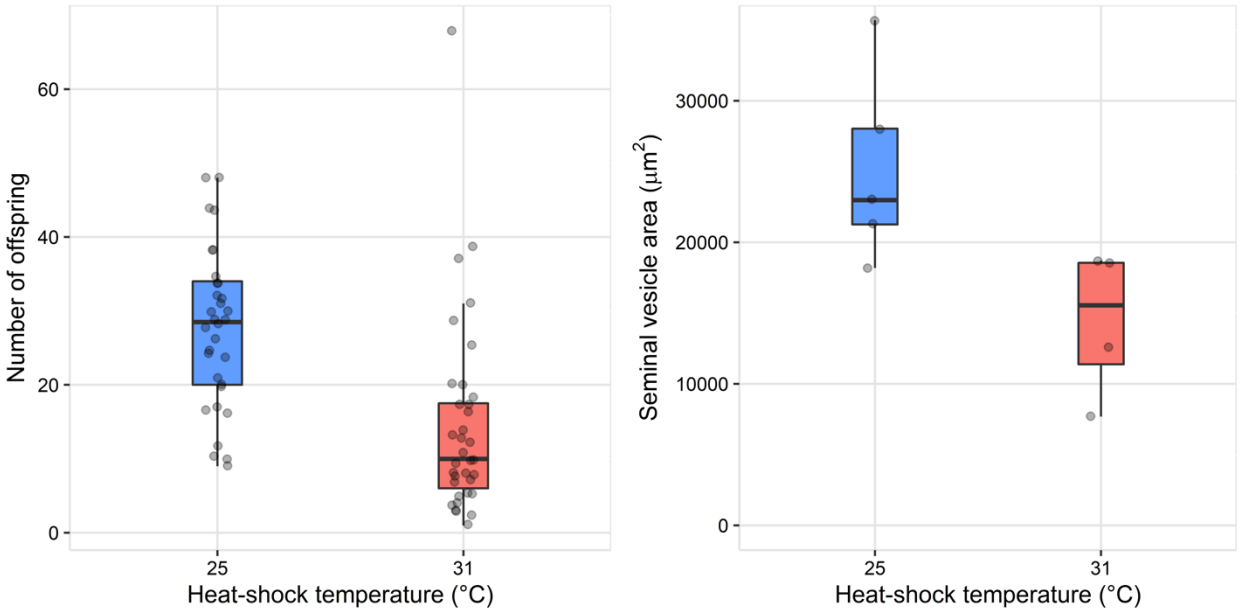



**Figure 4.1.** Survival and fertility of *Drosophila melanogaster* (Dahomey) after a 48h thermal stress during the pupal stage over a range of temperatures. The temperature at which 50% of individuals are dying due to thermal stress relative to the benign survival rate ($LT_{50}$) is designated by a black dashed line (33.6°C). The temperature at which fertility loss of surviving individuals reaches 50% relative to the benign fertility rate ($TFL_{50}$) is shown using the pink dashed line (31.5°C). Shaded areas represent the standard errors of the means.

## Experiment 1: Establishing a proxy for male fertility loss using *D. melanogaster* (Dahomey)

We found that the $LT_{50}$ was 33.6°C, whereas the $TFL_{50}$ of *D. melanogaster* was 31.5°C. As the confidence intervals do not overlap between $LT_{80}$ and $TFL_{80}$, we conclude that the $TFL_{50}$ is significantly lower than the $LT_{50}$. We subsequently decided to use 31°C as our 'sub-fertile' stress temperature, as it represents a temperature where just over 50% of males are fertile, and 31°C results in high survival with almost no death due to thermal stress (Figure 4.1). Therefore, we decided that this temperature would be an ideal candidate for exploring variation in sterility response.

When examining offspring number at benign (25°C) and stress (31°C) temperatures, we found a main effect of temperature on offspring number ($\chi^2_{(1)}$= 17.613, p< 0.001; Figure 4.2a). The stressful temperature of 31°C reduces offspring number by about 48% as compared with controls. We also found a main effect of temperature on seminal vesicle surface area ($\chi^2_{(1)}$= 6.765, p= 0.009; Figure 4.2b). High temperatures reduce seminal vesicle area by around 43% as compared with controls.

