Thermal impacts on insect reproduction

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Thesis submitted for the degree of Doctor of Philosophy

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September 2025

Thesis abstract

Humans are driving significant changes to Earth's climate, resulting in both an increase in the mean global temperature and a rise in the incidence of extreme heat. Heatwaves are becoming more common and intense, which is particularly concerning given that they are predicted to have a greater ecological impact than increases in mean temperature alone.

Insects are the most diverse and abundant group of animals on the planet, and underpin a host of essential ecosystem processes. Their ectothermic physiology makes them particularly susceptible to thermal stressors such as heatwaves. Throughout this thesis, *Tribolium castaneum* is utilised to assess the impacts of ecologically relevant heatwave exposures on survival, reproduction and gene expression. This species serves as a model to represent how insects may broadly respond to heatwaves under current and future climate change scenarios.

Throughout this thesis, it is demonstrated that *Tribolium castaneum* is resilient to less intense heatwaves of 42°C and 44°C. However, as temperatures increase from this point, the resilience of key focal groups (virgin males, virgin females, mated males, and mated females) fails dramatically. Widespread reductions in survival and fertility were observed in response to heatwave exposures of 46-50°C, temperatures which are projected to become commonly seen across large areas of this species' global distribution under climate change. Most concerningly, it is demonstrated that mated females show the greatest vulnerability to such heatwave exposures. Mated females exhibited reductions in survival under less intense heatwave conditions than the other focal groups and consistently showed worse survival than all other focal groups in response to identical heatwave conditions. This vulnerability is further investigated through the examination of transcriptional responses to a 48°C, 5-hour heatwave exposure. Under these conditions, it is demonstrated that post-mating, females fail to mount a molecular heat stress response, providing a potential mechanism underlying the vulnerability of mated females to heatwaves.

Acknowledgements

Firstly, I would like to thank Professor Matthew Gage, without whose belief in me and this project, I would never have had the opportunity to embark on this journey. It is only a shame he did not see the fruits of his guidance.

I would also like to thank Professor Alexei Maklakov and Professor Tracey Chapman, who kindly stepped in to supervise me following Matt's passing. Their willingness to take me on and their guidance have been invaluable.

A special thanks to Dr Ramakrishnan Vasudeva, whose mentorship has been central to my growth as a scientist, from learning the basics of all things *Tribolium* to navigating the complexities of publishing and the art of networking at conferences. His unwavering ability to commit his time, energy and patience to those around him is genuinely remarkable.

To the rest of the *Tribolium* lab: George, Mike, Tash, and Ginny, thank you for your support, advice, and collaboration, which made the lab an enjoyable place to work. I am also grateful to the project students who worked alongside me, and to the wider Biology community at UEA, who made this experience so rewarding.

Thanks to my friends, especially the Kois, whose laughter, kindness and encouragement are irreplaceable.

To my family, Mum, Dad, Vicky and Josh. Thank you for your endless love and support. A particular thanks to Josh, with whom I had the rare privilege of collaborating. Let the record show I submitted first.

Finally, to my wife, Bethany. You have endured more stories and frustrations about beetles than anyone should have to in ten lifetimes. Your support and love have carried me through this PhD.

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1. General introduction

1.1 Climate change and extreme weather conditions

Significant changes to Earth's climate have already been observed as a result of human activities, including increased greenhouse gas emissions, land surface changes, and particulate air pollution (Garnett, 2009; Ramanathan and Feng, 2009; Paoletti et al., 2010). The most prominent cause of climate change is increasing global carbon dioxide levels (IPCC, 2013; Anderson, Hawkins and Jones, 2016), which have increased from 280ppm in pre-industrial times to current levels of over 410ppm (IPCC, 2023). These human-driven changes are predicted to result in a mean global surface temperature increase of up to 1.9°C higher than in pre-industrial times by 2028 (World Meteorological Organisation, 2024), and have caused a mean sea surface temperature increase of 0.11°C per decade since 1971 (IPCC, 2014). Furthermore, the rate of temperature increase is accelerating as levels of carbon dioxide emissions continue to rise, resulting in the 2015-2019 period being on average 0.2°C hotter than the previous 5 years (World Meteorological Organisation, 2020). By 2100, the mean global surface temperature is projected to increase dramatically by a 'best estimate' of a further 2.7°C under intermediate greenhouse gas emission scenarios (SSP2-4.5; IPCC, 2023).

In addition to increases in global mean temperatures, the incidence of extreme heat conditions is also rising. Heatwaves (classically defined as an increase of daily maximum temperatures by 5°C or more for five consecutive days (Frich et al., 2002)) are predicted to become more common and intense throughout the 21st century across both terrestrial (Frich et al., 2002; Meehl, 2004; Trenberth, 2018) and marine environments (Frölicher, Fischer and Gruber, 2018; Oliver et al., 2018). Research has suggested that less predictable and more extreme climate events, such as heatwaves, may be more harmful to biodiversity than increases in mean temperature (Parmesan, Root and Willig, 2000; Ruthrof et al., 2018; Smale et al., 2019; Seneviratne et al., 2021). Biologists are therefore exploring how these more extreme conditions impact on living systems.

1.2 Insects and their importance

Insects are the most numerous and diverse animal group, with an estimated 5.5 million species existing worldwide (Stork, 2018), occupying a wide range of niches and playing crucial roles in most ecosystems. Insects can be broadly defined as having several shared characteristics: three pairs of jointed legs, a three-section body, a chitinous exoskeleton, compound eyes and a pair of antennae (Grimaldi and

Engel, 2005). Within this group, over 1 million species have been classified so far, with a majority of the diversity being found in the following Orders: Coleoptera (386,500 species), Lepidoptera (157,500 species), Diptera (155,500 species), Hymenoptera (117,000 species) and Hemiptera (103,500 species)(Stork, 2018).

The rich diversity across insects means that they play key roles across a wide range of biological functions. From a human perspective, many of these insects provide vital ecosystem services such as nutrient cycling (Maldonado et al., 2019), biological control (Southon et al., 2019) and pollination (Klein et al., 2007). Pollination alone is estimated to be worth over $200 billion annually to the global economy (Gallai and Vaiisiere, 2009; Potts et al., 2016). Conversely, insects are vectors of disease (Peterson et al., 2005), and cause damage to buildings (Robinson, 2005), crops, livestock and agricultural yields (Arora and Sandhu, 2017). In post-harvest stored-product facilities alone, yield losses range from 9% to upwards of 20% due to pest insects (Phillips and Throne, 2010).

If the human global population continues to grow, biodiversity and ecosystem services are expected to come under even greater strain (Scholes, 2016). Should key ecosystem roles (both positive and negative from a human perspective) be impacted by environmental change, the ability for humans to feed themselves into the future could be drastically impacted (Novais et al., 2018; Lehmann et al., 2020). From a research perspective, we therefore need to understand how insects are affected by climate change across a range of levels, particularly in relation to pollinator and pest species.

1.3 Insect susceptibility to climate change

Insects are ectothermic and rely on their external environment for thermoregulation (Resh and Cardé, 2009). Insects also have complex and often multi-stage life cycles, which can involve extreme physiological and biochemical changes, as well as alterations to both body plans and size (Resh and Cardé, 2009; Merkey et al., 2011). Undergoing such rapid, diverse and complex changes through different life stages could make insects particularly vulnerable to extreme weather and its associated abiotic stressors (Franke et al., 2014; Sales et al., 2021).

With anthropogenic climate change occurring at accelerating rates (World Meteorological Organisation, 2024), there is emerging evidence that it has caused species extinctions (Ceballos et al., 2015), with insects highlighted as being particularly at risk (Dunn, 2005; Wiens, 2016). There are general concerns that the ability of insects to ‘out evolve’ the predicted temperature increases is limited, and

may not occur at rates needed to match climate change (Kellermann et al., 2012). Research has shown that in some species, insect populations lack sufficient genetic variation in traits related to heat stress responses, thereby constraining their ability to adapt genetically under current warming trajectories (García-Robledo and Baer, 2021).

Recent large-scale studies have shown dramatic global insect declines, for example, Van Klink et al. (2020) showed a 9% decline in global terrestrial insect abundance per decade and Hallmann et al. (2017) showed a 75% decline in total flying insect biomass in German protected areas over 27 years. Furthermore, Van Klink et al. (2020) suggest that an 11% increase in freshwater insect abundance per decade may be partly due to climate change. It has also been shown that species that were most abundant in the past have exhibited the strongest declines (van Klink et al., 2023). These general trends may not accurately reflect the true extent of climate change impacts, as different groups of insects are affected differently, and some species are more ecologically relevant to humans.

Sánchez-Bayo and Wyckhuys (2019) conducted a comprehensive review of historical insect biodiversity declines, concluding that Lepidoptera, Hymenoptera, and Coleoptera have been the most affected Orders in terrestrial ecosystems. Furthermore, climate change has often been cited as the primary driver of declines in butterfly and bee populations (Sánchez-Bayo and Wyckhuys, 2019). Forister et al. (2021) demonstrated an increased susceptibility of butterflies to climate change due to physiological stress and altered species interactions associated with autumn warming, a critical season for insect diapause and migration (Gallinat, Primack, and Wagner, 2015). A 1.6% annual loss of butterfly abundance was estimated between 1977 and 2018 across 262 species in 72 locations in the American West (Forister et al., 2021). Similarly, using bumble bee occurrence data for 66 species across North America and Europe, Soroye, Newbold and Kerr (2020) linked the increasing frequency of abnormally hot days under climate change to increased local extinction rates and reduced site occupancy between baseline levels (1901-1974) and recent data (2000-2014). The probability of site occupancy declined on average by 17% in Europe and 46% in North America, with much of this loss due to substantial declines in warming southern population margins, coupled with a lack of compensating colonisation in cooler northern margins.

In addition to taxa-level susceptibility to climate change, studies have highlighted variation in sensitivity to change across different thermal niches. Counterintuitively, tropical systems are expected to suffer more under climate

change because insect species are adapted to a less variable thermal environment, reducing plasticity and the potential to adapt (Chevin and Hoffmann, 2017). Deutsch et al. (2008), for example, showed that tropical insects had a warming tolerance (the difference between CT_{max} values and current mean annual surface air temperature) one-fifth of the values seen in mid-latitude insects. Similarly, Van Heerwaarden and Sgrò (2021) showed that tropical species of *Drosophila* went extinct at temperatures 1.6°C less than widespread species (all species were collected from the same location) under incremental temperature increases of 0.2°C per generation. Tropical lowland forest ectotherms in particular tend to be adapted to narrow temperature ranges (Huey et al., 2012), which is especially concerning because these are the regions where insect biodiversity is especially high (Novotny and Miller, 2014; Stork, 2018). In their review, Sánchez-Bayo and Wyckhuys (2019) states that the impacts of climate change have been most evident in tropical regions and on endemic species. These groups have seen reduced genetic diversity due to strong selection pressures of climate change hindering dispersal capacity and host selection (Hill, Griffiths and Thomas, 2011), which is expected to particularly impact invasive species (Pauls et al., 2012).

1.4 Adaptive and plastic potential of insects

Adapting to or buffering against changing and variable climate conditions are paramount for species survival (Seebacher, White and Franklin, 2014). However, ectothermic insects are limited in their physiological abilities to control thermoregulation and are strongly subject to environmental temperatures. In order to survive and maintain fitness in a thermal environment that is changing unnaturally (Abram et al., 2016), they must either move towards more favourable conditions, adopt behavioural strategies that allow cooling, or adapt (Crozier et al., 2008; Platts et al., 2019; Radchuk et al., 2019).

With a rapidly rising global surface temperature (World Meteorological Organisation, 2024), species are under pressure to respond and adapt to novel thermal regimes. Inherent variation in the anatomy, physiology and genetics of a species can dictate the potential and extent of these responses (Platts et al., 2019). Adaptation to climate change can be split into two general strategies: 1) phenotypic plasticity or 2) genetic selection and evolution. Phenotypic plasticity is commonly described as a change in phenotype in response to environmental changes, which is not associated with any underlying change in genotype (Kellermann and van Heerwaarden, 2019). Plastic responses can occur rapidly, producing changes in phenotype in response to sublethal environmental stimuli over a scale of minutes to

hours (Kellermann and van Heerwaarden, 2019). Such reactions to extreme temperature can often include transcriptional responses to changes in temperature, whether an increase or a decrease. In *Monochamus alternatus,* various HSP70 isoforms show rapid upregulation in response to heat shock stress, whose expression remains elevated for over 48 hours (Li et al., 2022). Another example of this can be seen in cold tolerance in the soybean pod borer, *Leguminivora glycinivorella*. Exposure of adults to temperatures of -5°C for 1.5 hours resulted in a rapid increase in HSP70 expression by up to 80 times compared with levels observed in individuals maintained at 25°C (Wang et al., 2014). Longer-term responses can also be seen, often with more extreme phenotypic changes, such as phase polyphenism in locusts, which may take generations to occur fully (Song et al., 2017). Many insect traits, which increasing temperatures may impact, exhibit some phenotypic plasticity (Sgrò, Terblanche, and Hoffmann, 2016). Examples of plastic responses to increasing temperatures include shorter larval development in *Neodiprion sertifer* (Kollberg et al., 2013), delayed reproduction and cryptic wing colours in *Bicyclus anynana* (Rodrigues and Beldade, 2020) and increased number of generations per year due to delayed induction of diapause in *Paludum fabricius* (Harada et al., 2011).

Plastic changes often include morphological and physiological alterations to improve overall survival, but specific responses are also known, such as gamete plasticity to improve reproductive fitness. Vasudeva et al. (2019) showed in adult red flour beetles (*Tribolium castaneum*) that sperm and egg size and function were thermally plastic, conferring reproductive advantages in novel thermal environments. There is therefore evidence for some thermal plasticity that allows insects to cope with climate change and thermal extremes. Conversely, van Heerwaarden and Sgrò (2021) have recently highlighted limitations in plastic responses to thermal stress. Across 10 species of *Drosophila*, males showed no capacity to plastically shift their TFLs (Thermal fertility limits, the temperature at which thermally induced sterility occurs) under incrementally increasing temperature regimes (+0.2°c per generation). Furthermore, males only showed limited ability to plastically improve their CTmax (critical thermal maximum, the temperature at which all females went into a heat coma) by a maximum of 0.1°c per 1°c of warming. A recent meta-analysis has also highlighted that beneficial plasticity is generally weak. Weaving et al. (2022), assessed 74 studies of 102 insects, and showed that thermal limit plasticity is pervasive but weak, with CT_{max} increasing by only 0.09°C for every 1°C of acclimation. Such a small degree of adjustment is unlikely to buffer insects

against the rapid pace of global temperature increases, coupled with more frequent and intense heatwaves.

Genetic adaptation through evolutionary selection occurs over a much slower multi-generational timescale than short-term plastic responses to changes in temperature (Sgrò, Terblanche and Hoffmann, 2016). The ability and potential for populations to thrive by adapting to increasing temperatures under climate change is complex and not guaranteed. Strong selection by one environmental stressor has the potential to cause fitness trade-offs, and there is an obvious requirement for genetic variation to be present in the gene pool for selection and adaptation in novel thermal environments (Meester, Stoks and Brans, 2018). It has been proposed that evolutionary adaptation to a broad range of temperatures is physiologically costly, and not uniformly evolved (Kaspari et al., 2014). Different species of ants from the same Family living in close proximity have been shown to have vastly different CT_{max} values (maximum temperature at which an organism can perform activities requiring muscular control such as the ability to self-right), due to subtle habitat niche differences (Kaspari et al., 2014). Furthermore, Porcelli et al. (2016) showed responses to a 6-day heatwave could vary significantly between populations in *Drosophila subobscura*, based on geography and their variable natural conditions. In general, the evolutionary potential for traits such as heat resistance to be selected and evolve within populations at a sufficiently rapid rate to combat extinction under climate change is so far poorly established, with several studies showing population extinctions under both gradual and extreme temperature increases (Plesnar-Bielak et al., 2012; Schou et al., 2014; van Heerwaarden and Sgrò, 2021). Adaptation rates, especially in reproductive traits, may be assisted by sexual selection, with polyandry allowing a greater chance of population persistence under experimental temperature increases compared to monandry (Grazer and Martin, 2011; Plesnar-Bielak et al., 2012; Parrett and Knell, 2018).

1.5 Insect responses to climate change

In response to increasing temperatures, significant range shifts have been seen across different insect taxa, with many species expanding their range towards the poles or by increasing altitudes (Chen et al., 2010; Pureswaran, Roques and Battisti, 2018). As high as 61% of all montane insects have been reported to be moving upwards with warming temperatures, although very few are successfully tracking such temperature changes (McCain and Garfinkel, 2021). A heavy focus has been put on assessing range shifts in ecologically important species. Such examples include elevation shifts of 52-68 metres across 208 lepidopteran species

in Malaysia over 42 years (Chen et al., 2010) and an uphill shift in range in up to 90% of the 49 dung beetle species found across two mountain systems in France and Spain over 20 years (Menéndez et al., 2013). While several insect species have successfully expanded their occupied ranges (Hill et al., 2002; Jepsen et al., 2008), limitations have been identified for all species in altering their ranges. Kerr et al. (2015) showed, across 67 bumblebee species, that failure to track warming conditions in the northern part of their geographical range had occurred, while simultaneously losing up to 300km of southern range, indicating obvious challenges for some species to expand their range into new habitats or areas. Another issue is whether physical limits are blocking range shifts, with insurmountable obstacles such as the peaks of mountain ranges, water bodies or different vegetation types limiting dispersal (Hill et al., 2002; Chen et al., 2010).

Even in species that successfully undergo range shifts, trade-offs may occur. Reduced genetic diversity in the leading edge of a range can occur due to recurring founder events, which alter genetic distribution and potentially reduce population viability or cause extinctions (Pauls et al., 2012; Gilbert, Peischl, and Excoffier, 2018). Climate change has also caused multiple species' ranges to overlap, and rates of hybridisation have increased (Sánchez-Guillén et al., 2015). Range shifts also cause dramatic changes to the number of species associated with particular areas. For example, 11 of the 16 species of bumblebee initially associated with a mountain system of the Northern Iberian Peninsula went locally extinct over 20 years due to range shifts (Ploquin, Herrera and Obeso, 2013).

As temperatures continue to increase, biological and ecological mismatches are likely to impact on a wide range of insects, including both pests and pollinators (Renner and Zohner, 2018). Some pollinators will show reduced fitness under higher temperatures and may be expected to alter their phenology and/or ranges in synchrony with pollinator-associated plants (Parmesan, 2006). However, due to differences in interactions between increasing temperatures and both pollinators and their associated plants, temporal (where flowering times and pollinator emergence or maturation times do not align) and spatial (where thermal niches clash with unsuitable conditions for either the plant or their pollinator, such as the presence of other species or habitat changes) mismatches are likely to become more common and widespread (Hegland et al., 2009), which could potentially reduce crop yields. Whilst documentation of spatial mismatches between plants and their insect pollinators has been historically lacking (Morton and Rafferty, 2017), modelling strongly suggests that this will be frequent in the future under climate

change scenarios (Kolanowska, 2025; Watteyn et al., 2025). Changes in temperature may also cause a decoupling between pest ranges and their natural predators (War et al., 2016), shifts in life histories that cause demographic and ecological mismatches between insect pests and crops (Renner and Zohner, 2018), and shifts in pest species ranges, densities and growth rates (Sharma, 2014; War et al., 2016). All of these factors may alter how we manage pests, potentially leading to reduced yields and production. A telling example here comes from the modelling of *Tribolium castaneum* ranges under predicted climate change. By 2100, range expansions are estimated to increase yearly economic losses of up to $3.16 billion in cocoa bean production alone (Jung et al., 2020).

Utilisation of a diverse range of behavioural strategies to prevent thermal damage will also be crucial to enable survival and reproduction for many insect species. A range of studies in recent years has shown how insect systems can improve their thermal tolerance through direct and indirect behavioural strategies. For example, aphids show increasing dropping probabilities in response to higher temperatures (Ma and Ma, 2012), parasitoid wasps increase foraging patch residency time in order to maintain lipid reserves in a warming environment (Denis et al., 2013), and butterflies show changes in habitat association with hotter conditions (Davies et al., 2006). These behavioural adaptations to buffer against increased temperatures may come at a cost to fitness, with impacts on other behavioural aspects such as reduced feeding or predator avoidance being seen (Ma et al., 2018). For example, treehoppers may respond to extreme heat by altering behaviours to rapidly escape from hot microclimates, a strategy that improves survival but comes at the expense of reproduction (Leith et al., 2024).

In parallel with ecological studies, lab-based work has increased to investigate how thermal stress impacts insect systems. Not only has this allowed greater understanding of concepts such as thermally increased chances of death (eg. Blanckenhorn et al., 2014 and Porcelli et al., 2016) and species extinctions (eg. van Heerwaarden and Sgrò, 2021), but it has also allowed a greater focus on the underlying sub-lethal mechanisms by which thermal stress can impact insects (eg. Chirault et al, 2015 and Sales et al., 2018).

1.6 Reproduction as a sub-lethal mechanism for insect declines

To effectively understand current and future insect losses, it is essential to elucidate the underlying mechanisms by which increasing temperatures impact insects. As

insects are ectothermic, they are subject to a more variable internal thermal environment than organisms that can control this environment through physiological means and temperature homeostasis (Abram et al., 2016). Insect responses to temperature are often framed in the context of thermal performance curves, where an individual's biological performance increases towards an optimum temperature for a variety of traits before declining after exceeding this optimum (Huey and Stevenson, 1979; Deutsch et al., 2008). Within such a framework, T_{opt} represents the temperature at which each given trait is maximised, with performance declining as temperature moves away from this value, in both directions. As previously discussed, not only do increased temperatures and heatwave events cause death in insects, but there is also a wide variety of sub-lethal impacts that may harm the ability of insects to thrive and persist into the future. One sub-lethal aspect which is gaining recognition as a limiter for population viability under climate change for ectotherms is thermal damage to reproduction (Grazer and Martin, 2012; Walsh et al., 2019)

Unlike in endotherms, where reproductive biology, associated sensitivities, and adaptations to protect against them have long been a topic of research (Galil and Setchell, 1988; Mieusset and Bujan, 1995), much less is understood about thermal reproductive sensitivities in insects and ectotherms generally. The majority of research on thermal effects focuses on the survival consequences rather than sublethal damage to fertility and reproductive success under increased temperatures (Hoffmann, 2010). More recently, however, there has been a focus on research into thermal reproductive sensitivities, revealing effects across multiple species in both males and females (e.g. Porcelli et al. (2016), Sales et al. (2018), Canal Domench and Fricke (2023) and Ratz et al. (2024) for males, and Delisle, Bernier-Cardou and Laroche (2016), Bauerfeind et al. (2018), Weaving, Terblanche and English (2024) and Meena et al. (2024) for females).

Classically, a key method to distinguish an organism's response to a range of temperatures has been achieved through assessment of critical thermal limits, typically assessing the CT_{min} and $CT_{max,}$ representing the critical thermal minima and maxima, respectively, whereby individuals lose the ability to perform essential functions, for example, self-righting (Walsh et al., 2019). Whilst these limits are beneficial for assessing acute thermal tolerance, they may not account for sub-lethal fitness costs, which are particularly important to assess as different traits can have different thermal performance curves and will not share the same optima or limits as critical thermal limits.

One key aspect of reproductive success under differing thermal conditions is Thermal Fertility Limits (TFL), where a species has evolved to reproduce between a minimum (TF_{MIN}) and maximum (TF_{Max}) temperature (Walsh et al., 2019). By definition, temperatures outside of these ranges will induce sterility (Walsh et al., 2019). Temperature-induced sterility has been shown to be both temporary (Sales et al., 2021) and permanent (Jørgensen, Sørensen and Bundgaard, 2006), depending on the species and individual context. Male TFLs have been shown to better predict species distribution than previously used Critical Thermal Limits (CTLs), and may help to predict changes in distribution under future climate change (Parratt et al., 2021, Van Heerwaarden and Sgrò, 2021). Due to an inability to thermoregulate physiologically, ectotherms may be particularly vulnerable to fertility damage from extreme temperatures (Kingsolver, Diamond and Buckley, 2013). As temperatures move towards a species' TFL, some degree of intermediate fertility loss is usually observed before reaching the limit, showing that even non-sterilising temperature changes may impact fertility (Chakir et al., 2002). At some point between these TFLs, a thermal fertility optimum (TF_{OPT}) will occur. A critical challenge in assessing the thermal impacts of climate change on reproduction will be to evaluate which groups of animals will be driven away from their TF_{OPT}, and whether these species have traits that render them more vulnerable to these changes. As previously discussed, across 10 species of *Drosophila*, no ability to plastically change TFLs under gradually warming conditions was detected (Van Heerwaarden and Sgrò, 2021).

Thermal performance curves are also important for understanding how heatwaves may impact organisms. Due to a heatwaves' fluctuating thermal profile, and the fact that the relationship between temperature and individual trait performance is non-linear, the biological impact of heatwaves may be greater than expected under the mean temperatures experienced within them (Martin and Huey, 2008). This is consistent with Jensen's inequality, where variation around a mean can alter average biological outcomes when the relationship between temperature and performance is nonlinear. This is particularly relevant where individuals are found close to the upper end of various traits' thermal performance curve, where performance often falls off dramatically, for example, in tropical species which are adapted to a narrow range of temperatures (Deutsch et al., 2008). Under such scenarios, short periods of intense heat experienced during a heatwave may cause higher reproductive trait losses than expected under increased mean temperatures.

Heatwave impacts on insect reproduction may therefore be modulated not only by the temperatures experienced, but by duration, timing and accumulation of damage.

The dominant mechanisms of insect reproduction involve oviparity, viviparity and parthenogenesis, with oviparity being the most common (Bilinski et al., 2018). With the exception of parthenogenesis, insect reproduction largely follows a basic pathway. Reproduction begins with gametogenesis, followed by mating, female storage of sperm, fertilisation, and embryogenesis, and ends with the production of viable offspring (Resh and Cardé, 2009). The universality of these basic reproductive steps enables us to understand where thermal sensitivities occur along this path and then examine the impacts of future climate change by studying several model systems across the group. Going through each of these stages in oviparity, I summarise what we know about the impact of increasing and extreme temperatures on insect reproduction.

1.7 Reproductive stage sensitivities to heat

1.7.1 Gametes

1.7.1.1 General principles

Along with the majority of multicellular life, gamete dimorphism is displayed widely in insects; a single sperm cell is much smaller and therefore much less energetically and materially costly to produce than a single egg cell (Monro and Marshall, 2016). This variability between gametes, together with their widely different functions, organelles and mechanisms of production, may produce different interactions with increased temperatures. The impacts of environmental conditions on gametes have the potential to alter individual fitness throughout their entire lifespan (Porcelli et al., 2016). There is evidence that both sperm and eggs can adapt through phenotypic plasticity to changes in temperature, thereby increasing fertility and fitness (Vasudeva et al., 2019). Studies assessing the impacts of temperature on reproduction have historically failed to isolate female from male effects, and thereby have also failed to consider gamete-level sensitivities, although newer research is aiming to fill this knowledge gap (Dougherty et al., 2024).

1.7.1.2 Male gametes

Due to the small size of spermatozoa, the male gamete has limited cytosolic content and, consequently, a reduced level of intracellular antioxidants for preventing damage (Dada, 2017). Furthermore, sperm are highly sensitive to oxidative damage caused by increased temperatures due to the high presence of polyunsaturated fatty acids in their plasma membranes (Hamilton et al., 2016). Germ cells responsible for the initial production of spermatozoa also have high mitotic activity, which can make them more susceptible to environmental stressors such as heat (Du, Ashok Agarwal and Sabanegh, 2014).

The majority of insects are internal fertilisers, in which sperm operate externally to the male but within the female reproductive tract, often after a period of storage in the female's specialised spermathecal storage organ (Resh and Cardé, 2009). Both male and female reproductive tracts provide a range of environments (within males during spermatogenesis and storage, and within females after copulation and insemination) by which sperm may be subject to damage from a series of abiotic stressors, with temperature long being known to be an important factor (Appell, Evans and Blandy, 1977).

Even among mammals possessing adaptations to reduce heat stress (such as the cooled scrotum), higher external temperatures have been associated with deleterious effects on sperm (Hamilton et al., 2016). Increasing temperature in the testis has been shown to cause local hypoxia and death, as well as increased apoptosis of sperm cells (Lue et al., 1999; Hamilton et al., 2016). As well as the death of mature sperm, spermatogenesis has been shown to be heavily impacted by increasing temperatures (Setchell, 1998; Hansen, 2009).

An array of underlying mechanistic responses to increased temperatures have been highlighted in mature adult sperm which may impact function and viability, such as: increased DNA fragmentation (Zhang et al., 2015), chromatin structure abnormalities and epigenetic changes (Sailer et al., 1997; Rahman et al., 2018), RNA profile changes (Lymbery, Evans and Kennington, 2020), and disruption of mitochondrial function (Gong et al., 2017).

Following past attention to thermal fertility effects in endothermic mammalian and avian systems, ectotherms have been investigated more recently. Within insects, there has been an increasing focus on assessing and understanding the impacts of elevated temperatures on fertility and sperm form and function. A wide

variety of impacts on sperm traits which negatively impact fitness have been shown, including spermatogenesis (Canal Domenech and Fricke, 2023), sperm motility, mobility and morphology (Uy et al., 2015; Porcelli et al., 2016; Iossa et al., 2019), reduced sperm number (Vasudeva, Deeming and Eady, 2014; Gasparini et al., 2017; Sales et al., 2018), Sperm viability (Sales et al., 2018; Martinet et al. 2020; McAfee et al., 2020; Campion et al., 2023), reduced eupyrene sperm ratios (Iltis et al., 2020), altered flagellar function (Humphries, 2013), and reduced sperm length and size (Vasudeva, Deeming and Eady, 2014; Iossa et al., 2019). Furthermore, increased spermatophore detritus, possibly associated with apoptosis, has been recorded (Sales et al., 2018). Accompanying sperm impacts, studies have also shown impacts on other reproductive organs and processes. Testes volume has been shown to be consistently decreased in response to heat stress (Sales, Vasudeva and Gage, 2021 Hu et al., 2022), and increased developmental temperatures have been shown to impair accessory gland growth (Canal Domenech and Fricke, 2023). Importantly, recent studies have shown that male sterility occurs at temperatures lower than are required to reach lethal limits, and therefore, are a better predictor of susceptibility to climate change and extreme heat than lethal limits (Parratt et al., 2021).

The impacts of heatwaves and extreme temperatures are damaging to sperm function. Sales et al. (2018) demonstrated that exposure to a 5 day heatwave in *Tribolium castaneum* adults halved male fertility, and a subsequent heatwave caused complete male sterility. Similarly, Porcelli et al. (2016) showed that a 23.5°C heat shock (5.5°C above a non-stressful temperature for *Drosophila subobscura*) for 6 days caused a 40% reduction in motile sperm, as well as a subsequent reduction in fertility of up to 80%. Evidence of heatwaves having such drastic impacts on reproduction in individual species supports the growing claims that extreme temperature conditions and heatwaves may be more detrimental to biodiversity than mean temperature increases (Ruthrof et al., 2018; Smale et al., 2019), and highlights sperm damage as one mechanism by which these impacts may occur.

Whilst increased temperatures cause a wide variety of cellular and phenotypic impacts discussed above, there is considerable evidence in endotherms that many of these may be short-term impacts, and may be reversible once conditions become more favourable again (Lue et al., 1999). Recently, this has been shown in insects. Sales et al. (2021) showed male recovery to even extreme heatwaves in *Tribolium castaneum*: individuals exposed to a 42°C 5-day simulated heatwave as mature adults incurred an initial 58% fitness loss, but complete

recovery occurred 15 days after exposure. Individuals who experienced two heatwave bouts as mature adults took 20 days to recover, while those exposed to one heatwave bout as immature adults showed complete sterility and recovered only after 28 days. Conversely, individuals who were subject to the same simulated heatwave conditions as larvae produced normal levels of offspring by the time they were mature adults. Similarly, Meena et al. (2024) demonstrated that male *Drosophila melanogaster* also show recovery of fertility after heat stress. However, they showed that males that were heat-stressed did not fully recover after 5 days of recovery. This highlights short-lived species as potentially more vulnerable to heat-induced sterility.

Together, these studies demonstrate the variable impacts of heatwave conditions on sperm and subsequent recovery, highlighting critical weaknesses in the face of current and future extreme climate conditions. Although additional species need to be examined, these findings show that some insects can recover. However, species that produce all their sperm before maturity and have no continuous spermatogenesis (such as most Lepidopterans; Friedländer (1997)) may suffer complete male lifetime sterility from heatwave damage.

1.7.1.3 Female gametes

Eggs have also shown to be sensitive to heat stress prior to fertilisation. Thermal stress has been shown to alter follicular growth (Roth et al., 2000), gene expression (Argov, Moallem and Sklan, 2005), egg maturation (Berger, Walters and Gotthard, 2008) and the ability for immature eggs to produce and secrete steroids (Hansen, 2009). Within insect models, it has been predicted that oogenesis is not as sensitive as spermatogenesis to heat (David et al., 2005). Due to their larger size, limited number, and energetic costs of production, ova receive greater investment in maintaining and repairing function than spermatozoa (Hayward and Gillooly, 2011).

Increasing temperatures have been shown to have a variety of impacts on insect egg traits, causing fitness reductions, including reduced fat content (Bauerfeind et al., 2018), increased egg metabolic rates (Potter, Davidowitz and Woods, 2009), as well as several other papers suggesting potential damage to general egg quality but not quantified (e.g. Blanckenhorn et al., 2014).

The ability to produce more eggs may also be damaged by increases in temperature. Oocyte deterioration has been shown as a response to temperatures of just 25°C (7°C lower than yearly maximum temperatures within their range) in the

hemlock looper moth, *Lambdina fiscellaria* (Delisle, Bernier-Cardou and Laroche, 2016). Temperature increases have also been associated with females having a decreased initial mature egg load (Jerbi-Elayed et al., 2015; Denis et al., 2013) and maturing fewer eggs over a lifetime (Berger, Walters and Gotthard, 2008; Denis et al., 2013). In the yellow fever mosquito, *Aedes aegypti,* increasing the temperature to ~5°C above their environmental mean maximum temperature (from ~30°C to 35°C) as adults resulted in a 15% reduction in the number of ovarioles per female. However, these temperatures were not definitively linked to reductions in fitness; instead, they were associated with a levelling off of fecundity (Bader and Williams, 2012). Recent work has shown that pre-mating heat stress can impair female reproductive output. Meena et al. (2025) showed that virgin *Drosophila melanogaster* females subject to a short-duration heat exposure (4 hours at 36°C) reduced reproductive performance to a greater degree than males at this stage, indicating oocytes as being vulnerable to high-intensity thermal stress.

Conversely, some studies have shown that females may also be resilient to reproductive damage and fitness declines in response to heatwaves compared to males. Sales et al. (2018) demonstrated that male fertility could be halved and subsequently eliminated by 5-day continuous heatwaves, whereas virgin females showed no loss of fertility. Similarly, females may show resilience under fluctuating heat stresses. *Menochilus sexmaculatus* showed no change in egg viability in response to fluctuating temperature intervals of 15°C and 35°C (8°C above their optimum temperature in constant conditions) (Singh, Mishra and Omkar, 2018).

1.7.2 Mating behaviour

1.7.2.1 Mating frequency

Thermally-induced changes in mating behaviour may impact an individual's ability to successfully produce offspring, as well as alter the number of offspring individuals produce or sire over their lifespan. Increases in temperature can have a wide variety of impacts on mating frequency within insects through two main avenues: either by directly affecting reproductive physiology or indirectly by influencing how often individuals seek out mates through behavioural trade-offs.

Increased temperatures may often impact the function of systems which are indirectly related to mating and reproduction. In *Drosophila melanogaster*, increases in temperature between 18°C and 25°C increased the rate at which females remated due to quicker degradation of anti-aphrodisiac sex peptides found in sperm

(Best et al., 2012). Negative impacts of temperature are often associated with a reduced ability to detect pheromonal cues. In the parasitoid wasp *Aphidius colemani,* individuals displayed both delayed courtship and reduced mating probability after increasing temperatures (Jerbi-Elayed et al., 2015). Similarly, another parasitoid wasp, *Aphidius avenae,* showed a reduction in the proportion of times females mated due to males displaying no courtship behaviour as females had thermally damaged pheromone production or release (Roux et al., 2010). Increasing developmental temperatures decreased the frequency of copulation events in the Indian meal moth, Plodia interpunctella (Iossa et al., 2019). In the Hemlock looper, *Lambdina fiscellaria*, the probability of individuals mating after an introduction and 24-hour mating window increased from 53% at 10°C, to 86% at 15°C, and 83% at 20°C, where it then rapidly dropped to 38% at 25°C (Delisle, Bernier-Cardou and Laroche, 2016).

Indirect impacts from increased temperatures largely include changes in energy or time expenditure and associated trade-offs. in the English grain aphid, *Sitobion avenae*, increases in temperature caused individuals to more commonly utilise leaf dropping thermoregulatory behaviour, reducing the amount of time individuals spent looking for mates (Ma et al., 2018). Similarly, increases in temperature are associated with longer patch residency times in parasitoid wasps, as more energy is spent on maintenance, resulting in reduced time spent actively searching for mates (Denis et al., 2013). Conversely, an increase in ambient temperatures meant that the Red-legged grasshopper, *Melanoplus femurrubrum*, did not have to alter metabolic rates as much in response to predation risk (which is energetically costly), thereby allowing a greater allocation of resources to mate finding rather than searching for food or avoiding predation (Schmitz, Rosenblatt and Smylie, 2016).

Following the same trend as other aspects of reproduction, the impacts of heatwaves can have dire consequences for an individual's ability to undertake mating events at normal rates. In the Melon fly, *Bactrocera cucurbitae*, the time taken to initiate mating behaviours was increased from 4.7 minutes at their optimal temperature (25°C) to nearly 323 minutes after a 3-hour 33°C heatwave and 459 minutes after a 3-hour 37°C heatwave (Zeng et al., 2018). Increases in the time between mating events have been seen in many insects after a heatwave: a 38% reduction in *Tribolium castaneum* male mating events after a 5-day heatwave at 7°C above optimum growth temperature (Sales et al., 2018); a 30-60% reduction in *Chrysomela aeneicollis* male mating frequency after a 4-hour simulated heatwave at

36°C (16°C above controls and 13°C above midday daily peak temperatures at the collection site)(Dick et al., 2013) and a 25-33% reduction in *Aphidius avenae* mating events after a 36°C, 1 hour heat shock, a temperature which is frequently experienced by parasitoids in summer (Roux et al., 2010).

1.7.2.2 Mate preference

Selection of a mate who will bestow the highest offspring fitness is key to successful reproduction through sexual selection. Selecting a good mate may confer both a direct fitness advantage to the female through factors such as male fertility and paternal resource provision (Kirkpatrick and Ryan, 1991), as well as conferring an indirect genetic advantage to offspring through passing on successful genes (Cally, Stuart-Fox and Holman, 2019). Increased sexual selection has been linked to driving adaptive evolution under thermal stress. Parrett and Knell (2018) showed that *Plodia interpunctella* populations facing strong sexual selection (through a male-biased sex ratio) had improved fecundity and offspring survival when compared to weak sexual selection (female-biased sex ratio) when subject to gradually increasing temperatures. This increased sexual selection allowed for greater promotion of beneficial alleles within those populations under a changing and stressful thermal environment.

Increases in temperature away from thermal optima have been shown to have a wide range of impacts on mate selection, including reducing intraspecific competition for mates (Johnson et al., 2015), altering male courtship intensity towards receptive females (Best et al., 2012), and even reversing which sex is ‘choosy’ (Westerman and Monteiro, 2016). In *Drosophila subobscura*, developmental heat stress has been shown to impact nuptial gift behaviours. Heat-stressed males were less likely to present a gift to a mate, and females were less likely to accept such gifts (Pembury Smith, Latkova and Snook, 2025).

Within mate selection, one sex often puts on a sexually selected display. 92% of insect species employ vibrational signals, which often act as a sign of fitness or species identity (Cocroft and Rodriguez, 2005). Temperature can interact with signals, as well as change mate preference in the receiving individual. In treehoppers, *Enchenopa binotata*, as temperatures increased from 18°C to 36°C, male vibrational signals increased in frequency. This increase in temperature was met with females increasing their preferential frequency in response (Jocson et al., 2019). This is a process known as ‘signal-preference temperature coupling’, and has been noted in several other insects (Symes, Rodríguez and Höbel, 2017).