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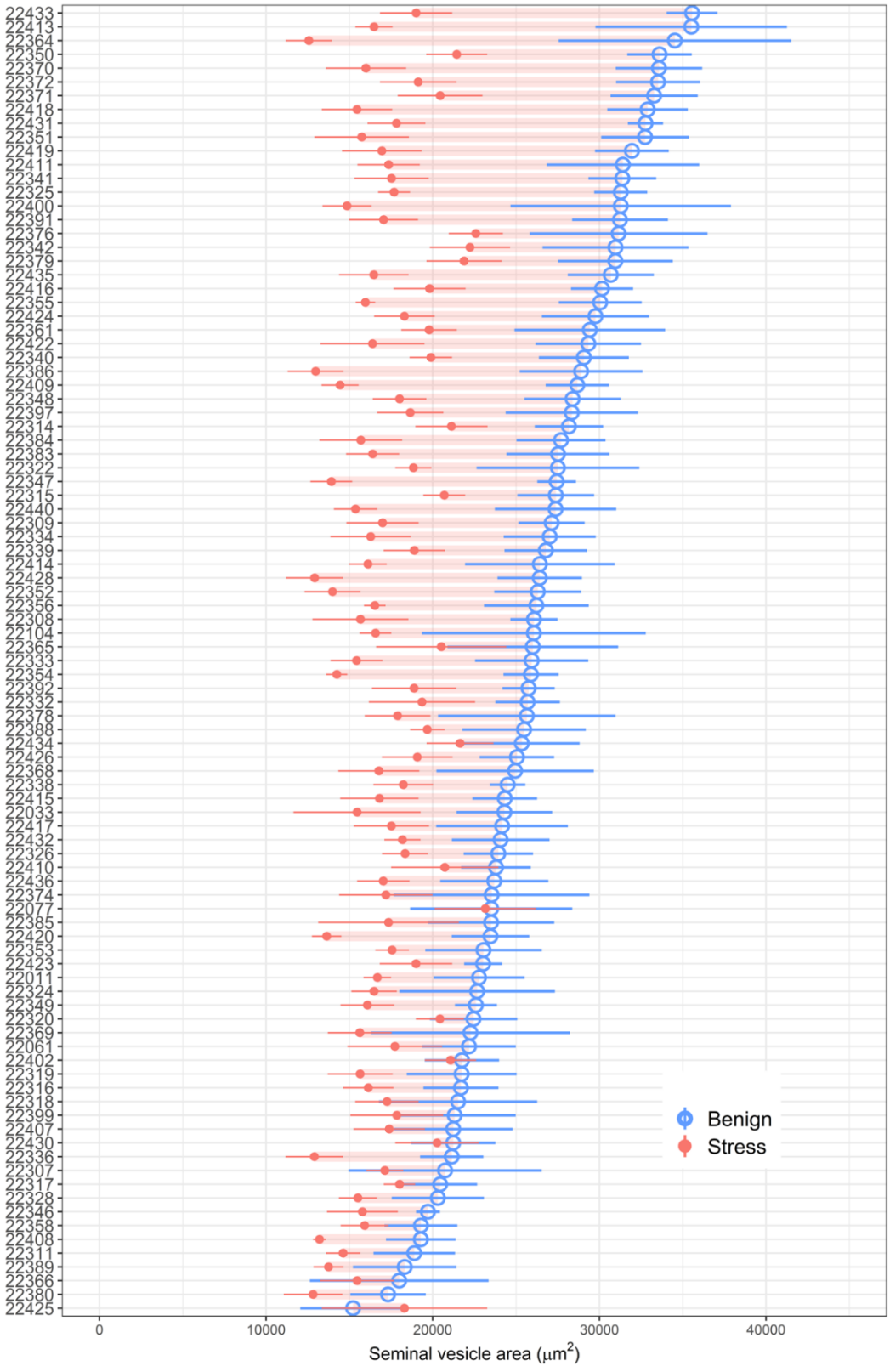
2512 **Figure 4.2.** Measures of *Drosophila melanogaster* (Dahomey) male fertility loss after

2513 thermal stress at benign (25°C) and stress (31°C) temperatures for 48h during the pupal

2514 stage. A) Number of emerging adult offspring from mating vials. B) Cross-sectional area of

2515 seminal vesicles dissected from sexually mature males.


Benign
Stress
0
10000
20000
30000
40000
Seminal vesicle area (μm²)

2516

**Figure 4.3.** Variation in benign and stressed seminal vesicle cross-sectional area across DSPR recombinant inbred lines (RILs). Y axis is arranged by benign seminal vesicle size. Points represent the mean seminal vesicle area, and error bars represent standard errors around the mean. Points are connected by a shaded orange line, outlining the difference between benign and stressed seminal vesicle areas within RILs. Lines with a larger distance between benign and stress points implicate a more severe sterility response.

## Experiment 2: Variation and heritability of fertility loss using the DSPR

### Phenotypic response of high temperatures on seminal vesicle size

We found a significant interaction between line and temperature on seminal vesicle size ($\chi^2_{(64)}$= 6.762, p< 0.001; Figure 4.3). Stress temperatures significantly reduce seminal vesicle size, but the size of the effect changes between lines. We also found a main effect of body size ($\chi^2_{(1)}$= 0.313, p< 0.001; Figure 4.3) on seminal vesicle area.

Heritability analyses are given in Table 4.1, with highest broad-sense heritability observed in benign seminal vesicle area. The 95% confidence intervals between benign and stressed individuals did not overlap in 75 of the 95 measured lines, resulting in over three quarters of lines (79%) showing significant reductions in seminal vesicle area due to thermal stress.