However, not all species display this: in the red mason bee, *Osmia bicornis,* males who maintained rather than increased their pulse duration in higher temperatures commonly experienced in nature (22-26°C compared to 17-21°C from controls) were preferentially mated over males who could not, showing no coupling in preferences between sexes (Conrad, Stöcker and Ayasse, 2017)

Most species of insects have set breeding periods and seasons, with temperature being an important environmental cue for the initiation and length of the breeding period (Pittendrigh and Takamura, 1987). In Northeast Finland, summers have lengthened by 5 days per decade between the late 1970s and 2020, significantly expanding the breeding seasons of boreal moths (Keret et al., 2020). Similarly, in 3 species of British grasshoppers, increasing mean temperatures by 5°C effectively increased their available breeding season by shortening the time between adult eclosion and first egg pod laying date by up to 4.1 days (Willott and Hassall, 1998).

While some species may improve population viability through longer breeding periods, others may experience reductions in reproductive success. In wild populations of the blueberry bee, *Osmia ribifloris*, increased daily maximum nest temperatures of 3.6°C and 6.6°C delayed male emergence by 9.8 and 23.1 days, whereas females were delayed by 23.0 and 4.9 days. This mismatched impact on genders caused a reduced effective breeding season due to short individual lifespans (CaraDonna, Cunningham and Iler, 2018). In the parasitic wasp, *Nasonia vitripennis*, a 1-day heat shock during development delayed spermatogenesis, leading to reductions in sperm production between 67% at +9°C and 91% at +13°C (both above standard lab rearing temperatures of 25°C) at emergence. Furthermore, females were unable to discriminate between males who had been impacted and those who had not (Chirault et al., 2015). In species with short lifespans, mismatches in emergence or sexual maturation timings could be damaging for individual fitness.

Another way in which species may react to increased thermal stress is through alterations of mating systems. Polygamy has the potential to enhance adaptation rates to novel and fitness-reducing temperatures, when compared to monogamy (Parrett and Knell, 2018). In the red flour beetle, *Tribolium castaneum*, there was no significant benefit of either polyandry or monogamy at standard maintenance temperatures. However, when temperatures were increased from 30°C to 34°C, polyandry produced favourable reproductive output, with nearly 30% more offspring (Grazer and Martin, 2011). In the bulb mite, *Rhizoglyphus robini,*

when populations were subject to a 4°C increase in temperatures (from 24°C to 28°C, a temperature seen by this species in midsummer), all populations maintained under a monogamous system went extinct. In contrast, those under a polygamous system survived (Plesnar-Bielak et al., 2012). Increased sexual selection has been linked to improved fecundity and offspring survival when subject to gradually increasing temperatures, with an 8% higher offspring survival rate being seen in *Plodia interpunctella* in polyandrous populations compared to monogamous populations. However, polygamous mating did not delay the rate of population extinctions when temperatures got excessively high (Parrett and Knell, 2018).

1.7.3 Fertility and fecundity

Ectothermic insect metabolism and growth rates are strongly linked to external temperatures (Deutsch et al., 2008). In the Asian tiger mosquito, *Aedes albopictus*, increasing mean temperatures from 21°C (the mean temperature across this species' range) to 25°C shortened development times but reduced fecundity under long photoperiods due to having lower body mass (Yee, Ezeakacha and Abbott, 2016), showing an indirect impact of increased temperature on fecundity.

Increasing mean temperatures have been shown to rapidly damage fecundity once they pass population optima. In the fruit fly, *Drosophila melanogaster*, the net reproductive rate was maximised at 23°C, but reached nearly zero by 31°C, showing a sharp drop-off (Kim, Jang and Lee, 2020). Similarly, in the tropical Maize stalk borer moth, *Busseola fusca*, the number of eggs laid at 30°C was one-third of those laid at 26°C (standard laboratory conditions for this species; Glatz, du Plessis, and Van den Berg, 2016). Heat stress has also been associated with reduced first clutch size and female reproductive investment (Bauerfeind et al., 2018). These examples further highlight the concern for many tropical species that live in habitats with minor variation about their optimal temperatures, as shown in Deutsch et al. (2008), due to their adaptation to a narrow thermal environment and the speed at which average temperatures may increase to a point at which species decline and extinctions are seen rapidly.

The impact of thermal stress is highly dependent on the timing of the event in relation to each organism's reproductive cycle (Pilakouta et al., 2023). In the burying beetle, *Nicrophorus vespilloides*, a 25°C heatwave for 3 days dramatically reduced breeding success and increased breeding bout times when it occurred during either mating periods; however, it had limited impacts outside of this critical window (Pilakouta et al., 2023).

Heatwave conditions have recently been shown to have a significant impact on fecundity, reducing the number of viable offspring produced in the red flour beetle, *Tribolium castaneum*, primarily through damage to sperm production and function (Sales et al., 2018). However, due to the transient nature of these heatwave events, reductions in fecundity in insect species that reproduce continuously over longer periods may be temporary, as individuals recover from damaged reproductive function (Sales et al., 2021). Furthermore, it has been shown that polyandry allows females to avoid such male-specific damage by mating with several males (Vasudeva et al., 2021). It is yet unknown whether this fecundity is saved by using the 'best' sperm from multiple males, or by using sperm from the 'best' male. If the second option is true, this may limit the number of successfully breeding males in the population, reducing effective population sizes, which could have knock-on effects, such as loss of fitness through genetic drift, particularly in small populations (Frankham, 2005). Research into the mechanisms underpinning post-copulatory dynamics shows that males subject to heatwaves deliver ejaculates of reduced density, which are less able to spread throughout the female reproductive tract (Sales et al., 2024).

Increases in environmental temperatures can also heavily impact the ability of eggs to hatch. In the Maize stalk borer moth, *Busseola fusca*, increases in temperature from 26°C (standard laboratory conditions for this species) to 30°C reduced the number of fertile eggs from 49% to 0% (Glatz, du Plessis and Van den Berg, 2016). Similarly, in the Asian long-horned beetle, *Anoplophora glabripennis*, hatch rates were highest at 25°C, but by the time temperatures had reached 30°C this rate had halved, and at some point, between 30°C and 35°C this hatch rate had reached 0% (Keena, 2006).

Eggs have also been shown to be particularly susceptible to heat shock damage. In the alligator weed flea beetle, *Agasicles hygrophilia*, a 37.5°C (12.5°C above standard optimum growth conditions for this species) 1-hour heat shock exposure decreased egg survival from 92% to 24%, by 5 hours this survival rate was 0% (Jia et al., 2018). Similarly, in the Asian lady beetle, *Harmonia axyridis,* a 37°C (12°C above standard lab conditions for this species and a temperature often seen in summer over their range) 1-hour heat shock reduced hatch rates from 92.6% to 47.1% (Zhang et al., 2014).

1.7.4 Transgenerational and population impacts

Not only is it important for individuals to undergo successful mating events, but these events must also produce healthy offspring that go on to survive and successfully reproduce themselves. Increasing temperatures may cause transgenerational impacts, whereby reductions in fitness resulting from thermal stress are observed in both individuals exposed to heat and their offspring. Transgenerational impacts of varying environmental stressors on offspring fitness have been a growing research interest, primarily focused on assessing paternal influences (e.g. through sperm competition in zebrafish (Zajitschek et al., 2014) and diet in guppies (Evans et al., 2017)). Transgenerational impacts have been shown in insects too (Zajitschek, Zajitschek and Manier, 2017). In the Mediterranean field cricket, *Gryllus bimaculatus,* a 4°C increase in temperature exposure to males during development increased offspring hatch rates and survival, but exposure as adults reduced offspring hatch rates and survival (Gasparini et al., 2017). Sales et al. (2018) showed that negative transgenerational impacts were directly related to sperm damage (achieved by comparing thermal stress impacts on offspring from: 1) males before copulation, 2) females before copulation, and 3) females after copulation and insemination). Females who were subject to an artificial heatwave pre-copulation faced no negative reproductive impacts. Offspring produced by sperm that had experienced a 5-day heatwave at 7°C above optimum growth temperature had significantly reduced lifespans, and the male offspring showed a 25-40% reduction in mating frequency and reproductive output. In *Drosophila melanogaster*, heat stress during early embryogenesis has been shown to impact the sperm proteome of that generation, and consecutive generations of heat stress resulted in transgenerational carryover of disruption to ribosomal proteins, metabolism and chromatin in sperm (Khan and Mishra, 2024).

Temperature increases can also impact offspring sex ratios in insects. Cui et al. (2008) investigated the impacts of 1-hour heat shocks of varying intensity on adults and how they affected offspring sex ratios in two whitefly species. In one species, *Bemisia tabaci,* sex ratio stayed roughly even until 41°C, above which it decreased to 35% female by 45°C (temperatures seen in greenhouses over their range). However, in another species of whitefly, *Trialeurodes vaporarorium*, sex ratio did not change as heat shock temperatures increased (Cui et al., 2008). In the fungus gnat, *Bradysia odoriphaga,* exposure of parents to a benign temperature of 25°C resulted in twice as many male offspring being produced. After being subjected to 33°C for 1 hour, the ratio changed to nearly even, and at 37°C for 1

hour, the ratio shifted to twice as many female offspring being produced (Cheng et al., 2017).

1.8 Study system

The red flour beetle, *Tribolium castaneum*, is a species of beetle from the Tenebrionidae Family, originating from the Indian subcontinent. It is found natively under wood and bark of trees (Dawson, 1977). However, in modern times, this species is better known globally as a pest of stored products, where it is considered one of the most common secondary pests of stored plant commodities worldwide (Sallam, 2013).

Within the field of research, Tribolium castaneum serves as a widely used model organism in the study of evolution and ecology (Pointer, Gage, and Spurgin, 2021; Campbell et al., 2022). It possesses many qualities that make it ideal for experimental work, such as being inexpensive to maintain under conditions that can easily be replicated in a standard laboratory environment. They can be reared in large numbers and have a short generation time of ~30 days under conditions of 30°C and *ad libitum* fodder (Pointer, Gage, and Spurgin, 2021). These traits make it an ideal species for undertaking extensive experiments that test a wide range of conditions, while producing statistically robust data.

Tribolium castaneum can be easily sexed during the pupal stage by visual identification of sexually dimorphic genital papillae (Shukla and Palli, 2012). This makes them an ideal species for undertaking experiments which focus on sex- and mating status-specific aspects of their biology, as they can be readily maintained in single-sex groups long before they reach sexual maturity.

The genome of *Tribolium castaneum* is well-characterised, being both fully sequenced and annotated, making it a suitable choice for genetic experiments (Herndon et al., 2020). In addition, it has been widely used in transcriptomic and gene expression profiling studies, demonstrating its value and ease as a model for exploring molecular and physiological responses to a wide variety of environmental conditions (Park et al., 2008; Leader, Naseem and Halberg, 2024).

Within the specific framing of this thesis, *Tribolium castaneum* has been extensively used for several decades at the University of East Anglia, where prior research in this system has focused strongly on the effects of temperature, heatwaves and thermal stress on reproductive biology. This existing body of work provides an important foundation for the experiments presented here because it

shows that reproduction in *T. castaneum* can be highly sensitive to thermal conditions, and that this sensitivity may occur before adult survival is strongly compromised. In terms of thermal performance, this suggests that reproductive traits in *T. castaneum* may have upper thermal limits that differ from those associated with adult survival. While many studies in this system have not explicitly estimated complete thermal performance curves, CTmax, TFmax or TFopt values, they provide clear evidence that fertility-related traits decline under high-temperature conditions and can therefore be interpreted within the broader framework of thermal fertility limits.

In particular, Sales et al. (2018) demonstrated that exposure to a 5 day continuous heatwave in *Tribolium castaneum* adults halved male fertility, and a subsequent heatwave caused complete male sterility. This work showed that male reproductive systems and function display thermal sensitivity in this species, and showed that heatwaves can induce reproductive output costs under sub-lethal heatwave conditions. Within the context of thermal limits, Sales et al. (2018) showed that prolonged exposure to sub-lethal heat (42°C for 5 days) can push male reproductive performance beyond fertility limits, at least temporarily, highlighting further that thermal fertility limits exist as a biologically crucial threshold for population persistence, which is distinct from thermal survival limits.

This was followed up by Sales et al. (2021) where it was shown that male fertility can recover, even after exposure to such prolonged heatwaves. In response to exposure to a singular simulated 42°C 5-day heatwave, adult males incurred a 58% drop in reproductive output. However, within just 15 days of this exposure, reproductive output under future matings had completely recovered. Conversely, recovery was much slower in individuals who were subject to repeated incidences of these 42°C 5-day heatwaves. Individuals who were exposed to two heatwave bouts took longer than 20 days to recover their reproductive output in future matings. These findings have shown that heatwave impacts are not simply down to temperature alone, but can interact with exposure duration and history of exposure.

Most recently, work has shown that heatwaves reduce the ability of sperm to occupy the female reproductive tract in this species (Sales et al., 2024), showing that thermal stress experienced through heatwave exposure can disrupt post-mating processes, such as sperm transfer and storage. This has important implications for interpreting thermal fertility limits in this species, as reproductive losses may result from thermal effects on both sexes rather than male fertility alone. Furthermore, if multiple reproductive processes differ in their thermal sensitivity, singular estimates

of thermal fertility limits may mask variation among reproductive traits, reflecting only on those traits with the limits most quickly reached.

Alongside this work on heatwaves, *Tribolium castaneum* has also been used to investigate broader areas of thermal reproductive biology. Vasudeva et al. (2019) showed that gamete traits are thermally plastic in this species and can respond to changing thermal environments to improve reproductive outcomes through impacts on both sperm and eggs. This highlights that not only does acute heat stress experienced by adults play a factor in reproductive success, but also thermal conditions experienced throughout different stages of their life cycle, during gamete development. Other work has focused on evolution under warming regimes (Sales, Gage and Vasudeva, 2024) and genetic rescue of thermally adapted lines (Lewis et al., 2024).

Bringing together the practical advantages of using this study system and the foundation of prior work on thermal stress and reproduction in this species, *Tribolium castaneum* is positioned as an ideal system to address the questions posed in this thesis. This thesis therefore extends existing knowledge beyond prolonged, male-centric heatwave exposure research and examines how short-duration exposure to heatwaves may impact insect survival, reproductive morphology, reproductive output and gene expression across different sex and mating status contexts.

1.9 Thesis aims

This thesis aims to investigate how ecologically relevant heatwave exposures affect survival, reproduction, and gene expression in *Tribolium castaneum*, serving as a model for insects in general. The central question of this thesis is whether reproductive traits are affected under sub-lethal heatwave exposures, and whether particular focal sex- and mating status-groups are especially vulnerable to this thermal stress

In Chapter 3, I ask whether short and repeated 42°C heatwave exposures impact survival and reproductive output in *Tribolium castaneum*. Specifically, I test whether heatwave exposure affects reproduction when applied to virgin males, virgin females, both virgin males and females, or mated females, under simulated heatwave temperatures, which have previously been shown to reduce fertility in this species under continuous heatwave scenarios.

In Chapter 4, I ask whether short, ecologically relevant heatwave exposures compromise male survival, reproductive output and reproductive traits. I focus on males here, as their reproductive function, and particularly sperm, is broadly susceptible to thermal stress in insects. I test heatwave temperatures up to 50°C, which represent extreme conditions currently experienced across this species' range and are expected to become more common due to climate change.

In Chapter 5, I ask whether survival and reproductive responses to more intense heatwave exposures differ according to sex and mating status. Specifically, I compare virgin males, virgin females, mated males and mated females' survival and reproductive responses under short heatwave exposures of up to 50°C, allowing me to identify which focal groups and fitness-related traits are the most vulnerable to extreme heat.

In Chapter 6, I ask how mated female survival and reproductive output are affected by heatwave temperature and exposure duration. I also ask whether mated female heatwave vulnerability is specifically linked to ejaculate receipt, rather than other aspects of mating, such as male exposure or harassment.

In Chapter 7, I ask whether transcriptional responses to heatwave exposure differ according to sex, mating status and body part in *Tribolium castaneum*. Using RNA sequencing, I compare gene expression responses in the head, thorax and abdomen of virgin and mated males and females to attempt to identify potential molecular pathways underlying reproductive and survival sensitivities to heatwaves.

Finally, I summarise these findings in the context of the current knowledge base and identify priorities for future work in this field.

2. General methods

2.1 Overview

This chapter outlines the general experimental procedures used throughout this thesis. These procedures provide the shared methodological foundation for the data chapters, including stock maintenance, standard rearing conditions and the general structure of experimental heatwave exposures. Detailed experimental designs, sample sizes, response variables and statistical analyses are provided within each data chapter, where they are linked directly to the chapter-specific questions and hypotheses. The purpose of this chapter is therefore to describe the common procedures that were used repeatedly across experiments and to clarify how they support the overall aim of assessing heatwave impacts on survival, reproduction and gene expression in *Tribolium castaneum*.

2.2 Stock maintenance

T. castaneum beetles used in this thesis were from the Krakow Super Strain (KSS) outbred stock line. This line was produced by interbreeding 11 different strains of *Tribolium castaneum:* GA-1, GA-2, Tiw-1, Col-2, Japan #2, cSM, San Bernardino, Jerez, Banos, Schegel Farm and Bloomington (Dickinson, 2018). Stock populations were maintained in 12 x 12 x 12 cm plastic tubs, half-filled with fodder (a 9:1 volume ratio of organic flour and yeast), and topped with an even layer of oats for traction. Populations were maintained at constant standard conditions of 30 ± 1°C and 60 ± 10% RH under a 16:8 light: dark photoperiod in the Controlled Environment Facility at the University of East Anglia. Stock populations were maintained as non-overlapping generations by removing ~300 mature adults selected at random from the current stock population every 35 days, and allowing them to mate and oviposit for 7 days in a fresh tub before the removal of adults by mechanical sieving, leaving only the fodder with oviposited eggs in this new population.

The use of a single outbred stock line and standardised maintenance conditions aimed to ensure that differences among treatment groups can be interpreted in relation to experimentally imposed heatwave exposure, sex and mating status, rather than differences in prior rearing conditions. Maintaining individuals at 30 ± 1°C provided a standard benign temperature from which heatwave responses could be assessed.

2.3 Experimental heatwave treatments

Throughout the thesis, experimental heatwave treatments of various lengths and intensities are applied to individual or single-sexed groups of beetles, who are kept in 6cm Petri dishes filled with 3 grams of fodder and topped with an even layer of oats. Within the thesis, several focal groups are exposed to heatwave conditions in various configurations: virgin males, virgin females, mated males, and mated females. Figure 2.2 illustrates a generalised method for exposing virgin beetles, along with the subsequent timings for assaying survival and 20-day reproductive output, as required. Likewise, Figure 2.1 illustrates a generalised method for exposing mated beetles. These schematics are intended to illustrate the common structure of heatwave exposure experiments and the subsequent measurement of response variables. The exact conditions of each heatwave treatment are provided in the material and methods section for each Chapter.

The general heatwave exposure framework displayed in Figures 2.1 and 2.2 was designed to test how short-duration extreme thermal stress affects different fitness-related traits. Throughout the thesis, exposure temperature, exposure duration, and the reproductive state of the exposed individual were manipulated to address specific questions. In Chapter 3, 42°C heatwave exposures were used to test whether short (2 h, 5 h and 10 h), repeated (3 consecutive days of 10 h of exposure or 5 consecutive days of 10 h of exposure), or separated heatwave events (2 separate bouts of 5 consecutive days of 10 h of exposure) affect reproductive output across different reproductive contexts. This chapter, therefore, used the general heatwave framework to ask whether thermal stress applied to virgin males, virgin females, both virgin individuals, or mated females produced different reproductive outcomes. In Chapter 4, the heatwave framework was applied specifically to virgin males across a broader range of temperatures (42°C – 50°C) and durations (2 h – 10 h) to test whether male survival, reproductive output, testicular volume, and sperm length differed in their thermal sensitivity. In Chapter 5, the same general structure was extended across virgin males, virgin females, mated males and mated females to test whether sex and mating status altered survival and reproductive responses to short, intense heatwave exposures. In Chapter 6, the heatwave framework was applied specifically to mated females across a broad range of temperatures (42°C – 50°C) and durations (2 h – 10 h) to test whether mated female survival and reproductive output were affected by increasing heatwave intensity. The design was then further modified (focusing only on heatwave exposure conditions of 48°C for 5 h) by experimentally preventing females from

receiving an ejaculate while retaining exposure to males and attempted mating. This allowed heatwave vulnerability linked specifically to ejaculate receipt to be separated from other aspects of mating, such as male exposure, harassment or attempted copulation. Finally, in Chapter 7, the heatwave framework was adapted for transcriptomic analysis, utilising a single heatwave condition (48°C for 5 h) before RNA sequencing to test whether transcriptional responses to thermal stress differed according to sex, mating status and body part under conditions that had previously been shown to produce clear sex- and mating-status-specific survival and reproductive effects. These repeated but modified applications of the same general heatwave exposure framework allowed the thesis to compare survival, reproductive and transcriptional responses while keeping the underlying experimental logic consistent across chapters.

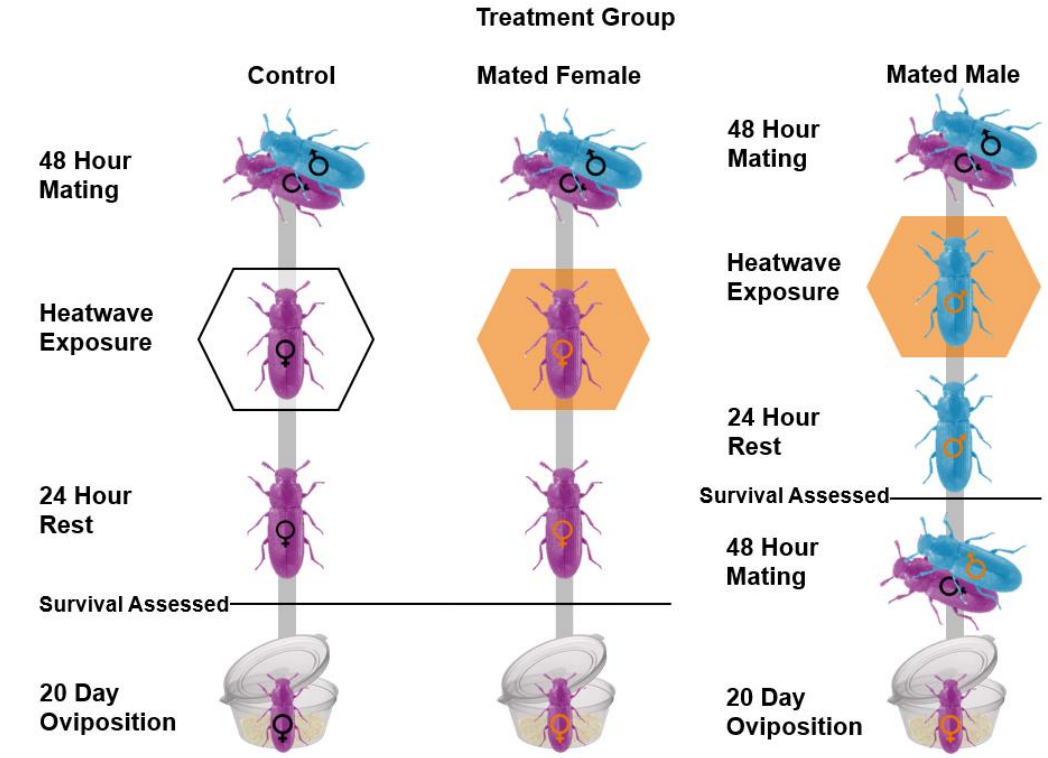

Figure 2.1: General methodology for exposing mated beetles to heatwave conditions. The figure shows the sequence of treatments across different focal groups. Each treatment group represents the various groups of individuals subjected to heatwave treatment: mated females only, mated males only, and controls.

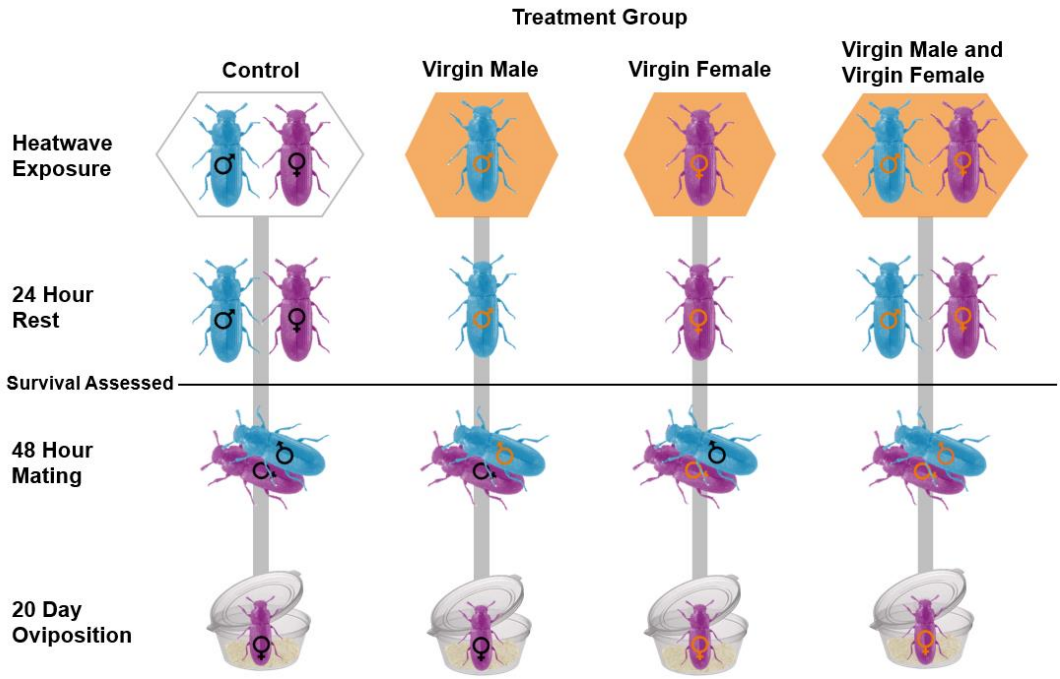

Figure 2.2: General methodology for exposing virgin beetles to heatwave conditions. The figure shows the sequence of treatments across different focal groups. Each treatment group represents the various groups of individuals subjected to heatwave treatment: virgin males only, virgin females only, and both a virgin male and a virgin female and controls.

3. Sex- and mating status-specific impacts of short and multi-day heatwave exposures on reproduction in *Tribolium castaneum*

3.1 Abstract

The prevalence of extreme weather events, such as heatwaves, is increasing rapidly under anthropogenic climate change, and these events are expected to have a greater impact on biodiversity than increases in mean temperature alone. Previous work in *Tribolium castaneum* has shown that continuous multi-day heatwaves can strongly reduce male fertility, but less is known about how short daily heat exposures, repeated heatwaves and recovery periods affect reproduction across different sex and mating-status groups. Here, I assess the impacts of 42°C experimental heatwaves lasting 2 h, 5 h or 10 h, as well as 3 or 5 consecutive days of 10 h exposure, and a double heatwave treatment separated by a recovery period, on survival and reproductive output in *T. castaneum*. This was achieved by subjecting four focal groups to heatwave conditions: i) virgin males, ii) virgin females, iii) both virgin males and females, and iv) mated females. I show that survival and reproductive output were generally resilient to 42°C heatwave exposure, with no significant reductions after single-day exposures, 3 consecutive days of exposure, or the double heatwave treatment. However, mated females exposed to 5 consecutive days of 10 h heatwave conditions showed reduced reproductive output, suggesting that vulnerability may emerge when repeated thermal stress accumulates across several days. These findings suggest that *T. castaneum* reproductive output is broadly resilient to intermittent 42°C heatwave exposure, while also identifying mated females as a potentially overlooked heat-sensitive group under repeated thermal stress.

3.2 Contributors

Ramakrishnan Vasudeva and Laura Davey assisted with sexing experimental individuals.

3.3 Introduction

As outlined in the general introduction, it is well established that heatwaves are becoming increasingly prevalent and severe (Perkins-Kirkpatrick and Lewis, 2020). Research on the impacts of heatwaves is crucial, as such extreme climatic events are changing more rapidly than global mean temperatures alone (Seneviratne et al., 2021) and it has been suggested that extreme temperature conditions and heatwaves may be worse for biodiversity than increases in mean temperatures (Ruthrof et al., 2018; Smale et al., 2019).

As insects are the most abundant class of animals on Earth (Stork, 2018), and reproductive capability is often the key trait in determining population vulnerability to thermal stress (Parratt et al., 2021; van Heerwaarden and Sgrò, 2021), understanding the consequences of heatwaves for insect reproduction is therefore critical. Across the Insecta class, male reproduction may be particularly susceptible to heatwave impacts, whereas female reproduction has often been suggested to be more resilient (Iossa, 2019). This male reproductive susceptibility, measured through thermal fertility limits, has been shown to better predict species distribution and extinction risk than measures of critical thermal limits (Parratt et al., 2021; van Heerwaarden and Sgrò, 2021). Within *Tribolium castaneum*, it has been shown that a continuous five day, 42 °C heatwave caused a greater than 50% reduction in male reproductive output and a second successive heatwave led to near-complete reproductive output loss (Sales et al., 2018). In contrast, no significant impact on virgin female reproductive output was seen after a single heatwave. Males exposed to heatwaves exhibited reduced sperm counts, lower sperm viability, and impaired sperm transfer (Sales et al., 2018).

A recent systematic map testing the relationship between temperature and animal reproduction found that it was uncommon for experimental studies to test temperature effects independently in males and females (Dougherty et al., 2024). Work testing sex-specific susceptibilities in insects could be particularly insightful, given the diverse range of sex-specific life history strategies that the Insect class employs (e.g., haplodiploidy, eusociality, and unequal brood care). Within this framework, both the sex of the individuals being subjected to a heatwave and their mating status may have significant effects on both individual and population-level reproductive outcomes. Here, I assess the impacts of heatwave conditions on four key groups: i) virgin males, ii) virgin females, iii) both virgin males and females together and iv) mated females. This enables the assessment of key sex-specific susceptibility to be studied, and considerations for populations to be made.

Recent work has highlighted the need to consider timing-dependent impacts of extreme climate events such as heatwaves (Cinto Mejía and Wetzel, 2023), given that heatwave timing may affect different aspects of reproductive competence, including the production and maintenance of gametes, mating and associated behaviours, post-fertilisation processes and offspring fitness. Experiments explicitly testing timing-dependent heatwave impacts have begun to filter through into the literature, with insightful work showing that heatwave timing can have massive implications in *Nicrophorus vespilloides*, whereby a heatwave a few days before or after mating had no negative impacts on reproductive success, parental care or offspring fitness, yet experiencing a heatwave during mating periods had negative implications for brood size, breeding attempt success and offspring fitness (Pilakouta et al., 2023). Testing the impacts of heatwave conditions on virgin and mated females in this Chapter acts as a proxy for some timing-dependent impacts, considering that newly mature females will be virgins, yet females will spend the large majority of their lives as mated individuals in this species, considering their highly polyandrous life strategy (Pai and Yan, 2003).

While many studies provide crucial evidence on the impacts of heat stress on male fertility, one critical aspect that must be further considered is the comparative complexity of natural thermal regimes. One such example of heatwave complexities that must be considered is diurnal temperature fluctuations, which may allow for partial or complete reproductive recovery and repair of thermally induced cellular damage. (e.g., spermatogenesis, repair of heat-induced cellular damage). In *Propylea japonica*, the negative impacts of daytime heat stress on survival and development were offset by cooler night-time temperatures, which allowed thermal recovery (Bai et al., 2019). By manipulating daytime maxima and nighttime minima, it was demonstrated that nighttime recovery plays a critical role in shaping thermal performance, including survival and heat tolerance. Such findings highlight the importance of studying not only the duration and severity of heatwaves but also the structure of thermal variation to understand the impacts of natural heatwaves on insect reproduction.

This Chapter investigates the impacts of ecologically relevant single-day (2 h, 5 h, 10 h) and multi-day heatwave exposures (3 and 5 consecutive days of 10 h exposure) on reproductive output in *Tribolium castaneum* in virgin males, virgin females and mated females. Rather than applying continuous heat stress, this experiment utilises extreme temperatures that occur for relatively short periods each day, as seen during heat peaks in natural environments (Holmes et al., 2015;

Oliveira et al., 2021). The temperature used in this study (42 °C) occurs commonly across the natural range of *T. castaneum* (Hinton, 1948). In addition to testing the impacts of such temperatures in a single day exposure, a subset of treatments incorporates a simple day–night thermal fluctuation, where elevated daytime temperatures are followed by cooler nighttime periods to allow for potential physiological recovery. This design enables me to test whether the detrimental impacts on male fertility observed under sustained heat stress (e.g., Sales et al., 2018) are also present when the heat stress is brief and intermittent, and whether recovery windows between exposures moderate reproductive damage. By capturing both the intensity and temporal structure of heatwaves, the study provides a more nuanced understanding of how climate-induced temperature variability may affect insect reproduction under realistic environmental conditions.

In this Chapter, I ask whether short and repeated 42°C heatwave exposures affect survival and reproductive output in *Tribolium castaneum*, and whether these effects differ between individuals of different sexes and mating status. I predict that longer or repeated heatwave exposures would be more damaging to both survival and reproduction than shorter, single-day heatwave exposures.

3.4 Materials and methods

3.4.1 Line maintenance and experimental individuals

T. castaneum beetles used in these experiments were from the Krakow Super Strain (KSS) outbred stock line. This line was produced by interbreeding 11 different strains of *Tribolium castaneum:* GA-1, GA-2, Tiw-1, Col-2, Japan #2, cSM, San Bernardino, Jerez, Banos, Schegel Farm and Bloomington (Dickinson, 2018). Stock populations were maintained in 12x12x12 cm plastic tubs, half-filled with fodder (a 9:1 volume ratio of organic flour and yeast), and topped with an even layer of oats for traction. Populations were kept at constant standard conditions of 30 ± 1°C and 60 ± 10 % RH under a 16:8 light: dark photoperiod. Experimental individuals were obtained as the offspring of ~300 mature adults selected at random from the stock line. These individuals were allowed to mate randomly and oviposit for 7 days in a fresh tub before the removal of adults by mechanical sieving, leaving only the fodder with oviposited eggs in this new population.

Individuals were sexed at the pupal stage by visual identification of sexually dimorphic genital papillae (on day 18 of development) and then kept in single-sex groups of 20 individuals for a further 10 days to allow for development to sexual

maturity. Groups were kept in 6cm Petri dishes filled with 3 grams (g) of fodder and topped with an even layer of oats. Once matured, females were identified with a dot of Uni Posca non-toxic marker (Uni-ball, Tokyo, Japan) on the dorsal thorax.

3.4.2 Experimental treatment groups

The sex-specific impacts of heatwaves on survival and fertility were tested by selectively exposing combinations of focal individuals to heatwave conditions in the following combinations: virgin males only, virgin females only, virgin males and females, and mated females only. These treatment groups allowed reproductive consequences of heatwave exposure to be compared according to whether thermal stress occurred before mating in males, before mating in females, before mating in both sexes, or after mating in females.

3.4.3 Experimental heatwave treatments

Heatwave conditions were applied using an A.B. Newlife 75 Mk4 forced-air egg incubator (A.B. Incubators, Suffolk, UK). Virgin beetles were exposed in single-sex groups of 20 individuals in 6cm Petri dishes filled with 3g of ambient temperature fodder and topped with a layer of oats. Mated female beetles were exposed as individuals in a 6cm Petri dish, filled with 3g of ambient temperature fodder and topped with a layer of oats.

The impact of single-day and multi-day heatwaves was tested. For single-day heatwaves, focal beetles were exposed to heatwave conditions (42°C ± 1°C, ± 10 % RH) for one of the following durations: 2 hours (h), 5 h or 10 h, with controls (30 ± 1°C, 60 ± 10 % RH) run in parallel. For multi-day heatwaves, focal beetles were exposed to heatwave conditions (42°C ± 1°C, ± 10 % RH) for 10 h on either 3 or 5 consecutive days. In between heatwave exposures, the beetles were maintained at 30 ± 1°C and 60 ± 10 % RH for 14h. Controls were run in parallel. Heatwave treatments began at 10 am.

An additional group of virgin males were subject to two bouts of multi-day heatwave conditions, hereafter referred to as the double heatwave group. Virgin males were exposed to heatwave conditions (42°C ± 1°C, ± 10 % RH) for 10 h on 5 consecutive days. In between heatwave exposures, the beetles were maintained at 30 ± 1°C and 60 ± 10 % RH for 14h. After the initial 5 d heatwave exposure, these beetles were maintained at 30 ± 1 °C and 60 ± 10 % RH for 7 d before undergoing an identical heatwave exposure. Controls were run in parallel. This treatment structure allowed the effects of exposure duration, repeated daily exposure and recovery

between heatwave events to be assessed while keeping heatwave temperature constant at 42°C.

Two additional groups of virgin males were subject to identical heatwave conditions to the 2 h and 5 h groups, but their fodder was preheated to 30°C before individuals were subjected to heatwave conditions. This was done to test whether artificially cooler conditions of ambient temperature fodder (in comparison to elevated temperature conditions that may be experienced in the buildup to a heatwave), potentially buffered against the impacts of short duration heatwave conditions.

3.4.4 Virgin treatment groups

48 h after eclosion, single-sex groups of virgin males and/or females were subject to either heatwave conditions or control conditions as described above. After this, all individuals underwent a 24 h rest period at 30 ± 1 °C and were then visually assessed for survival. All survivors were then moved into a 7 ml mating vial (filled with 2.4 g of fodder and topped with an even layer of oats) with an age-matched member of the opposite sex from a KSS stock population for 48 h at 30 ± 1 °C. After the mating period, females were removed and placed into a reproductive output assay. These mating vials were retained to ensure successful ejaculate transfer, assessed by the visual presence of larval tracks/offspring.

3.4.5 Mated treatment group

48 h after eclosion, virgin females were moved into a 7 ml mating vial (filled with 2.4 g of fodder and topped with an even layer of oats) with an age-matched member of the opposite sex from a KSS stock for 48 h at 30 ± 1 °C. After this, individual females were placed into a 6cm Petri dish (filled with 3g of fodder and a layer of oats) and were subject to either heatwave conditions or control conditions as described above. After this, all individuals underwent a 24 h rest period at 30 ± 1 °C. Individuals were then visually assessed for survival at this time, and surviving females were placed into a reproductive output assay.

3.4.6 Reproductive output assay

Females from the above experimental groups were moved into individual 6cm Petri dishes (filled with 3g of fodder and topped with an even layer of oats) and allowed to oviposit. Oviposition occurred across two separate ten-day blocks (twenty days in total) to reduce cannibalism associated with overlapping offspring developmental life stages (Park et al., 1965). After twenty days, the females were removed, and the oviposited eggs were allowed to develop until maturity for an additional 35 days under

standard developmental conditions (30 ± 1°C, 60 ± 10 % RH). The reproductive output of each pair was then assessed as the number of mature adults produced from twenty days of oviposition (equating to ~ 51% of a female's lifetime reproductive output; pg. 31, Dickinson, 2018).

3.4.7 Statistical analysis

All data were analysed using R version 4.4.1 (R Core team, 2024) in RStudio Version 2024.04.2+764(Posit team, 2024). Plots were created using "ggplot2" (Wickham, 2016). Data manipulation was done using "tidyverse" (Wickham et al., 2019). The impact of heatwave temperature on survival for each of the heatwave conditions was initially assessed using a binomial GLM. Firth's penalised logistic regression from the brglm2 package was used where near-separation or complete separation issues caused model convergence issues (Kosmidis, 2023). Fit of the models were then assessed using "performance" (Lüdecke et al., 2021) and residuals were visually evaluated.

Reproductive output data were first censored where data were missing or individuals escaped from or died during the reproductive output assay (N = 49). Zero-inflated negative binomial GLMs (ZINB GLM; Zeileis, Kleiber and Jackman, 2008) were used to analyse all data after running and comparing Poisson, negative binomial, zero-inflated and zero-inflated negative binomial models for over- or under-dispersion and comparing goodness of fit using "performance" and "lmtest" (Zeileis and Hothorn, 2002). Zero-inflated negative binomial GLMs were structured with 20-day reproductive output as the response variable and treatment group was the predictor variable. Separate models were fitted for the 2 h, 5 h, 10 h, 3-day, 5-day and double heatwave treatments. For each model, treatment effects were assessed in both the count component, representing reproductive output among non-structural zero observations, and the zero-inflated component, representing the probability of excess zeros.

The impact of fodder temperature was assessed using non-parametric Wilcox tests.

3.5 Results

3.5.1 Survival

Survival rates for all groups across all heatwave durations were high (>95% except for virgin females subject to a 2 h heatwave in preheated fodder, who had a survival

of 85%; however, the sample size was low (n = 20)), and no significant impacts on survival were observed in any group as a result of heatwave exposure.