We found a statistically significant but weak correlation between body size and seminal vesicle size (Pearson's correlation coefficient= 0.24; t= 5.924, d.f.= 543, p< 0.001), suggesting that body size is may influence our estimate of heritability for SV size. We thus

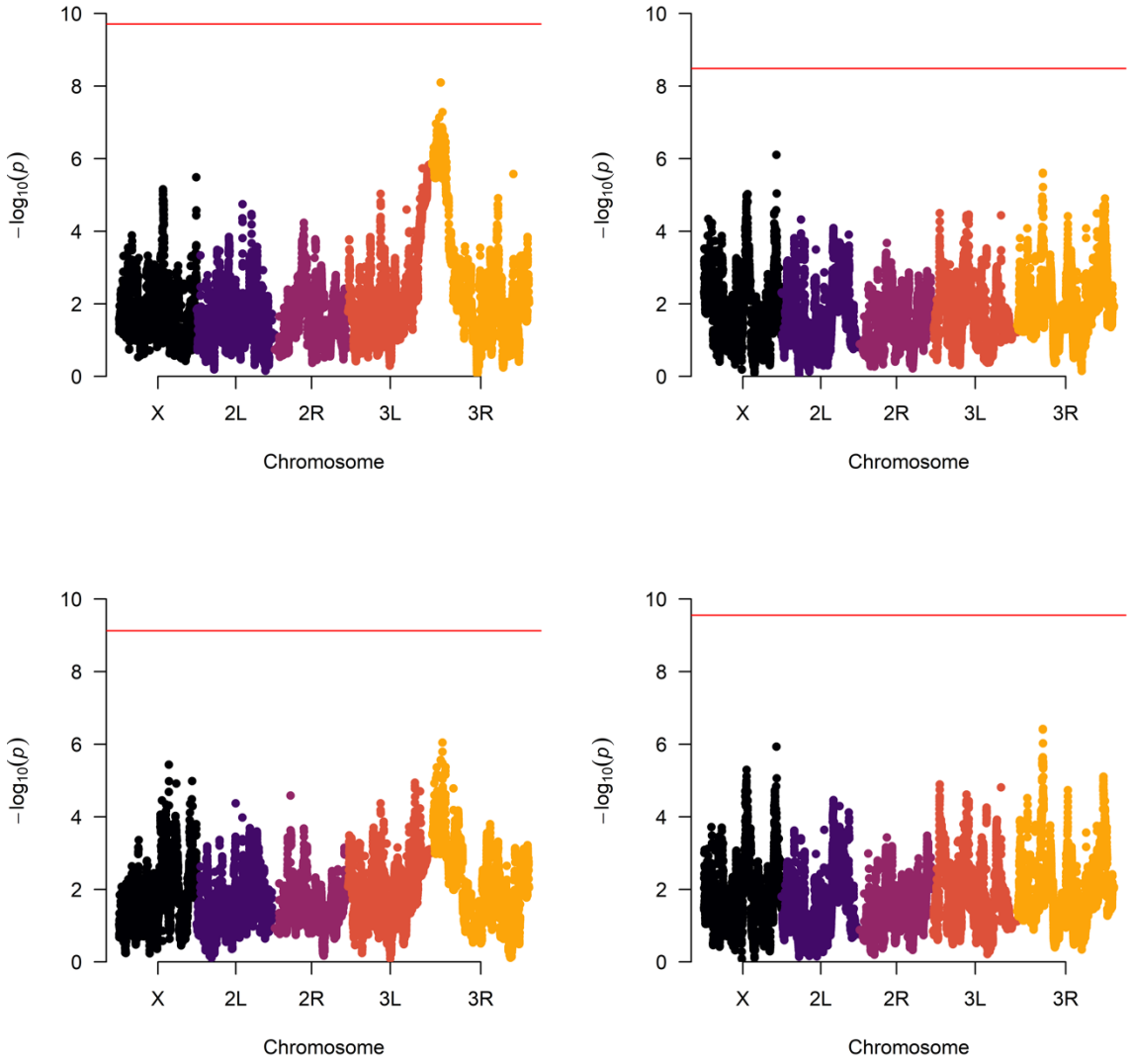
estimated heritability in SV size at both temperatures while also accounting for body size and found that heritability was unchanged under benign temperatures and reduced but still high under stress temperatures. These results suggest that the high heritability in SV size is not heavily impacted by body size.

**Table 4.1. Heritability analyses**

| $H^2$ Estimate | Number of lines | 25°C | 31°C | Difference |
|---|---|---|---|---|
| SV size $H^2$ | 95 | 0.546 | 0.433 | 0.477 |
| SV size accounting for body size $H^2$ | 65 | 0.534 | 0.367 | |
| SV difference accounting for benign size $H^2$ | 94 | | | 0.416 |

## Genome-wide association studies

For the GWAS performed for each of the different measures of seminal vesicle size, there were no LOD peaks that reached the level of significance required from the permutation tests (Figure 4.4). Therefore, no genetic variants for sperm production or protection of sperm loss at high temperatures were identified.

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2549 **Figure 4.4.** Genetic variants influencing four different measures of seminal vesicle size.

2550 Top left: a) benign SV size, top right: b) stressed SV size, bottom left: c) difference in

2551 benign and stressed SV size, bottom right: d) SV size difference accounting for starting SV

size. The red horizontal line represents calculated permutation levels of significance for each GWAS.