3.5.2 Reproductive output

3.5.2.1 Impact of a 2 hour heatwave exposure

Exposure to heatwave conditions of 42°C for 2 hours did not significantly impact reproductive output in any treatment group (Fig. 3.1). Reproductive output in groups where virgin males (ZINB GLM: count component; z = -0.902, p = 0.367, zero-inflated component; z = -0.460, p = 0.646), virgin females (ZINB GLM: count component; z = -0.899, p = 0.368, zero-inflated component; z = -0.517, p = 0.605), both virgin males and virgin females (ZINB GLM: count component; z = -1.369, p = 0.171; zero-inflation component: z = 0.359, p = 0.719) or mated females (ZINB GLM: count component; z = -0.931, p = 0.352; zero-inflation component: z = -0.006, p = 0.995) were subject to heatwave conditions for 2 h did not differ significantly from controls. Similarly, virgin males and females provided with preheated fodder at 30°C showed no significant differences in reproductive output compared to controls after being subject to heatwave conditions for 2 h (virgin males: ZINB GLM; count component: z = 0.010, p = 0.992; zero-inflation component: z = -1.024, p = 0.306; virgin females: ZINB GLM; count component: z = -0.679, p = 0.497; zero-inflation component: z = 1.047, p = 0.295).

To assess whether fodder temperature alone influenced reproductive output, groups with ambient versus 30°C preheated fodder were compared within virgin male and female groups. Due to the non-normality of data (Shapiro-Wilk $p < 0.05$), non-parametric tests were used. No significant difference was detected in females (Wilcoxon rank-sum test: W = 389, p = 0.397) or males (Wilcoxon rank-sum test: W = 632, p = 0.204), indicating that initial fodder temperature did not affect reproductive output response to heatwave conditions.

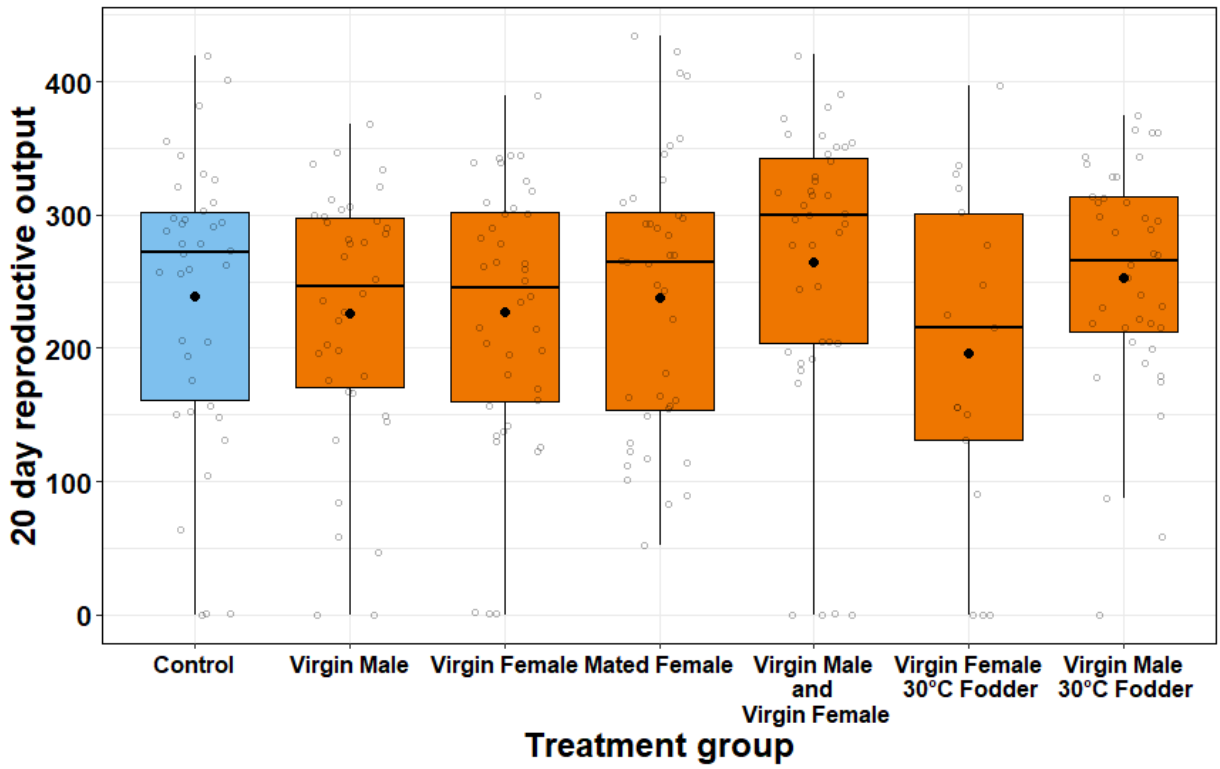

*Figure 3.1: Impact of 2 hour, 42°C heatwave conditions on reproductive output. Sample sizes for each group left to right: 38, 38, 40, 40, 39, 17, 40. Boxplots contain a median line, a mean dot and an interquartile range box. Significance values representing a comparison between 30°C and the experimental heatwave condition are denoted by stars: * = P <0.05, ** = P < 0.01, *** = P < 0.001.*

3.5.2.2 Impact of a 5 hour heatwave exposure:

Exposure to heatwave conditions of 42°C for 5 hours did not significantly impact reproductive output in any treatment group except from where both virgin males and females were subject to heatwave conditions (Fig. 3.2). Reproductive output in groups where virgin males (ZINB GLM: count component; z = -0.123, p = 0.902; zero-inflation component: z = 0.000, p = 1.000), virgin females (ZINB GLM: count component; z = 0.460, p = 0.646; zero-inflation component: z = 0.000, p = 1.000), or mated females (ZINB GLM: count component; z = 0.092, p = 0.926; zero-inflation component: z = 0.002, p = 0.998) were subject to heatwave conditions for 5 h did not differ significantly from controls. Similarly, virgin males and females provided with preheated fodder at 30°C showed no significant differences in reproductive output compared to controls after being subject to heatwave conditions for 5 h (virgin males: ZINB GLM; count component: z = 0.958, p = 0.338; zero-inflation component: z = 0.002, p = 0.998; virgin females: ZINB GLM; count component: z = 0.826, p = 0.409; zero-inflation component: z = 0.002, p = 0.998). Where both virgin males and females were subject to heatwave conditions, a 21% increase in the reproductive output of

these pairs was seen in comparison to controls (ZINB GLM: count component; z = 2.072, p = 0.038; zero-inflation component: z = 0.000, p = 1.000).

To assess whether fodder temperature alone influenced reproductive output, groups with ambient versus 30°C preheated fodder were compared within male and female groups. Due to the non-normality of data (Shapiro-Wilk p < 0.05), non-parametric tests were used. No significant difference was detected in females (Wilcoxon rank-sum test: W = 736, p = 0.400) or males (Wilcoxon rank-sum test: W = 648, p = 0.145), indicating that initial fodder temperature did not affect reproductive output response to heatwave conditions.

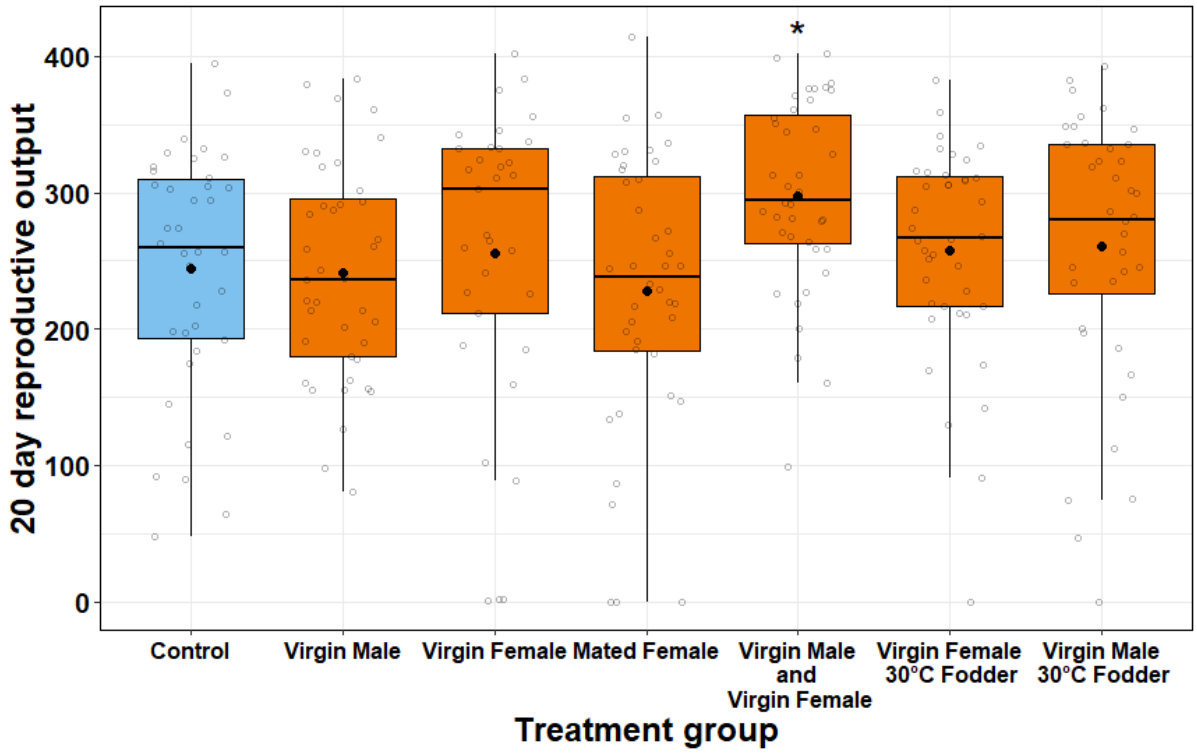

*Figure 3.2: Impact of 5 hour, 42°C heatwave conditions on reproductive output. Sample sizes for each group left to right: 39, 40, 34, 40, 40, 40, 40. Boxplots contain a median line, a mean dot and an interquartile range box. Significance values representing a comparison between 30°C and the experimental heatwave condition are denoted by stars: * = P <0.05, ** = P < 0.01, *** = P < 0.001.*

3.5.2.3 Impact of a 10 hour heatwave exposure

Exposure to heatwave conditions of 42°C for 10 hours did not significantly impact reproductive output in any treatment group except from where mated females were subject to heatwave conditions (Fig. 3.3). Reproductive output in groups where virgin males (ZINB GLM: count component; z = -0.403, p = 0.687; zero-inflation component: z = 0.006, p = 0.995), virgin females (ZINB GLM: count component; z = -1.761, p =

0.078; zero-inflation component: z = 0.006, p = 0.995), or both virgin males and virgin females (ZINB GLM: count component; z = 1.869, p = 0.062; zero-inflation component: z = 0.006, p = 0.995) were subject to heatwave conditions for 10 h did not differ significantly from controls. Where mated females were subject to heatwave conditions, a 29% decrease in the reproductive output of these pairs was seen in comparison to controls (ZINB GLM: count component; z = -3.258, p = 0.0011; zero-inflation component: z = 0.007, p = 0.995).

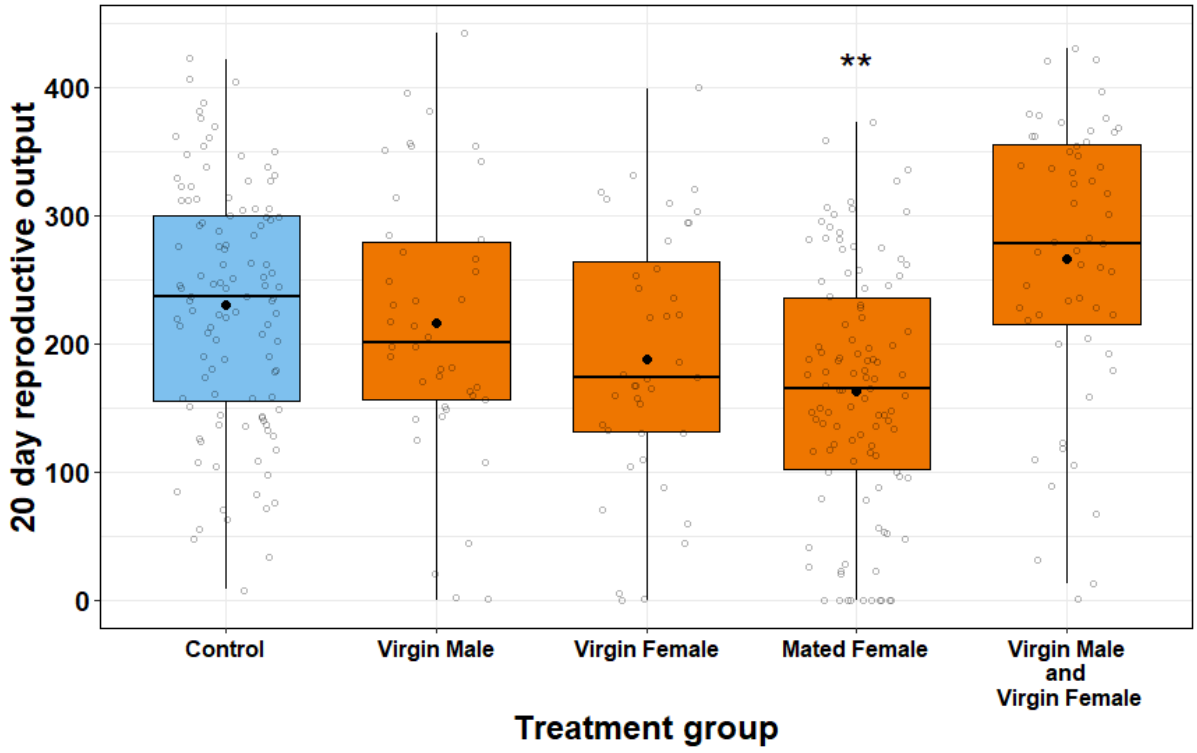

*Figure 3.3: Impact of 10 hour, 42°C heatwave conditions on reproductive output. Sample sizes for each group left to right: 113, 43, 40, 106, 56. Boxplots contain a median line, a mean dot and an interquartile range box. Significance values representing a comparison between 30°C and the experimental heatwave condition are denoted by stars: * = P <0.05, ** = P < 0.01, *** = P < 0.001.*

3.5.2.4 Impact of 3 consecutive days of 10 hour heatwave exposures

Exposure to heatwave conditions of 42°C for 10 hours on 3 consecutive days did not significantly impact reproductive output in any treatment group (Fig. 3.4). Reproductive output in groups where virgin males (ZINB GLM: count component; z = -0.242, p = 0.809; zero-inflation component: z = 0.526, p = 0.599), virgin females (ZINB GLM: count component; z = -0.042, p = 0.967; zero-inflation component: z =

0.779, p = 0.436), both virgin males and virgin females (ZINB GLM: count component; z =0.497, p = 0.619; zero-inflation component: z = 0.142, p = 0.887) or mated females (ZINB GLM: count component; z = -0.364, p = 0.716; zero-inflation component: z = -0.373, p = 0.709) were subject to heatwave conditions of 42°C for 10 hours on 3 consecutive days did not differ significantly from controls.

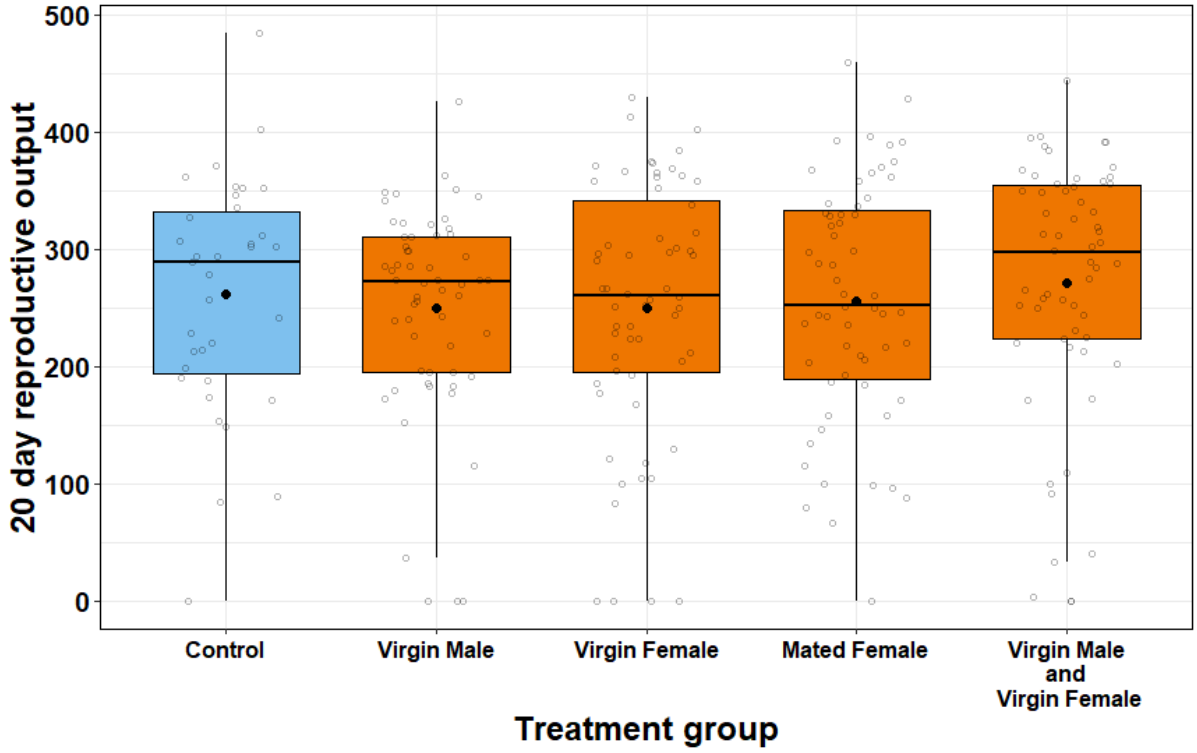

*Figure 3.4: Impact of 3 consecutive days of 10 hour, 42°C heatwave conditions on reproductive output. Sample sizes for each group left to right: 35, 58, 60, 59, 59. Boxplots contain a median line, a mean dot and an interquartile range box. Significance values representing a comparison between 30°C and the experimental heatwave condition are denoted by stars: * = P <0.05, ** = P < 0.01, *** = P < 0.001.*

3.5.2.5 Impact of 5 consecutive days of 10 hour heatwave exposures

Exposure to heatwave conditions of 42°C for 10 hours on 5 consecutive days did not significantly impact reproductive output in any treatment group except from where mated females were subject to heatwave conditions (Fig. 3.5). Reproductive output in groups where virgin males (ZINB GLM: count component; z = -1.470, p = 0.142; zero-inflation component: z = -0.253, p = 0.800), virgin females (ZINB GLM: count component; z = -1.423, p = 0.155; zero-inflation component: z = 0.253, p = 0.801), or both virgin males and virgin females (ZINB GLM: count component; z =-1.801, p = 0.072; zero-inflation component: z = 0.628, p = 0.530) were subject to heatwave conditions of 42°C for 10 hours on 5 consecutive days did not differ significantly from

controls. Where mated females were subject to heatwave conditions, an 18% decrease in the reproductive output of these pairs was seen in comparison to controls (ZINB GLM: count component; z = -2.567, p = 0.0103; zero-inflation component: z = -0.240, p = 0.810).

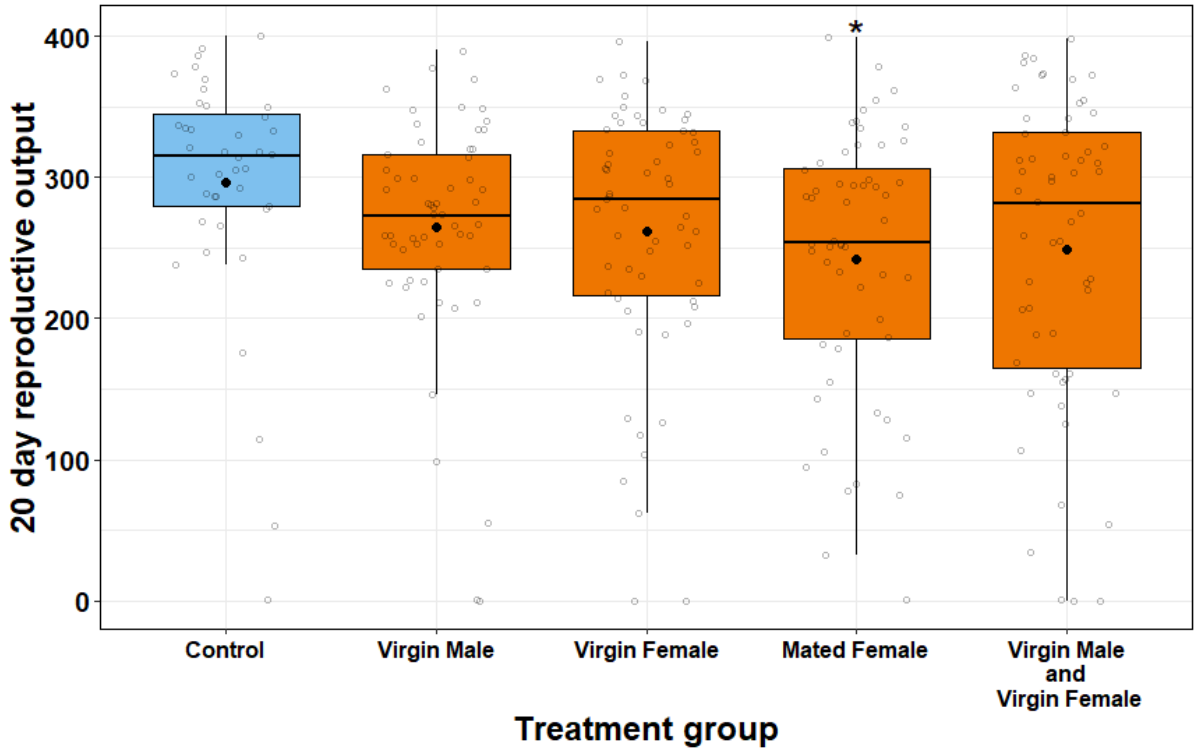

*Figure 3.5: Impact of 5 consecutive days of 10 hour, 42°C heatwave conditions on reproductive output. Sample sizes for each group left to right: 40, 57, 59, 56, 59. Boxplots contain a median line, a mean dot and an interquartile range box. Significance values representing a comparison between 30°C and the experimental heatwave condition are denoted by stars: * = P <0.05, ** = P < 0.01, *** = P < 0.001.*

3.5.2.6 Impact of a 'double heatwave' exposure

Exposure of virgin males to heatwave conditions of 42°C for 10 hours on 5 consecutive days, followed by a 7-day rest, and then another exposure to heatwave conditions of 42°C for 10 hours on 5 consecutive days did not significantly impact reproductive output (Fig. 3.6; count component; ZINB GLM: z = 0.288, p = 0.773; zero-inflation component: z = 0.003, p = 0.998).

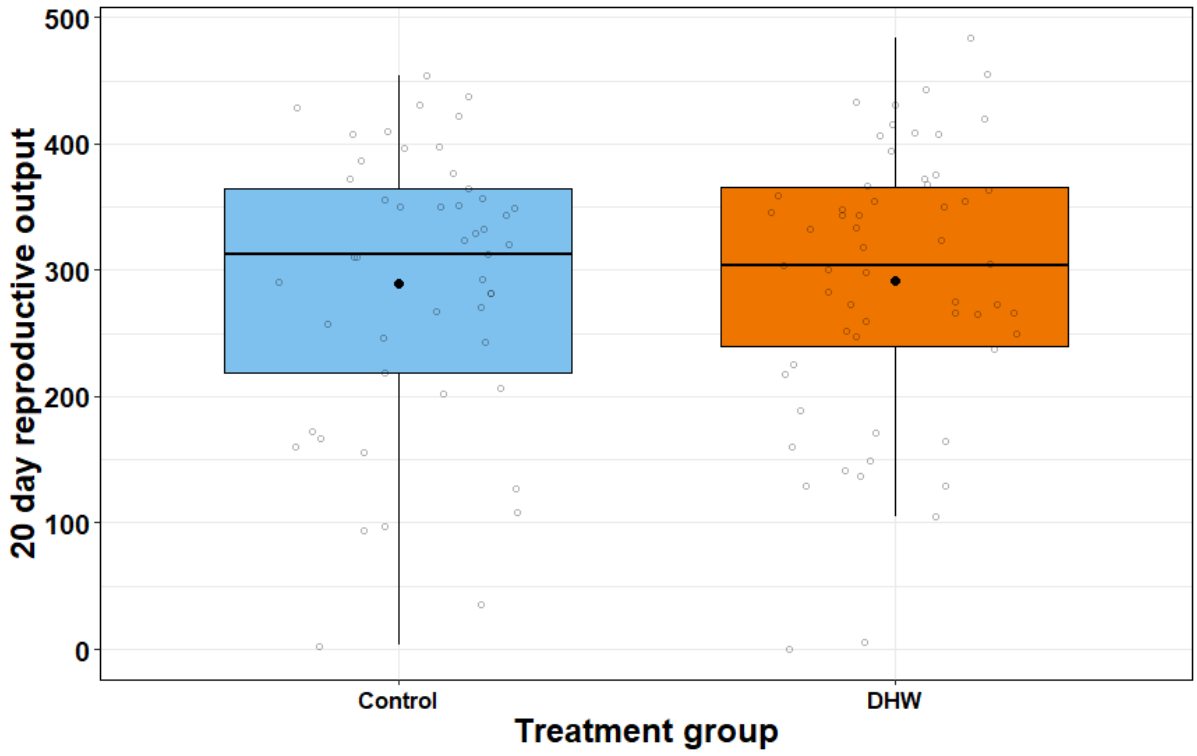

*Figure 3.6: Impact of a double heatwave on virgin male reproductive output. Sample sizes for each group left to right: 49, 59. Boxplots contain a median line, a mean dot and an interquartile range box. Significance values representing a comparison between 30°C and the experimental heatwave condition are denoted by stars: * = P <0.05, ** = P < 0.01, *** = P < 0.001.*

3.6 Discussion

In *T. castaneum*, survival was highly resilient to the heatwave conditions tested. All groups showed survival rates greater than 95%, except for virgin females subjected to a 2 h heatwave in preheated fodder, which had a survival rate of 85%. However, the sample size of the latter treatment was relatively low. This resilience is unsurprising given that the heatwave temperature used here (42°C) is only 7°C above this strain's population productivity optimum (Sales et al., 2018), and pest species often have a wide thermal tolerance (Deutsch et al., 2008). Furthermore, across *T. castaneum's* global distribution, 42°C represents a temperature that is commonly experienced, and may represent >10°C less than thermal maxima experienced across their distribution (Campbell et al., 2022; Christidis et al., 2023). Whilst I also show that no apparent sex-specific differences in survival emerged in response to the heatwave conditions tested, given that survival was very high across all groups, any sex-specific differences in survival could be masked by the lack of a severe enough thermal stressor. It is therefore possible that both sexes were operating well within

their thermal limits, and that more extreme heatwaves may be required to reveal subtle differences in thermal sensitivity. I assess the impacts of 2, 5 and 10 h heatwaves with temperatures ranging from 42-50°C on survival in Chapter 5.

Many experimental studies have failed to test thermal exposures independently in males and females, so the full extent of sex-specific responses to heatwaves is currently not well understood (Dougherty et al., 2024). In other studies assessing sex-specific survival responses to heatwaves, there have been variable outcomes, with some studies showing similar survival reductions (e.g. Tsetse flies; Weaving, Terblanche and English, 2024), some showing females being more susceptible (e.g. Soil mites; Neelam Porwal et al., 2025), and others showing males being more susceptible (e.g. alfalfa leafcutting bees; Hayes and López-Martínez, 2021).

Similarly to survival, reproductive output was highly resilient to the heatwave conditions tested. Even the most extreme conditions, where virgin males were subject to a double heatwave before mating, did not result in a significant change in the number of offspring produced. These results are markedly different from those shown in *T. castaneum* by Sales et al. (2018), whereby a 5 d continuous heatwave at 42°C resulted in a 50% reduction in reproductive output when virgin males were the focal group, and a double heatwave resulted in nearly zero reproductive output. Likewise, a 33% reduction in reproductive output was seen when mated females were the focal group subject to a 5 d continuous heatwave. In this Chapter, however, no detectable decrease in reproductive output was observed in virgin males. As discussed in the introduction, it appears that the inclusion of recovery windows between daily exposures may have allowed sufficient physiological repair to mitigate reproductive damage, consistent with evidence that diurnal cooling periods can buffer the impacts of heat stress (Bai et al., 2019). The absence of reproductive output impacts shown here suggests that *T. castaneum* may possess greater resilience to heatwaves than previously thought.

Whilst no consistent trends in reproductive output reductions were seen, the extent to which such heatwave conditions may impact reproductive morphology, even if this doesn't result in noticeable reductions in reproductive output, is currently unclear and requires future investigation. It is well known that extreme temperatures can disrupt normal reproductive development and maintenance of gametes. This is particularly relevant for males, where it has been shown that extreme heat can reduce testes size, alter sperm morphology, and decrease sperm survival in several insect

taxa (Vasudeva, Deeming and Eady, 2014; Uy et al., 2015; Porcelli et al., 2016; Sales et al., 2018; Iossa et al., 2019; Martinet et al., 2020; Campion et al., 2023; Sales et al., 2024). Any morphological impacts on sperm (even if they do not manifest in reproductive losses initially) may have more severe consequences in species that produce most or all of their sperm during development, as is seen in some insects (Dumser, 1980). In such species, repeated damage incurred from subsequent heatwaves, particularly given their increasing frequency, could have compounding effects on lifetime reproductive function due to the limited capacity for further sperm generation. To assess the potential for reproductively 'silent' damage to male reproductive morphology, I assess the impacts of the short heatwave conditions tested here (alongside more extreme temperatures) on male reproductive morphology (testes volume, sperm length and sperm length variation) in *T. castaneum* in Chapter 4.

Whilst inconsistent across all groups, mated females who were subject to longer heatwave exposures (a single 10 h heatwave exposure or 5 days of heatwave exposures, but not 3 days) showed a significant decrease in reproductive output (Figs. 3.3 and 3.5). This contrasts with previous work by Sales et al. (2018), which showed that virgin males were the group most greatly impacted by heatwave conditions in this species. However, in that study, mated females also showed a marked, yet lesser, reduction in reproductive output. Due to the inconsistency in responses of mated females shown here, caution is needed when interpreting thee results; nevertheless, they do suggest potential vulnerability of females to heatwaves. Considering that females will spend the majority of their lives as a mated individual (Pai and Yan, 2003), and factoring in their key roles in storing sperm from multiple males and laying fertilised eggs, any severe impacts on mated females are likely to have drastic impacts on population structure and potentially persistence. The impacts of heatwaves on mated females are explored in Chapters 5, 6 and 7 of this thesis, under more severe heatwave exposures, to better understand the potential vulnerabilities of this group in *T. castaneum*.

To assess whether indirect effects of fodder temperature may confound reproductive outcomes, I assessed whether preheating fodder to 30 °C before heatwave exposure impacted reproductive output losses when compared to groups that were placed in ambient temperature fodder before being placed in the simulated heatwave conditions (measured at ~19°C). No differences were seen between males or females in pre-heated or ambient temperature fodder before either a 2 h or 5 h heatwave. This indicates that the thermal properties of the fodder

may not substantially alter the experimental beetles' reproductive outcome. Consequently, consistent heating of the substrate may not be necessary when designing future experiments involving similar heatwave profiles, particularly when thermal exposures are brief and conducted in controlled environments where temperature equilibrates quickly.

In summary, this Chapter provides important insights into the implications of heatwave exposures on survival and reproductive output in *T. castaneum* using more ecologically relevant heatwave conditions than have been previously tested. It is demonstrated that heatwaves ranging from 2 h to 5 d have limited, if any, impacts on survival and reproductive output in this species, and highlights some crucial areas for further investigation in future Chapters focused on the ability for this species to withstand higher temperature heatwaves under such ecologically relevant scenarios and the degree to which this may impact reproductive systems.

4. Impacts of ecologically relevant heatwave exposures on male survival and reproduction in *Tribolium castaneum*

4.1 Abstract

Heatwaves are becoming more common and severe. Previous work has highlighted male insects as being particularly vulnerable to multi-day continuous heatwaves, yet our understanding of short duration heatwave impacts on insects is limited. Here, we assessed the impacts of short, simulated heatwave exposures (2, 5 and 10 h) using ecologically relevant temperatures (42, 44, 46, 48 and 50°C) on survival, reproductive output, testes volume and sperm length in *Tribolium castaneum*. We show that reproductive output is compromised at lower temperatures than survival, especially during the shortest heatwaves, supporting the notion that thermal fertility limits are lower than thermal viability limits. Furthermore, testes volume was reduced by 40% after a 10 h exposure at 42°C and sperm length decreased by 2.7% after an exposure of 42°C for just 2 h. This highlights that even short heat exposure can impact male fertility and reproductive trait morphology at temperatures below viability limits.

4.2 Contributors

Ramakrishnan Vasudeva helped with sexing experimental individuals alongside dissections and taking measurements of sperm length and testes volume. Kay Dragoi, Josie Hibble, and James King assisted with the maintenance of experimental lines, sexing experimental individuals, and collecting data on reproductive output.

4.3 Introduction

Significant changes to Earth's climate are being seen due to human activities, with mean global temperature predicted to reach up to 1.9°C higher than pre-industrial times by 2028 (World Meteorological Organisation, 2024). In addition to increases in mean global temperature, incidences of extreme heat are also rising. Heatwaves are becoming broadly more common and intense, including in regions where they were previously rare (Meehl and Tebaldi, 2004; Perkins-Kirkpatrick and Lewis, 2020). In particular, short-duration heatwaves and their impacts on ecosystems and the environment are expected to increase in frequency (Trenberth, 2018). Understanding the ecological and evolutionary implications of these heatwaves is critical as climatic extremes are shifting more rapidly than mean temperature (Seneviratne et al., 2021).

With anthropogenic climate change occurring at accelerating rates in all geographical regions (World Meteorological Organisation, 2024), there is emerging evidence that it has caused species extinctions (Ceballos et al., 2015), with insects highlighted as being particularly at risk (Dunn, 2005; Wiens, 2016; Wagner et al., 2021). Understanding the mechanisms underpinning current and future insect declines under climate change is becoming an increasingly important research focus, with extreme heat often linked to decreasing insect survival (Ma, Ma and Pincebourde, 2020). However, sub-lethal impacts of heat on biological functions are less well understood. Recent research has shown that thermal fertility limits (TFLs) are reached at lower temperatures than critical thermal limits in some species (CTLs; Parratt et al., 2021). Furthermore, male TFLs have been shown to predict species distribution and extinction risk better than CTLs (Parratt et al., 2021; van Heerwaarden and Sgrò, 2021).

Many studies have assessed the effect of extreme heat on multiple aspects of male insect reproduction including spermatogenesis (Canal Domenech and Fricke, 2023), sperm motility, mobility and morphology (Uy et al., 2015; Porcelli et al., 2016; Iossa et al., 2019), sperm number (Vasudeva, Deeming and Eady, 2014; Sales et al., 2018), sperm viability (Sales et al., 2018; Martinet et al., 2020; Campion et al., 2023), sperm storage (Sales et al. 2024), testes volume (Vasudeva, Deeming and Eady, 2014; Sales, Vasudeva and Gage, 2021), courtship and mating behaviour (Grandela et al., 2023; Ratz et al., 2024) and parental care (Pilakouta et al., 2023). These studies have highlighted the range of fertility traits that can be affected. However, research on the impact of short-duration heatwaves remains limited (Dougherty et al., 2024), which is a particular concern given that shorter and more intense heatwaves are increasing in frequency (Trenberth, 2018).

Using *Tribolium castaneum*, a widely used model in evolution and ecology (Pointer, Gage and Spurgin, 2021; Campbell et al., 2022*),* Sales et al. (2018) showed that subjecting virgin males, but not virgin females, to a simulated heatwave before mating severely reduced reproductive output. Subjecting males to heatwave conditions of 42°C for 5 continuous days halved male fertility, with a further heatwave sterilising most individuals, which was linked to decreased sperm viability and number. Such long-term heatwaves were also shown to reduce testes volume by half, which recovered to control levels ~25 days post heatwave exposure (Sales, Vasudeva and Gage, 2021). Under future climate projections, ’extremely hot days’ with temperatures over 50˚C are expected to become increasingly common within areas of *T. castaneum’s* global distribution (Campbell et al., 2022; Christidis et al.,

2023). It is important to understand the consequences of such intense conditions under ecologically relevant scenarios, as peak temperatures within a heatwave may only be experienced for a few hours in a single day (Holmes et al., 2015; Oliveira et al., 2021). Therefore, there is a pressing need to investigate how exposure to various short-duration heatwave conditions may impact male survival and fertility and understand the potential implications this may have on insect populations.

This has been further emphasised by recent studies applying the Thermal Death Time (TDT) framework, which integrates both the duration and intensity of thermal exposures to predict biological damage. While initially focused on describing survival thresholds, such approaches are increasingly being used to understand sublethal impacts on fertility, highlighting that different traits may have distinct thermal sensitivities and damage accumulation rates (Jørgensen et al., 2019; Ørsted et al., 2024; Rezende et al., 2014). This Chapter aims to contribute to the growing understanding of such interacting factors by testing the impacts of ecologically relevant simulated heatwaves with varying duration (2, 5 or 10 hours [h]) and intensity (42°C, 44°C, 46°C, 48 °C or 50 °C) on male survival, reproductive output, testes volume and sperm length using *T. castaneum.*

In this chapter, I ask whether short, ecologically relevant heatwave exposures compromise male survival, reproductive output and reproductive morphology in *Tribolium castaneum*. I predict that reproductive output, testes volume and sperm length would be affected at less severe heatwave conditions than those required to substantially reduce survival.

4.4 Materials and methods

4.4.1 Line maintenance and experimental individuals

T. castaneum used in these experiments were from the Krakow Super Strain (KSS) outbred stock line, which was maintained as described in Chapter 2. Experimental individuals were sexed at the pupal stage by visual identification of sexually dimorphic genital papillae (on day 18 of development) and then kept in single-sex groups of 20 individuals for a further 10 days to allow for development to sexual maturity. Groups were kept in 6cm Petri dishes filled with 3 grams (g) of fodder and topped with an even layer of oats. Once matured, females were identified with a dot of Uni Posca non-toxic marker (Uni-ball, Tokyo, Japan) on the dorsal thorax.

4.4.2 Experimental heatwave treatments

Heatwave conditions were applied using either an A.B. Newlife 75 Mk4 forced air egg incubator or A.B. Newlife 75 Mk 4 Moving Air Incubator (A.B. Incubators, Suffolk, UK). These conditions were selected based on previous research, which established the reproductive optimum of males (see Sales et al. 2018). Virgin male beetles were either exposed to heatwave conditions (42°C, 44°C, 46°C, 48 °C or 50 °C ± 1 °C, 60 ± 10 % RH) or control conditions (30 ± 1°C, 60 ± 10 % RH) for 2, 5 or 10 h in single-sex groups of 20 individuals in 6cm Petri dishes filled with 3g of fodder and a layer of oats. Temperature was checked every 30 minutes using a digital thermometer integrated into the incubator and an additional mercury thermometer placed in the incubator. No recorded temperature was above or below 1°C from the set point. Using the same temperature and duration combinations across all exposed males allowed male survival and reproductive traits to be compared under identical heatwave conditions, and allowed the effects of increasing heatwave temperature and exposure duration to be assessed within a single experimental framework.

4.4.3 Survival and reproductive output assay

Virgin, sexually mature males (48–72h post eclosion) were subject to heatwave conditions with controls run in parallel (conditions described above). All individuals then experienced a further rest period at 30 ± 1 °C for 24 h. Females were sourced from the same population but were maintained constantly under standard conditions at an identical density. Individual males were visually assessed at this time, and survivors were paired with an age-matched virgin female for 48 h at 30 ± 1 °C in a 7 ml mating vial. These mating vials were filled with ~2.4 g of fodder and topped with a few pieces of oats for traction. These mating vials were retained to ensure successful ejaculate transfer, assessed by the visual presence of larval tracks/offspring.