## Discussion

We present here a good method for understanding how high temperatures impact male fertility in the absence of females. We demonstrate, as predicted, that high temperatures reduce the proportion of fertile males. We also find ~48% fewer offspring produced at the temperature which results in half of males being unable to produce any offspring ($TFL_{50}$). We link this lower offspring production to reductions in male seminal vesicle size (~43%), which suggests that fertility loss may be connected to a lack of mature sperm in males. As previous studies have demonstrated, reduced quantities of sperm after thermal stress can occur due to the impairment of spermatogenesis (David et al. 2005; Rohmer et al. 2004). Therefore, we conclude that seminal vesicle area is a useful proxy for fertility loss at high temperatures.

We then measured seminal vesicle size at benign and stressful temperatures across 95 RILs in the DSPR. Seminal vesicle size of benign males varied considerably and showed high heritability. However, we found that a reduction of seminal vesicle size due to thermal stress was common across lines. Of the 95 lines, 75 showed significant reductions in seminal vesicle size due to high temperatures. We assume that this reduction in seminal vesicle size will result in reduced fertility, should they share the link between seminal vesicle size and fertility we have demonstrated using Dahomey. Additionally, the difference between benign and stressed seminal vesicle area varied across lines and

showed high broad-sense heritability. These results suggest that there is standing genetic variation in sperm production at benign temperatures. The presence of standing genetic variation for sensitivity of fertility to high temperatures could, in theory, allow populations to rapidly evolve to better cope with high temperatures.

Our results suggest that some RILs are more robust to high temperatures than others. These findings are consistent with Zwoinska et al. (2020), which found that sensitivity of offspring production at high temperatures in 127 *D. melanogaster* DGRP lines has similarly high levels of phenotypic variation and broad-sense heritability. We thus suggest that our utilised proxy may have successfully recaptured variation in fertility loss at high temperatures. We found that benign seminal vesicle size was the trait that gave the highest heritability. This result differs from previous studies describing examples of heritability which are greater in stressful conditions (Hoffmann and Parsons 1991; Hoffmann and Merilä 1999; Zwoinska et al. 2020). One reason for this pattern is that stress reduces the number of genotypes that are viable by removing alleles with low fitness, which in turn increases the heritability of measured traits (Hoffmann and Merilä 1999). In our study, the stress treatment did not result in increased death which therefore does not inflate heritability measures.

We did not find any genetic variants associated with seminal vesicle size at benign temperatures, or its sensitivity to high temperatures. This result was consistent across the different measures run through the GWAS, with no SNPs reaching the calculated permutation levels of significance. This agrees with previous studies; we found no clear

candidate genes linked to sensitivity of fertility or sperm to high temperature (Bundgaard and Barker 2017; Zwoinska et al. 2020). It is possible that this lack of candidate genes may be a function of the relatively small number of lines sampled, resulting in a low mapping power of our DSPR panel (Turner et al. 2013; Zwoinska et al. 2020). Additionally, genetic variance in sperm production may be explained by more loci of small effect and so more difficult to detect with a lower genome coverage.

Our results reaffirm that local standing variation in the sensitivity of sperm production at high temperatures may not be enough to prepare species for increasing ambient temperatures (Zwoinska et al. 2020). However evolving higher upper fertility limits may be possible by using variation from high temperature areas rather than standing genetic variation in a population, with previous studies connecting local adaptation to improved fertility at high temperatures (Porcelli et al. 2016; Rohmer et al. 2004). This would in theory put endemic or isolated populations without access to populations from warmer climates more at risk to the consequences of rising temperatures. Although this field is still young, the emerging trend is that increasing upper fertility limits through evolution is unlikely to allow populations to cope with rising temperatures.

## Acknowledgements

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## CRediT authorship contribution statement

**Benjamin S. Walsh**: methodology, validation, formal analysis, investigation, data curation, writing- original draft, writing- review and editing, visualisation. **Tom A. R. Price:** conceptualization, resources, writing- original draft, writing- review and editing, supervision, project administration, funding acquisition. **Mollie Manier:** conceptualization, resources, writing- original draft, writing- review and editing, supervision, project administration.

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# General discussion

There are studies showing heat-induced sterility of insects going back nearly 100 years (Cowles 1945; Young and Plough 1926). However, there was little movement in the field until the late Jean R. David and colleagues began to publish some key papers building on this early work (Araripe et al. 2004; Chakir et al. 2002; David et al. 2005; Petavy et al. 2001; Rohmer et al. 2004). There was a resulting flurry of papers published in the early 00s, demonstrating heat-induced sterility in some *Drosophila* species, culminating with a review which called for more research on this 'neglected' topic (David et al. 2005). Outside of *Drosophila*, there were many studies that examined how heat affected reproduction (Table 0.1), but these effects of temperature on fertility across taxa were generally isolated, and had not been brought together in the context of climate change. I made it an early goal of my PhD project to help build the field by writing a key review that would link projects on the effect of temperature on reproduction, and provide biologists from diverse academic backgrounds inspiration for exploring this phenomenon. At the time of writing our review has surpassed 85 citations in two years, so I am beyond happy and proud of the impact of the review so far.

Writing the review allowed me to consider which unanswered questions I found particularly interesting. Our correspondence with Graziella Iossa quickly revealed the fact that this field is predominantly male-focused, probably due to the apparent sex-specificity of heat-induced sterility (Appendix 1; Iossa 2019; Walsh et al. 2019). I found that while the majority of studies on fertility limits focus on males, there are very few studies which test males and females together. Therefore, Chapter 1 focused on comparing the effect of thermal stress on males and females, and examining the possible influence of sex-specific sterility on sexual selection. The finding that males are more sensitive than females was not surprising, but was necessary to demonstrate. I also felt that there had been little discussion around the population-level consequences of only one sex being sterilised by high temperatures. Therefore, we discussed the idea that heat-induced sterility could

have subtle effects on the operational sex ratio of populations and may even influence mating behaviour. I am really pleased that this was published as part of a fascinating special issue in Current Zoology, edited by Murielle Ålund and Natalie Pilakouta.

After exploring sex-specific differences in fertility limits, I was interested in examining male heat-induced sterility in more depth. In Chapter 2, I firstly examined whether the life-stage at thermal stress affects the extent of heat-induced sterility. We found that the temperatures required to sterilise pupae and adults differ markedly, and that the response over time also shifts. I find this interesting because it means that researchers should always consider which life-stage or age individuals should be stressed at, ensuring this makes ecological sense. For example, pupae are immobile and cannot fly away to escape thermal stress, whereas adults may be able to find sanctuary from heat. Secondly, I was interested in whether individuals can plastically mitigate the costs of high temperatures through 'heat-hardening'. I wanted to first demonstrate how hardening can improve individual survival as previously shown (Moghadam et al. 2019). That way I was specifically testing whether this positive response was flexible enough to protect fertility, with the prior knowledge that it was already helping survival. To my surprise, I found that while heat-hardening improves survival, it does not also protect fertility. Either heat-hardening to protect fertility is not mediated by the same process as for survival, or the response is much weaker and therefore more difficult to measure. Our results suggest that plasticity cannot save populations from heat-induced sterility in this species. Chapter 2 was published in Ecology and Evolution at the end of 2021 marking the fourth publication of my thesis, an achievement that I am proud of.

Heat-induced sterility of males has been attributed to sensitivity of sperm to high temperatures (Rohmer et al. 2004; Sales et al. 2018). However, in insects and many other animals sperm can be stored by females for long periods of time. It has even been shown that sperm stored by overwintering females can be protected in specialised storage organs (Giraldo-Perez et al. 2016). In Chapter 3, I showed that sperm is not safe from high

temperatures in females. I did this by heating mated females from two fruit fly species, showing that it substantially reduces their capacity to produce offspring. Ideally, I would have directly measured sperm loss or death in females through dissecting their seminal receptacle and approximating stored sperm numbers, or completing a live/dead assay. However, due to complications as a result of the COVID-19 pandemic, I was unable to complete this follow-up experiment, instead allocating my remaining lab time on my final chapter. However, I am confident my experimental design demonstrated that sterility in females is due to sperm loss and not direct effects on females. Overall, this chapter shows that quantities of stored sperm in females are unlikely to provide a reservoir of viable sperm to buffer the consequences of heat-induced sterility on males.

My final chapter was addressing a complex question to answer. Firstly, whether there is standing variation in sensitivity of fertility to high temperatures across populations. Secondly, examining whether there is a genetic basis to this variation. The ultimate goal of this project was to unveil genes that heavily influence how sensitive a genotype is to fertility loss. The key component of this project that I wanted to achieve initially was finding a proxy for male fertility loss that could be measured in absence of females. I decided to use seminal vesicle size, partially due to some personal observations, while visiting my supervisor Rhonda Snook in Stockholm, which was an incredibly rewarding trip. As we practised dissections, we found that seminal vesicles from heated males had clearly reduced seminal vesicle size. While this project did not find any candidate genes that are linked to sensitivity of fertility, I did find substantial variation in loss of seminal vesicle size. This indicates that there may be possible evolutionary avenues to increase fertility limits. I would love to build upon this work, first by validating the measures further through testing fertility of lines that show high and low sensitivity to temperature. While not critical, phenotyping a greater number of lines would improve the power of the analysis overall and provide greater coverage of the DSPR panel.

When exploring my results as a whole, one may conclude that my findings are not particularly promising for biodiversity. Firstly, as with previous studies we continue to find fertility loss in *Drosophila*. Fertility loss occurs whether you heat males as pupae or adults, and whereas female fertility is seemingly robust to high temperature stress, the sperm females carry is not. Across multiple projects in my thesis, I generally find little evidence that populations are going to be able to mitigate or prevent heat-induced sterility. While this seems like pretty worrying news, the field of thermal fertility limits is still young, leaving many unanswered questions. Therefore, I would like to now touch on some future research questions that I would be interested in exploring, given the opportunity.