Females from the above mating assays were then moved into individual 6cm Petri dishes (filled with 3g of fodder and topped with an even layer of oats) and allowed to oviposit. Oviposition occurred across two separate ten-day blocks (twenty days in total) to reduce cannibalism associated with overlapping offspring developmental life stages (Park et al., 1965). After twenty days, the females were removed, and the oviposited eggs were allowed to develop until maturity for an additional 35 days under standard developmental conditions (30 ± 1°C, 60 ± 10 % RH). The reproductive output of each pair was then assessed as the number of mature adults produced from twenty days of oviposition (equating to ~ 51% of a female's lifetime reproductive output; pg. 31, Dickinson, 2018). Because reproductive

output was measured after exposed males were paired with untreated virgin females, this assay assessed the reproductive consequences of male heatwave exposure while keeping female thermal history constant.

*Table 4.1: Number of individuals subject to each heatwave temperature/duration condition for the survival assay, and number of survivors who subsequently went through the reproductive output assay. * = No individuals were assayed due to 100% mortality during the heatwave exposure.*

Survival Sample Size

Temperature (°C)	2 hours	5 hours	10 hours
30	58	60	60
42	40	40	60
44	35	38	60
46	34	40	60
48	70	69	60
50	70	61	60

Reproductive Output Sample Size

Temperature (°C)	2 hours	5 hours	10 hours
30	53	54	51
42	38	37	56
44	28	32	60
46	32	32	59
48	65	65	32
50	68	52	0*

4.4.4 Testes and sperm measurements

An additional cohort of males that experienced identical experimental conditions were allocated to quantifying testes volumes and total sperm length. This additional cohort allowed testes and sperm traits to be measured from males exposed to the same temperature and duration combinations used in the survival and reproductive output assays, without disrupting the reproductive output assay itself. These individuals were frozen at -20°C immediately after being subject to either heatwave or control conditions (as described above). All samples were blinded to the user by a random code at this point to avoid any unconscious biases during morphological measurements. Measurements were taken from individuals who survived the heatwaves for all experimental groups apart from those subject to 50°C for 10 h,

where all males had died during the exposure period. Testes dissections and measurements were carried out according to a standard protocol, as described in Sales, Vasudeva, and Gage (2021). Males were dissected in a saline buffer solution (0.9% NaCl), where the abdomen was isolated using dissecting forceps. The testes were excised from the dorsal abdomen and sealed under a 20 x 20 mm coverslip, maintained in 30 µl of buffer solution. Testes volume was calculated as the mean volume of all intact testicular follicles, divided by the total number of intact follicles. Images of testes were taken at 10x magnification using an Olympus BX41 microscope (Olympus Corporation, Tokyo, Japan), then captured with a GXCAM CCD camera (GT Vision, Suffolk, UK) and GXCapture (release 8.5) software. The testes of 10 males were measured per duration/ heatwave condition. To measure total sperm length, testes were isolated as described above. Testes were then ruptured using forceps, and sperm were dispersed within the saline solution, and were then allowed to dry. Images of sperm were taken using 600x magnification using an Olympus BX41 microscope (Olympus Corporation, Tokyo, Japan), then captured with a GXCAM CCD camera (GT Vision, Suffolk, UK) and GXCapture (release 8.5) software. Twenty individual mature intact sperm were measured per male (N = 10 males per duration/heatwave condition).

4.4.5 Statistical analysis

All data were analysed using R version 4.4.1 (R Core team, 2024) in RStudio Version 2024.04.2+764(Posit team, 2024). Plots were created using “ggplot2” (Wickham, 2016). Data manipulation was done using “tidyverse” (Wickham et al., 2019). The impact of heatwave temperature on survival for each of the three heatwave durations was initially assessed using a binomial GLM. Firth's penalised logistic regression from the brglm2 package was used where near-separation or complete separation issues caused model convergence issues (Kosmidis, 2023). Final survival models used Firth’s penalised logistic regression for the 2 h and 10 h exposure durations, and a standard binomial GLM for the 5 h exposure duration. Fit of the models were then assessed using “performance” (Lüdecke et al., 2021) and residuals were visually evaluated.

Reproductive output data were first censored where individuals escaped from or died during the reproductive output assay (N = 31). Zero-inflated negative binomial GLMs (Zeileis, Kleiber and Jackman, 2008) were used to analyse data after assessing models for over or under-dispersion and comparing goodness of fit using “performance” and “lmtest” (Zeileis and Hothorn, 2002). Models were fitted separately

for each heatwave exposure duration, with 20-day reproductive output as the response variable and heatwave temperature treatment as the predictor variable. For each model, treatment effects were assessed in both the count component, representing differences in reproductive output, and the zero-inflation component, representing differences in the probability of excess zeros.

Testes volume was analysed separately for each heatwave exposure duration, with testes volume as the response variable and heatwave temperature treatment as the predictor variable. Gaussian GLMs were initially fitted for each exposure duration, and model residuals were assessed visually and using diagnostic tests. Final models for the 2 h and 5 h exposure durations used Gaussian GLMs. For the 10 h exposure duration, where residuals from the Gaussian model showed evidence of heteroscedasticity, a Gamma GLM with an inverse link was used.

Average sperm length was analysed separately for each heatwave exposure duration, with mean sperm length per male as the response variable and heatwave temperature treatment as the predictor variable. Gamma GLMs with a log link were used for each exposure duration after comparison with inverse Gaussian models using AIC and BIC. Model fit and residuals were assessed using “performance” and “lmtest”.

The standard deviation of sperm length was calculated for each male as a measure of within-male sperm length variation. Sperm length variation was analysed using linear mixed models, with heatwave temperature treatment, exposure duration and their interaction included as fixed effects, and Male ID included as a random effect. Duration-specific models were then fitted with heatwave temperature treatment as the fixed effect and Male ID as a random effect, followed by Tukey-adjusted pairwise comparisons using “emmeans”.

4.5 Results

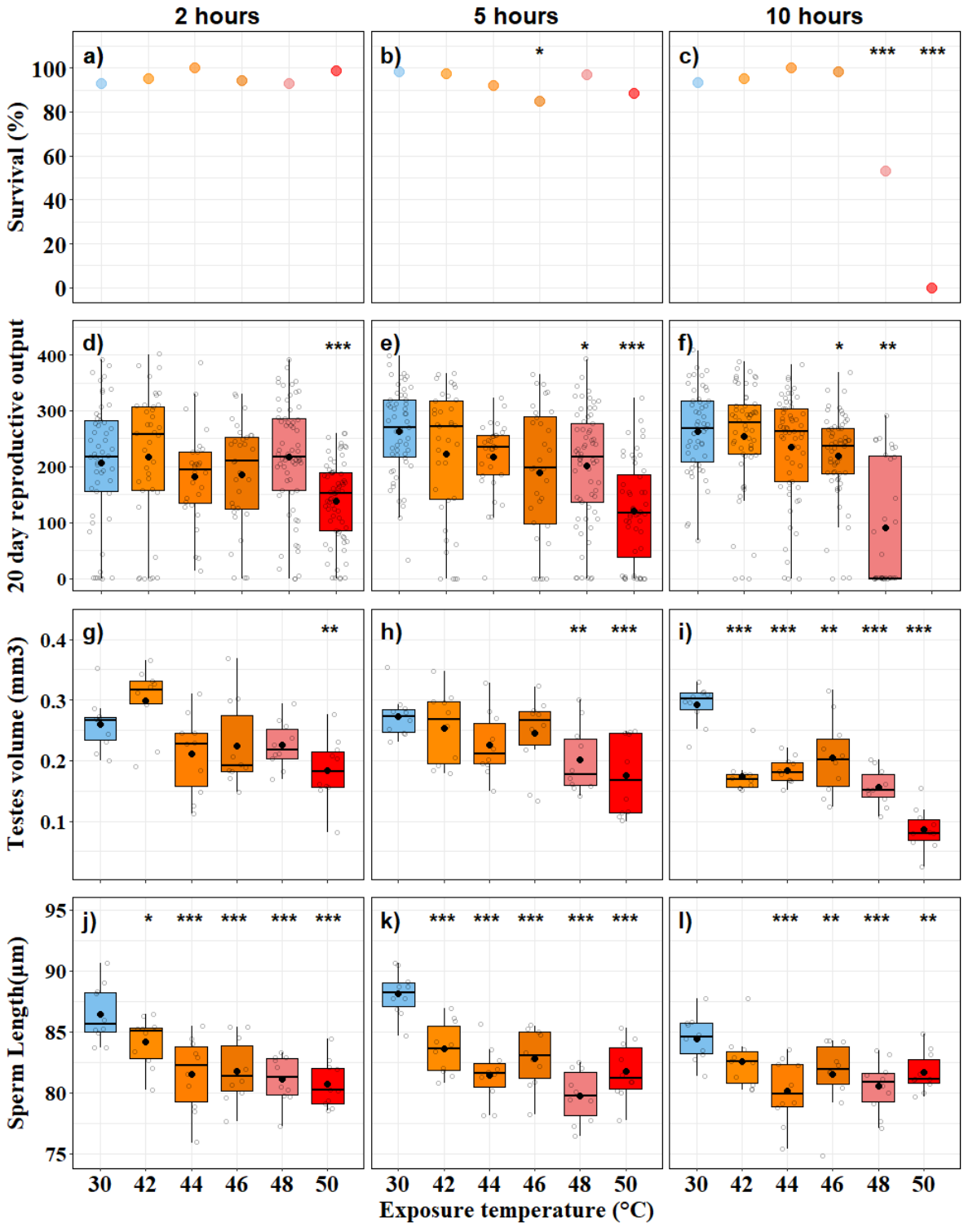

Figure 4.1: Impact of varying heatwave temperature/duration conditions on male survival (Fig. 4.1a-c), 20-day reproductive output (Fig. 4.1d-f), testes volume (Fig. 4.1g-i) and sperm length (Fig. 4.1j-l) following exposure to 30, 42, 44, 46, 48 and 50°C for 2, 5 and 10 h. For Fig. 4.1d-i, raw data points are plotted as open jittered circles. For Fig. 4.1j-l, the mean sperm length per male is plotted as open jittered circles. Boxplots contain a median line, a mean dot and an

*interquartile range box. Significance values representing a comparison between 30°C and the experimental heatwave condition are denoted by asterisks: * = p <0.05, ** = p <0.01, *** = p <0.001. The sample size for survival from left to right for each exposure duration: 2 hours (Fig. 4.1a): 58, 40, 35, 34, 70, 70; 5 hours (Fig. 4.1b): 60, 40, 38, 40, 69, 61; 10 hours (Fig. 4.1c): 60, 60, 60, 60, 60, 60. The sample size for reproductive output for each exposure duration: 2 hours (Fig. 4.1d): 53, 38, 28, 32, 65, 68; 5 hours (Fig. 4.1e): 54, 37, 32, 32, 65, 52; 10 hours (Fig. 4.1f): 51, 56, 60, 59, 32. The sample size for testes volume was 10 individuals per group. The sample size for sperm length was 20 individual sperm from 10 males per group.*

4.5.1 Survival

The survival of males exposed to heatwave conditions for 2 h or 5 h was high across all groups. Survival started to drop dramatically compared to controls in males exposed to higher temperatures for 10 h (Fig 4.1a-c). There was no effect of any temperature treatment on survival following a 2 h exposure. A 46°C 5 h exposure produced a 13.5% reduction in survival compared to controls (Binomial GLM: $z = -2.128$, $p = 0.03$). However, this difference was not observed at higher temperatures. Males exposed to heatwave conditions of 48°C and 50°C for 10 h showed a marked reduction in survival compared to controls (Firth's penalised regression: 48°C; $z = -4.302$, $p < 0.001$, 50°C; $z = -4.837$, $p < 0.001$, Fig 4.1c). At 48°C, there was a 42.9% reduction in survival, and at 50˚C, no males survived (compared to 93.3% survival in the control group at 30˚C).

4.5.2 Reproductive output

As exposure duration and intensity increased, increasingly negative impacts on reproductive output were observed. Heatwave exposure at 50˚C for 2 h reduced male reproductive output by 33% compared to controls (ZINB GLM; count component: $z = -4.302$, $p < 0.001$; zero-inflation component: $z = -0.412$, $p = 0.681$; Fig. 4.1d). Heatwave exposure at 48˚C and 50˚C for 5 h reduced male reproductive output by 23% and 55% respectively (ZINB GLM; count component: 48°C: $z = -1.985$, $p = 0.047$; 50°C: $z = -6.111$, $p < 0.001$; zero-inflation component: 48°C: $z = 0.007$, $p = 0.994$; 50°C: $z = 0.008$, $p = 0.994$; Fig. 4.1e). Heatwave exposure at 46˚C and 48˚C for 10 h reduced male reproductive output by 17% and 66% respectively (ZINB GLM; count component: 46°C: $z = -2.026$, $p = 0.043$; 48°C: $z = -2.837$, $p = 0.00455$; zero-inflation component: 46°C: $z = 0.007$, $p = 0.995$; 48°C: $z = 0.008$, $p = 0.994$; Fig. 4.1f). All individuals exposed to 50°C for 10 h died before the reproductive assay; therefore, no reproductive output data were collected.

4.5.3 Testes volume

Increasing heatwave duration and intensity resulted in a negative impact on testes volume. Heatwave exposure at 50˚C for 2 h reduced testes volume by 29% compared to controls (Gaussian GLM: t = -3.018, p = 0.004, Fig. 4.1g). Heatwave exposure at 48˚C and 50˚C for 5 h reduced testes volume by 26% and 36% respectively (Gaussian GLM: 48˚C: t = -2.847, p = 0.006; 50˚C: t = -3.865, p < 0.001, Fig 4.1h). All heatwave exposures for 10 h reduced testes volume, with even the lowest exposure temperature of 42˚C resulting in a 40% drop in testes volume (Gamma GLM: t = 4.47, p < 0.001, Fig. 4.1i). This reduction in testes volume remained relatively consistent over 44˚C (Gamma GLM: 37%, t = 4.077, p < 0.001) and 46˚C (Gamma GLM: 30%, t = 3.147, p < 0.001) and further reductions were seen at 48˚C (Gamma GLM: 47%, t = 5.356, p < 0.001) and at 50˚C, where testes volume was reduced by 70% (Gamma GLM: t = 8.754, p < 0.001).

4.5.4 Sperm length

Even the shortest and least intense heatwaves caused a decrease in total sperm length. All heatwave exposures for 2 h reduced sperm length compared to controls, with exposure at 42˚C for two hours reducing sperm length by 2.7% (Gamma GLM: t = -2.099, p = 0.04). This reduction in total sperm length generally worsened with increasing temperatures; 44˚C: 5.7% (Gamma GLM: t = -4.560, p < 0.001), 46˚C: 5.4% (Gamma GLM: t = -4.346, p < 0.001), 48˚C: 6.2% (Gamma GLM: t = -4.977, p < 0.001), 50˚C: 6.7% (Gamma GLM: t = -5.352, p < 0.001). Similarly, in all heatwave exposures of 5 h we observed reduced sperm length compared to controls, with 42˚C resulting in a 5.1% reduction (Gamma GLM: t = -4.367, p < 0.001), 44˚C: 7.6% (Gamma GLM: t = -6.581, p < 0.001), 46˚C: 6% (Gamma GLM: t = -5.162, p < 0.001), 48˚C: 9.5% (Gamma GLM: t = -8.344, p < 0.001) and 50˚C: 7.3% (Gamma GLM: t = -6.267, p < 0.001). All heatwave exposures for 10 h, except 42˚C, resulted in significantly reduced sperm length compared to controls. After a 44˚C exposure there was a 5.1% reduction in sperm length (Gamma GLM: t = -4.228, p < 0.001), 46˚C: 3.5% (Gamma GLM: t = -2.836, p = 0.006), 48˚C: 4.7% (Gamma GLM: t = -3.824, p < 0.001) and 50˚C: 3.3% (Gamma GLM: t = -2.684, p = 0.009).

All heatwave exposures for 2 h increased the standard deviation of sperm length when compared to controls, except for 42°C (LMM: t = 0.933, p = 0.356; Fig. 4.2). Variability increased by 61.1% at 44°C (LMM: t = 4.234, p < 0.001), 41.7% at 46°C (LMM: t = 2.881, p = 0.006), 43.3% at 48°C (LMM: t = 3.008, p = 0.004), and 53.1% at 50°C (LMM: t = 3.683, p < 0.001), relative to controls. In line with the 2 h

exposure results, a 5 h exposure at 42°C did not significantly affect sperm length variation when compared to controls (LMM: t = 0.415, p = 0.679). In contrast, exposure to 44°C resulted in a 51.3% increase (LMM: t = 3.459, p = 0.001). Variability remained high at more extreme temperatures, with increases of 57.2% at 46°C (LMM: t = 3.862, p < 0.001), 35.3% at 48°C (LMM: t = 2.390, p = 0.020), and 29.3% at 50°C (t = 1.974, p = 0.053), although the latter only approached significance. Exposure to heatwave conditions for 10 h significantly increased sperm length variability at all temperatures tested. Variability increased by 36.0% at 42°C (LMM: t = 2.792, p = 0.008), with larger increases at higher temperatures: 85.5% at 44°C (LMM: t = 6.637, p < 0.001), 72.5% at 46°C (LMM: t = 5.618, p < 0.001), 85.3% at 48°C (LMM: t = 6.614, p < 0.001), and 49.9% at 50°C (LMM: t = 3.883, p < 0.001), relative to controls.

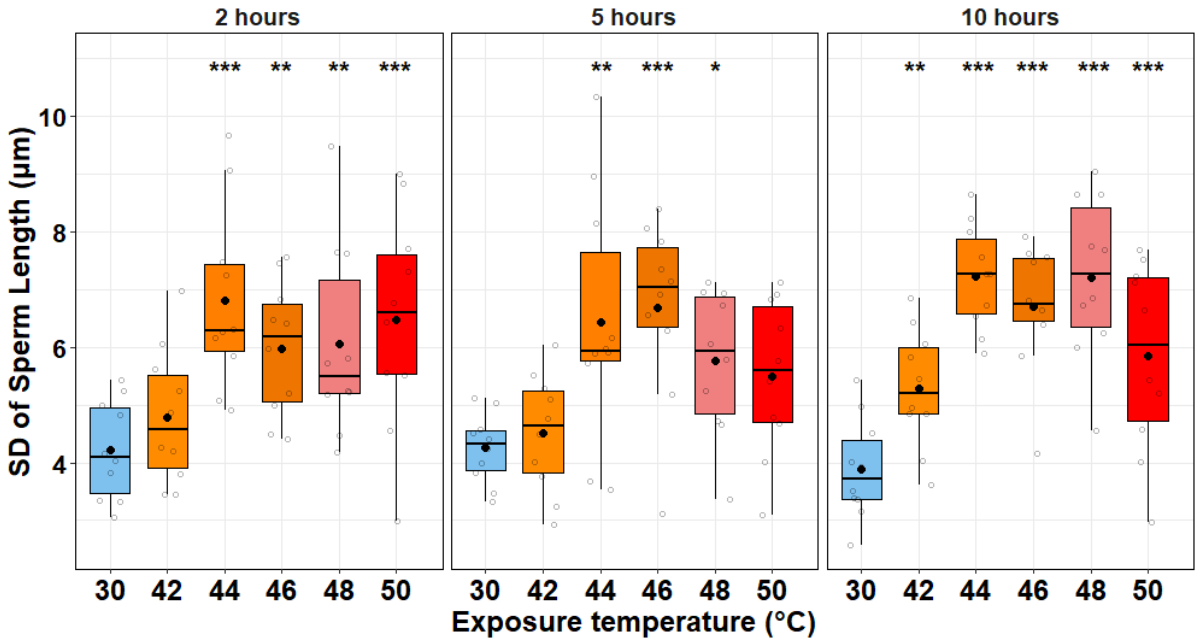

*Figure 4.2: Impact of varying heatwave conditions on the standard deviation of sperm length within males. The mean sperm length standard deviation per male is plotted as open jittered circles. Boxplots contain a median line, mean dot and interquartile range box. Significance values representing a comparison between 30°C and the experimental heatwave condition are denoted by asterisks: * = p <0.05, ** = p <0.01, *** = p <0.001. The sample size for sperm length variation consisted of 20 individual sperm from 10 males across all groups.*

4.6 Discussion

Here, I demonstrate that reproductive traits were affected at sublethal temperatures when male *Tribolium castaneum* beetles were exposed to heatwave conditions

ranging from 42°C to 50°C for 2, 5, and 10 hours. Some reproductive traits were also more thermally sensitive than others. For example, sperm length showed reductions at lower temperatures than reproductive output for all heatwave durations. These results are in line with the existing findings across taxa, which report sensitivity to heat in the reproductive traits of males (e.g. Canal Domenech and Fricke, 2023; Grandela et al., 2023; Meena et al., 2024; Ratz et al., 2024).