## Future research

I often think about the study group used in this thesis. As previously mentioned, fertility is affected by high temperatures across a wide range of taxa (Table 0.1), but fertility loss may be particularly extreme in insects, especially *Drosophila*. High temperatures can completely sterilise a large number of *Drosophila* species at sub-lethal temperatures, where males cannot produce a single offspring after thermal stress (Parratt et al. 2021; van Heerwaarden and Sgrò 2021). While *Drosophila* is inarguably the best studied taxonomic group for temperature impacts on fertility, current research might suggest that less severe reductions in fertility are observed in other insects (Sales et al. 2018), mammals (Pérez-Crespo et al. 2008), and birds (Hurley et al. 2018). To test this, I would be interested in running a meta-analysis examining the relative magnitude of fertility loss at high temperatures across taxa. Alternatively, a substantial but equally fascinating project could experimentally measure thermal fertility limits across a wide range of taxa for more direct comparison, utilising consistent methodology. It would be difficult to measure fertility limits across species with large differences in life-history traits and reproduction. Thus, examining fertility limits across insects would be more likely to be fruitful initially.

While many laboratory-based studies have demonstrated heat-induced sterility in artificial conditions, we still know surprisingly little about how this phenomenon manifests in nature. Presumably, those most likely to experience fertility losses in the wild would be species with relatively low thermal fertility limits living at their warmest edge of their range. I would be interested in collecting wild *Drosophila virilis* males and females during the summer. *D. virilis* is nearly cosmopolitan in distribution (Mirol et al. 2008), so is likely to experience vastly different temperatures across its species range. My PhD work has tested male and female *D. virilis* fertility limits in many different contexts, so the groundwork provided here could help identify heat-induced sterility in wild-caught flies. To do this, I would identify areas at the upper thermal edge of *D. virilis*' species range and trap wild flies across seasons. I would provide any caught males with laboratory-bred female partners and check for offspring production over the course of a number of weeks, similar to my methodological design in chapters 1 and 2. I would allow any caught females the chance to lay offspring to get an idea of the general female fertilisation rate. As in Chapter 3, I would also consider remating a sub-section of caught females in order to determine whether females unable to produce offspring are actually sterile, or simply do not carry viable sperm. I think that the key to making a project like this work is to ensure that we develop a throughput method to capture flies, and understand the general fertility rates of wild-caught males and females. Then, when we measure these same responses during and after a heat-wave, we can more easily identify whether temperature is affecting fertility. There are a few issues with this kind of experiment, however. Firstly, fruit-flies are generally much more difficult to capture during extreme weather, so ensuring capture rates are sufficient in hot weather will be a challenge. Also, the change in weather and/or seasonality may result in changes to the population dynamics of caught flies. For example, we may capture different sections of the populations during a heat-wave such as an ageing or young population. Regardless of these concerns, this would be among the first project to test wild populations for heat-induced sterility and would therefore be extremely valuable.

One of the problems with examining how climate change affects populations is how costly it can be to collect data across multiple years. However, we may be able to utilise pre-existing long-term datasets, which may have been originally collected for a different purpose. Many long-term projects measure reproduction in some way- whether that is overall population size, counting new births in a population, or even measuring individual reproductive rates. As long as approximations for the location and date of these data are also available, we would likely to be able to extract average temperatures of the area. I would examine whether changes in reproductive performance or population size correlate with the incidence of heat-waves. This technique could allow us to better understand the impact of climate change on fertility of larger, long-lived species such as mammals, birds, and fish, without the immense cost of initiating a new long-term research project.

## Practical uses of TFL research

While there are still so many unanswered questions, the findings I present in my thesis generally paint a negative picture for biodiversity. However, I want to finish my discussion touching on the potential practical uses of research into thermal fertility limits. I believe there are some important possible outcomes from this work.

Firstly, I believe that measuring thermal fertility limits across species will allow us to make better informed conservation decisions. As discussed in my introduction and as is shown in Parratt et al. (2021), including fertility limits into species' extinction risk can substantially change the rankings of species most vulnerable to the effects of high temperatures. Therefore, by continuing to measure fertility limits we may be able to more effectively and efficiently construct conservation plans that target the most vulnerable species as climate change progresses.

Another important role that this field could play is in combating food security crises. One of the original papers that inspired my review chapter found that summer infertility in

pigs is caused by DNA damage in sperm (Peña et al. 2019). This brought to my attention the fact that European breeds of boars, and probably other species, can be at risk to the effects of heat-induced sterility when raised in tropical locations for agricultural purposes. Heat-stress already costs the pig industry millions per year (St-Pierre et al. 2003), so increasing average and extreme temperatures are likely to exacerbate this issue, narrowing the areas where sensitive breeds can be used. By researching fertility limits, we could help identify resistant species or genotypes in hot areas, to improve efficiency. If we can better understand the genes involved in heat-resistance as I do in chapter 4, we may be able to create breeds or even hybrids that can succeed at higher temperatures.

The final example I would like to bring up here is gaining insight into the spread of vector-borne diseases. Thermal fertility limits can be used to predict current species distributions (Parratt et al. 2021; van Heerwaarden and Sgrò 2021). Therefore, we can use future climate scenarios to predict the change in distribution of populations as the globe warms. It follows that we could use fertility limits to explore the change in viable species range of vectors that bear disease, such as *Anopheles* mosquitos which transmit malaria (*Plasmodium sp.*). Hopefully, we could help use this information to more accurately model the change in species distributions. Ultimately, this could help prepare areas for the spread of disease, as rising temperatures force the invasion of vector populations into new areas.

This list of possible uses of research into fertility limits is not exhaustive. However, I want to finish on a more positive note by touching on a few of the major practical uses of this kind of research. Although the field of heat-induced sterility began almost 100 years ago, it is still in its infancy. However, it is picking up steam quickly as more researchers are finding and addressing interesting and important questions. I hope that my thesis has helped move towards these outcomes and shown that heat-induced sterility is a problem that species may struggle to cope with.

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# Appendices

## Appendix 1: Sex-Specific Differences in Thermal Fertility Limits

Graziella Iossa

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Critical thermal limits (CTLs) are established viability thresholds when studying the impact of climate change on natural populations. Novel 'thermal fertility limits' of species have been proposed alongside CTLs, to better assess the sublethal effects of rising temperatures on species persistence. However, sex-specific sensitivity of fertility to temperature also needs consideration.

Walsh et al [1] highlight the importance of monitoring and assessing fertility when investigating the impact of rising temperatures on natural populations. Models of the long-term impact of climate change on populations have focused on upper and lower 'critical thermal limits' (CTLs) beyond which critical biological functions and specifically, survival, are compromised (e.g. [2]). The authors note though, that individual fertility is typically compromised at lower temperature thresholds than CTLs, whereupon individuals are viable but infertile, and this can therefore jeopardise population persistence. In their article, they propose a framework for examining species 'thermal fertility limits' (TFLs) and the development of a standardised measure of fertility, similar to measures of

viability and other standardised measures of CTLs ([1]). An ecologically-relevant limit to fertility, they add, is represented by the point (i.e. temperature threshold) in which an organism is unable to produce viable adult offspring under controlled conditions. The introduction of TFLs is timely and pivotal to correctly estimate the effects of sublethal temperatures on the persistence of natural populations. However, to properly frame TFLs for researchers new to fertility studies, it is important to highlight a crucial difference in the way male and female primary reproductive traits respond to thermal stress. In the species studied to date the emerging pattern is that male gametogenesis appears more sensitive to thermal stress than female gametogenesis. These sex-specific differences in thermosensitivity can be observed in plants as well as animals, both for endotherms and ectotherms (Table 1).

For example, in *Drosophila spp.*, one of the better studied taxa both in terms of sexual traits and in terms of the effects of thermal stress on reproduction, upper TFLs are reached at lower temperatures for males than for females ([3]). *Drosophila buzzatii, D. simulans* and *D. melanogaster* males but not females exposed to heat stress are infertile, as females mated to unexposed males produce viable offspring (reviewed in [3]; [4]). Similarly, within developing flowers male reproductive organs appear more sensitise to temperatures $\geq$ 30°C than female reproductive organs. Reciprocal crosses between heat-stressed plants revelaed that the use of pollen produces significantly lower yield than when heat-stressed female plants are used as the receptor plant ([5]; [6]). Temperature-induced male infertility arises, at least in insects and mammals, from impaired

3027 spermatocyte and spermatid form and elongation leading to abnormal sperm form and
3028 function and reduced sperm motility ([7]; [3]). This is not to say that female reproductive
3029 organs are immune to heat stress. In mammals heat stress affects spermatogenesis but
3030 also oocyte function impairing fertilisation (reviewed in [7]). In the coral *Acropora*
3031 *digitifera*, an increase of 2°C in water temperature caused a significant decrease in sperm
3032 number and egg volume, but no change in egg number ([8]). The underlying physiological
3033 and biochemical mechanisms for this male-biased sensitivity and, more generally, the
3034 mechanisms implicated in the dysregulation of male and female reproductive function,
3035 are unclear and in need of further study ([3]; [6]).

3036 Accounting for these sex-specific differences in thermosensitivity is pivotal to model
3037 realistic scenarios for natural populations under heat stress. Female fertility, seemingly
3038 more resilient to heat-induced stress than male fertility, may buffer population
3039 persistence as temperatures continue to rise. This could happen via dispersal,
3040 immigration or be dependent on the level of mating promiscuity specific for that species
3041 or population. For example, polyandrous females may be able to reproduce where
3042 monoandrous females cannot. Moreover, the effects of male infertility might be
3043 compounded by the effects of heat stress on the likelihood of copulating [9]. In the design
3044 of quantitative point estimates of temperature limits for fertility that Walsh *et al.* [1]
3045 suggest to adopt, it will be important to consider that these estimates will likely vary
3046 between sexes (as well as depending on location, e.g. [10]). Equally, as TFLs can be
3047 defined as the temperature at which a determined proportion of individuals is sterile, at

either an upper $TF_{MAX}$ or lower $TF_{MIN}$ thermal stress limits [1], it is significant to emphasise that the proportion of infertile males and females at that point will differ. These sex-skewed differences on the proportion of infertile individuals will affect predictive models of distribution, abundance and persistence of populations under different climate change scenarios. As we work towards producing standardised measures for TFLs, it is important to understand the functional mechanisms responsible for these sex-skewed differences in thermosensitivity on fertility to inform robust predictions on the effects of climate change on population stability and persistence as well as capitalise on any buffering properties of reproduction.

**Table A1.1**. Examples of sex-specific differences in thermosensitivity on fertility

| Taxonomic group | Organism | Species | Measure | Sex affected | Refs |
|---|---|---|---|---|---|
| Insect | Fruit fly | Family: Drosophilidae | Offspring production, sperm motility | Males but not females | [11]; [3]; [10] |
| Vertebrate | Cow and bull | *Bos taurus* | Fertilisation | Both males and females | [7] |
| Poales | Barley | *Hordeum vulgare* | Gamete viability | Anthers but not ovules | [12] |
| Cnidarian | Coral | *Acropora digitifera* | Gamete number | Egg volume and sperm number but not egg number | [8] |

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# Appendix 2: Integrated Approaches to Studying Male and Female Thermal Fertility Limits

Benjamin S. Walsh, Steven R. Parratt, David Atkinson, Rhonda R. Snook, Amanda Bretman, and Tom A.R. Price

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In Walsh & Parratt *et al* (2019) [1] we call for research into the thermal fertility limits (TFLs) of species to better predict the impact of climate change, especially the increased frequency of heatwaves, on biodiversity. In a response to this, Graziella Iossa outlined the need to consider sex-specificity of TFLs within this framework [2]. Broadly, we agree with this; sexual specificity may be common in TFLs, and understanding this may be crucial to predicting a species' vulnerability to temperature. Ascertaining the extent and prevalence of sex-specific differences should be an essential goal of TFL studies.

However, although we agree that the limited current evidence suggests males are more sensitive to fertility loss than females, outside the plant literature there are relatively few studies that directly measure both male and female fertility simultaneously, most of which are included in Iossa's response (see [2, 3, 4]). We caution that it is too early to assume that male fertility is universally more vulnerable to temperature. We think the literature is heavily biased towards male gamete thermal tolerance, probably due to the idea that with a smaller investment required, cheaper male gametes are less robust. There are also some obvious morphological adaptations in male homeotherms to prevent

3108 sperm from experiencing thermal stress, such as the presence of external testes in many
3109 mammals. But we caution that until more studies investigate the temperature sensitivity
3110 of female fertility, we cannot assume females are more robust than males. Ultimately, we
3111 need more studies that directly examine the fertility of both sexes under similar
3112 conditions. An interesting approach might be to study TFLs in monoecious (i.e.
3113 simultaneous hermaphrodite) species, such as mangrove killifish [5], many gastropods,
3114 and many flowering plants. This would provide excellent tests of which gametes are most
3115 vulnerable to temperature extremes. Moreover, we do not know a great deal about
3116 which stages of gamete production are most vulnerable to thermal stress. In many
3117 species oogenesis develops to a late stage early in a female's life, whereas males develop
3118 sperm from basal cells throughout their lives. If more mature gametes are vulnerable to
3119 thermal stress, thermally induced female sterility might be more likely to be permanent,
3120 while males may recover fertility over time. Again, we need more detailed studies, across
3121 a variety of taxa.

3122 Where significant sex-specificity in TFLs exist, we need to consider how these differences
3123 manifest at the population level to understand species' vulnerability to climate change.
3124 For instance, in species where a few fertile males can fertilise large numbers of females, a
3125 small drop in thermally tolerant female fertility might have a similar effect as a
3126 catastrophic drop in male fertility at the same level of thermal stress. Theoretical
3127 methods that assess the contribution of each sex to population persistence may be
3128 invaluable in determining the relative importance of male and female TFLs**.** Analogous

models on sex ratio [6] suggest that even a small loss in fertile females may have a greater impact on population persistence than losing the majority of fertile males. Therefore, even if female fertility is less vulnerable to temperature than male fertility, it may still be more important for many organisms.

Iossa makes the interesting point that the importance of sexual specificity in TFLs may depend greatly on the mating system of the species; monandrous species may be more at risk to low male fertility than polyandrous species. However, even populations of polyandrous species may be vulnerable, if sterile males or inviable male gametes act as inhibitors by blocking fertilisation opportunities for fertile males. Indeed, the application of sterile insect release techniques to control disease vectors is based on the principle that a loss in male fertility can leave populations vulnerable [7].

Ultimately, while laboratory data will reveal the underlying biology of sexual specificity in thermal fertility tolerance, field studies will highlight the relevance of these data to natural processes. One possibility might be to test variation in both male and female gamete viability in broadcast spawners, by taking samples from the water column as average water temperatures continue to rise or during extreme temperature events. Researchers could also examine the impact of natural heat waves on the population dynamics and demography in closely related species with different mating strategies. Paternity analysis may also allow researchers to detect the effects of heat-induced sterility in polyandrous species – low male fertility may result in fewer fathers within

broods, whilst low clutch size or unhatched eggs might indicate both sexes are being affected.

Iossa's comments highlight some of the inherent complexity within TFL research, but also the need for integrated approaches to these important questions. Ultimately, it will take field, laboratory, and modelling studies across a broad range of organisms to determine if, when and where TFLs matter.

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