The survival of males exposed to heatwave conditions for 2 or 5 h was high across all groups, with no significant reduction compared to controls, except for a 5 h exposure at 46°C. However, this difference was not observed at higher temperatures. Survival started to drop dramatically compared to controls in males exposed to higher temperatures for 10 h (Fig. 4.1a-c). Males exposed to heatwave conditions of 48°C and 50°C for 10 h showed a marked reduction in survival. At 48°C, only 53% of males survived, and at 50˚C, none survived (compared to 93.3% in the control group). Here, I demonstrate that male *T. castaneum* survival was resilient to heatwave conditions until ~11˚C–13˚C above their optimum at 35°C (Sales et al., 2018) before hitting an upper limit when exposed to very intense heat (48°C and 50°C for 10 h).

By contrast, male reproductive output and reproductive morphology were less resilient across a range of short duration heat exposures. As exposure duration and intensity increased, we observed increasingly negative impacts on reproductive output. I found that temperatures as low as 46°C caused a significant reduction in reproductive output after exposure to heatwave conditions for 10 h. At 48°C and 50°C, there were significant reductions following heat exposure of only 5 h and 2 h, respectively. Heatwave exposure at 50°C for 2 h reduced male reproductive output by 33% compared to controls (Fig. 4.1d). Heatwave exposure at 48°C and 50°C for 5 h reduced male reproductive output by 23% and 55% respectively (Fig. 4.1e). Heatwave exposure at 46°C and 48°C for 10 h reduced male reproductive output by 17% and 66% respectively (Fig. 4.1f). All individuals exposed to 50°C for 10 h died before the reproductive assay, therefore, no reproductive output data were collected.

The relationship between heatwave duration and intensity shown in this Chapter further highlights the importance of considering both factors simultaneously when assessing the impact of heat on insects. This is particularly relevant when considering future climate change scenarios where both short-duration and more intense heatwaves are predicted to become more prevalent (Trenberth, 2018).

Moreover, there is a need to consider trait-specific responses to combinations of these factors.

Consistent with previous studies (Sales, Vasudeva and Gage, 2021; Hu et al., 2022), I found that testes volume was significantly impacted by heatwave exposure. I show that this effect was observed following exposure to very brief heatwave conditions. Males exposed to heatwave conditions of 50°C for 2 h showed reduced testes volume by 29% compared to controls (Fig. 4.1g). For 5 h heatwave exposure, 48°C and 50°C reduced testes volume by 26% and 36%, respectively (Fig. 4.1h). For 10 h exposures, all heatwave exposures reduced testes volume compared to controls, with even the lowest exposure temperature of 42°C resulting in a 40% drop in testes volume (Fig. 4.1i). This reduction in testes volume remained relatively consistent over 44°C (37%) and 46°C (30%), and further reductions were seen at 48°C (47%) and at 50°C, where testes volume was reduced by 70%. For both 2 h and 5 h heatwaves, the temperatures that resulted in significant reductions in testes volume were the same at which significant reductions in reproductive output occurred (50°C and 48°C respectively). For 10 h exposures, testes volume was significantly reduced at a lower temperature (42°C) than that at which reproductive output was first compromised (46°C). Broadly, these findings suggest that reductions in testes volume are associated with reduced male reproductive output and that testes volume is highly thermally sensitive.

Heat-related damage to insect sperm has been documented previously (Martinet et al., 2020; Sales, Vasudeva and Gage, 2021; Canal Domenech and Fricke, 2023; Lv et al., 2024). In *T. castaneum*, a five-day, 42°C heatwave reduced sperm survival by ~60% and sperm count by 75% (Sales et al., 2018). Here, I found that even the shortest and least intense heatwave exposures tested in this study caused a decrease in total sperm length. All heatwave exposures for 2 h reduced sperm length compared to controls (Fig. 4.1j), with exposure at 42°C reducing sperm length by 2.7%. This reduction in total sperm length generally worsened with increasing temperature, seeing decreases at 44°C (5.7%), 46°C (5.4%), 48°C (6.2%), and 50°C (6.7%). Similarly, for both 5 h and 10 h exposures, I observed reduced sperm length compared to controls (Fig. 4.1k-l). For 5 h, exposure at 42°C resulted in a 5.1% reduction, with further decreases at 44°C (7.6%), 46°C (6%), 48°C (9.5%), and 50°C (7.3%). For 10 h exposures, all heatwave exposures, except 42°C, resulted in significantly reduced sperm length compared to controls. After a 44°C exposure, there was a 5.1% reduction, with similar decreases at 46°C (3.5%),

48°C (4.7%), and 50°C (3.3%). It is unclear exactly what mechanism is resulting in sperm length reductions, and future work on this will be insightful.

Spermatogenesis occurs throughout the adult life stage in *T. castaneum,* but the exact duration of spermatogenesis and spermiogenesis is unknown. However, in other insects, these processes occur over a number of days (e.g. 10 days for spermatogenesis and 5 days for spermiogenesis in *Drosophila melanogaster*: Rohmer, 2004; Fabian and Brill, 2012 and 12 days for spermatogenesis in *Haematobia irritans*: Basso et al., 2011). Considering the short duration between the initiation of heat exposure and freezing of samples in this study (2-10 h), I suggest that the mechanisms underpinning sperm length change in response to heat exposure may be linked to disruption of spermiogenesis in nearly mature sperm or through direct impacts on mature sperm. This is supported by an observed increase in sperm length variation within individuals, associated with increasing temperatures of heatwave conditions (Fig. 4.2). These findings demonstrate that prolonged heat exposure consistently elevates sperm length variability, highlighting a potential sensitivity of sperm morphology to heatwave conditions. This increase in variability may have a role in the observed reductions in male reproductive output. Further work assessing the specific morphological changes associated with such sperm length variation may elucidate the mechanisms behind the impacts on sperm and male reproductive output observed in this study.

Broadly, I have highlighted that the thermal sensitivity of male reproductive traits is even greater than previously demonstrated (e.g., Sales et al. 2018; Sales, Vasudeva and Gage, 2021). These results showing trait-specific sensitivity also align with recent work on other taxa. For example, in *Drosophila suzukii*, traits (survival, coma induction and productivity) were shown to have different sensitivities to heat stress exposure, and the stress durations required to produce a 50% reduction in each trait often varied greatly (Ørsted et al. 2024). Their work, utilising thermal dose time models, builds on earlier work showing that the time–temperature relationship underlying thermal damage differs across traits (Jørgensen et al., 2019), and future work integrating such an approach would be insightful.

It would be interesting to expand future heatwave studies to test whether trait-specific variability changes after multiple days of exposure, with and without a rest phase. This may reveal the relative vulnerability of specific traits to natural heatwaves, whose intensity would vary over several days (Frich et al., 2002; Christidis et al. 2023). It is also unclear whether some traits can recover or harden after exposure to extreme conditions (e.g., 48˚C and 50˚C) under natural heatwave scenarios, as

potentially compounding effects from repeated exposures may be modulated by periods of non-stressful conditions (e.g., benign cooler nighttime and daytime temperatures), allowing time for physiological repair and fitness recovery (Bai et al., 2019). Previous work found that males can recover reproductive function after longer exposure to less intense experimental heatwave conditions (Sales, Vasudeva and Gage, 2021). Therefore, it will also be important to understand whether there is a general recovery of reproductive potential in males exposed to shorter but more intense heatwave conditions as used in this study.

This Chapter has focused on the effects of heatwave conditions that may be experienced by this species in its natural environment, and which are likely to be increasingly common in the future (Zittis et al., 2021; Campbell et al., 2022). I show that reproductive output is sensitive to sublethal short heatwaves. However, I recognise that the thermal homogeneity and potential lack of interacting factors in our study may have constrained strategies such as moving to more benign microhabitats (e.g. dropping behaviour in aphids; Ma and Ma, 2012), which may alleviate the impact of extreme heat (Terlau et al., 2023). In the future it will be necessary to explore these factors and assess whether different species can adapt to increasingly severe short-term heat exposures (Kellermann and van Heerwaarden, 2019) or whether they are likely to be overwhelmed by the transient and unpredictable nature of extreme thermal events (van de Pol et al., 2017).

5. Sex- and mating status-specific impacts of ecologically relevant heatwave exposures on survival and reproduction in *Tribolium castaneum*

5.1 Abstract

Heatwaves can affect survival and reproduction, but responses may differ due to factors such as sex and mating status influencing individual thermal sensitivity, as males and females invest differently in reproduction. Furthermore, females undergo dramatic physiological changes in response to reproduction. Here, I assess the impacts of ecologically relevant simulated heatwaves on virgin males, virgin females, mated males and mated females in *Tribolium castaneum*. Individuals were exposed to heatwave conditions of 42–50°C for 2, 5 or 10 h to assess survival, before a 48°C, 5 h exposure was used to test reproductive output across the four focal groups. I show that survival was unaffected by 42°C and 44°C heatwaves across all exposure durations, but declined under more intense conditions. After 2 h exposure, only mated females showed a significant survival reduction, and only at 50°C. After 5 h exposure, survival was reduced in virgin males and mated females at 46°C and above, whereas virgin females and mated males were only affected at 48°C and above. After 10 h exposure, all focal groups showed reduced survival at 48°C and 50°C, but mated females were the only group affected at 46°C. Reproductive output following a 48°C, 5 h heatwave was reduced in virgin males, mated males and mated females, whereas virgin females were unaffected. These findings demonstrate that both sex and mating status shape responses to heatwaves, with mated females showing the greatest survival vulnerability.

5.2 Introduction

Extreme weather events such as heatwaves are predicted to have a greater ecological impact than increases in mean temperatures alone (Seneviratne et al., 2021). This is concerning, given that heatwaves are becoming more common and intense due to anthropogenic climate change. Insects are among the most diverse and ecologically important groups on Earth (Deutsch et al., 2008; Stork, 2018), and their ectothermic physiology makes them particularly vulnerable to extreme heat. Because insects underpin key ecosystem processes such as pollination and nutrient cycling, understanding how they respond to heatwaves is crucial for predicting biodiversity and ecosystem resilience.

In Chapter 3, I broadly demonstrated that exposure to heatwave conditions of 42°C for between 2 hours (h) and 5 consecutive days (d) had no impact on survival and reproductive output in *Tribolium castaneum*, except for a tentative suggestion of potential reproductive vulnerability in mated females. Furthermore, survival rates across all treatments were very high. These results were somewhat unexpected, with *Tribolium castaneum* showing a high degree of thermal tolerance in comparison to previous studies, which had assessed virgin males as particularly susceptible to continuous 42°C 5 d heatwaves (Sales et al., 2018, Sales, Vasudeva and Gage, 2021). Given that previous literature has shown males to be more susceptible to continuous heatwaves than females (e.g., Sales et al., 2018), I then focused on potential male susceptibilities to short but more intense heatwave exposures in Chapter 4. There, I showed that higher temperature (46-50°C) heatwave exposures of between 2-10 h caused significant impacts on male reproductive output and reproductive morphology. I also showed that the most intense heatwave conditions (48/50°C for 10 hours) dramatically reduced virgin male survival. Although preliminary, the potential sensitivity of mated females highlighted in Chapter 3 poses a key question as to whether this is present and persistent under heatwave conditions identical to those used in Chapter 4. In this Chapter, I aim to begin addressing these key questions by focusing on elucidating sex- and mating status-specific survival sensitivities, before briefly examining reproductive sensitivity.

Should any such sensitivities exist, this may have dramatic implications for insect survival under future climate regimes, especially given the increasing prevalence of short-duration heatwaves (Trenberth, 2018). This would be particularly salient if female-specific sensitivities are seen, given that the broad majority of current literature has focused on male reproductive sensitivities, with an “emerging pattern” in the literature indicating that this is a key sensitivity to heatwaves among the Insect Class (Iossa, 2019). The ability of insect populations to persist and thrive relies on the survival and reproduction of both sexes, as stress acting on either males or females can disrupt mating and offspring production. However, in many species, such as *Tribolium castaneum*, females spend the vast majority of their lives as mated individuals (Pai and Yan, 2003), and therefore represent a particularly important demographic factor in determining population growth and resilience under heatwave scenarios. Such considerations may extend to other groups of insects, for example, in eusocial species where the survival and fertility of queens are essential for colony persistence (Keller and Chapuisat, 2017).

While the dynamics of social insects differ from those of many insect species, this example illustrates how the loss of reproductively successful females can impact populations under extreme heat exposure. Elucidating any potential sex-specific sensitivities to heatwaves may have profound implications for future research aimed at understanding how such trends may exist across insect taxa.

To assess whether any such sex-specific sensitivities exist in *Tribolium castaneum*, I first assess the impacts of a wide range of heatwave conditions (42-50°C for 2, 5 or 10 h) on survival in four focal groups: virgin males, virgin females, mated males and mated females. This full factorial experimental design enables a comprehensive understanding of sex-specific vulnerabilities, as well as determining whether mating status may influence resilience to heatwaves. This is particularly important to understand given the vast changes that occur post-mating, particularly in females, whereby individuals undergo dramatic changes in behaviour and physiology which are influenced by energetic investments in offspring production, hormonal changes and responses to proteins found within male seminal fluids (Chapman et al., 1995; Wolfner, 2009; Nanfack-Minkeu and Sirot, 2022).

Following-up on the outcomes of the first experiment, I then assessed the sex- and mating status-linked impacts of short heatwave conditions on reproduction. An exposure duration and temperature of 48°C for 5 h was selected as it induces mild survival costs in some of the focal groups assessed in this Chapter within *T. castaneum* (representing conditions bordering sub-lethality/lethality), is towards the lower end of temperatures assessed previously in Chapter 4 as inducing future reproductive losses in virgin males, and represents ecologically relevant heatwave conditions which are commonly experienced by this species in nature (Oliveira et al., 2021; Campbell et al., 2022; Christidis et al., 2023). Together, the findings from these two experiments aim to provide a comprehensive understanding of sex-specific and mating status-dependent survival sensitivities within *Tribolium castaneum* to heatwaves, as well as an initial examination of reproductive sensitivities. This data offers valuable insights into factors which may affect insect population resilience under current and future climate change, whereby short, intense heatwaves are becoming more common (Trenberth, 2018).

In this chapter, I ask whether survival and reproductive responses to short, intense heatwave exposures differ according to sex and mating status in *Tribolium castaneum*. I predict that heatwave sensitivity will generally increase with temperature and duration, but that virgin females will be broadly resilient based on previous work showing limited virgin female reproductive sensitivity to heatwaves.

Among the other focal groups, I predict the severity of heatwave impacts would differ according to sex and mating status, with males showing the greatest reproductive vulnerability.

5.3 Materials and methods

5.3.1 Line maintenance and experimental individuals

T. castaneum used in these experiments were from the Krakow Super Strain (KSS) outbred stock line, which was maintained as described in Chapter 2. Experimental individuals were sexed at the pupal stage by visual identification of sexually dimorphic genital papillae (on day 18 of development) and then kept in single-sex groups of 20 individuals for a further 10 days to allow for development to sexual maturity. Groups were kept in 6cm Petri dishes filled with 3 grams (g) of fodder and topped with an even layer of oats. Once matured, females were identified with a dot of Uni Posca non-toxic marker (Uni-ball, Tokyo, Japan) on the dorsal thorax.

5.3.2 Experimental treatment groups

The sex-specific impacts of heatwaves on survival and fertility were tested by selectively exposing combinations of focal individuals to heatwave conditions in the following combinations: virgin males only, virgin females only, mated males only, and mated females only. This focal-group structure allowed heatwave responses to be compared between sexes and between mating statuses while applying the same general exposure framework to each group.

5.3.3 Experimental heatwave treatments

Heatwave conditions were applied using replicate Octagon 20 Eco incubators (Brinsea Incubation Specialists, Somerset, UK). For the survival assay, virgin male, virgin female, mated male, or mated female beetles were exposed in single-sex and mating status groups in 6cm Petri dishes filled with 3g of ambient temperature fodder and topped with a layer of oats. The vast majority of pots had 10 individuals (n = 997), although due to sample size constraints, a small number of pots from control groups (n = 11) had between 8 and 9 individuals. For the reproductive assay, virgin male or female beetles were exposed in single-sex groups of 20 individuals in 6cm Petri dishes filled with 3g of ambient temperature fodder and topped with a layer of oats. Mated male and female beetles were exposed as individuals in a 6cm Petri dish, filled with 3g of ambient temperature fodder and topped with a layer of oats to allow for tracking of individual fertility measurements.

To assess survival, focal beetles were exposed to heatwave conditions (42°C, 44°C, 46°C, 48°C or 50 °C ± 1°C, ± 10 % RH) for one of the following durations: 2 h, 5 h or 10 h, with controls (30 ± 1°C, 60 ± 10 % RH) run in parallel. To assess reproductive output, focal beetles were exposed to heatwave conditions of 48°C ± 1°C and 60 ± 10% RH for 5 h, with controls (30 ± 1°C, 60 ± 10% RH) run in parallel. The survival assay used the full temperature and duration range to compare the heatwave conditions under which survival began to decline in each focal group, whereas the reproductive output assay used a single 48°C, 5 h heatwave exposure so that reproductive consequences could be compared across sex and mating-status groups under the same heatwave condition.

5.3.4 Virgin survival treatment groups

48 h after eclosion, single-sex groups of virgin males or virgin females were subject to either heatwave conditions or control conditions as described above. After this, all individuals underwent a 24 h rest period at 30 ± 1 °C and were then visually assessed for survival.

5.3.5 Mated survival treatment groups

48 h after eclosion, age-matched pairs consisting of 1 virgin female and 1 virgin male were moved into a 7 ml mating vial (filled with 2.4 g of fodder and topped with an even layer of oats) for 48 h at 30 ± 1 °C. Pooled single-sex groups of mated males or mated females were then subject to either heatwave conditions or control conditions as described above. After this, all individuals underwent a 24 h rest period at 30 ± 1 °C and were then visually assessed for survival.

5.3.6 Virgin reproductive output treatment groups

48 h after eclosion, single-sex groups of virgin males or virgin females were subject to either heatwave conditions or control conditions as described above. After this, all individuals underwent a 24 h rest period at 30 ± 1 °C and were then visually assessed for survival. All survivors were then moved into a 7 ml mating vial (filled with 2.4 g of fodder and topped with an even layer of oats) with an age-matched member of the opposite sex from a KSS stock population for 48 h at 30 ± 1 °C. After the mating period, females were removed and placed into a reproductive output assay. These mating vials were retained to ensure successful ejaculate transfer, assessed by the visual presence of larval tracks/offspring.

5.3.7 Mated reproductive output treatment groups

48 h after eclosion, virgin females were moved into a 7 ml mating vial (filled with 2.4 g of fodder and topped with an even layer of oats) with an age-matched member of the opposite sex from a KSS stock for 48 h at 30 ± 1 °C. After this, individual males and females were placed into a 6cm Petri dish (filled with 3g of fodder and a layer of oats) and were subject to either heatwave conditions or control conditions as described above. After this, all individuals underwent a 24 h rest period at 30 ± 1 °C. Individuals were then visually assessed for survival at this time, and surviving females were placed into a reproductive output assay. Males were moved into a new 7ml mating vial (filled with 2.4 g of fodder and topped with an even layer of oats) with an age-matched member of the opposite sex from a KSS stock for 48 h at 30 ± 1 °C. Females from these mating pairs were placed into a reproductive output assay.

5.3.8 Statistical analysis

All data were analysed using R version 4.4.1 (R Core team, 2024) in RStudio Version 2024.04.2+764 (Posit team, 2024). Plots were created using “ggplot2” (Wickham, 2016). Data handling was done using “tidyverse” (Wickham et al., 2019). Survival was analysed separately for each heatwave exposure duration, with survival status as the binomial response variable. Heatwave temperature treatment, focal group and their interaction were included as fixed effects. Models were initially fitted as GLMMs with a binomial error structure and logit link function, with pot number included as a random effect. However, for 2 hours, its explanatory power of variance was estimated to be 0 (compared to 0.37% for 5 hours and 0.11% for 10 hours); therefore, a binomial GLM with a logit link function was used instead for this time period. For the 5 h and 10 h exposure durations, GLMMs with pot number as a random effect were retained. Post-hoc Tukey tests were used to compare the focal groups within each heatwave treatment temperature and to compare survival across temperature treatments within each focal group.

Reproductive output data were first censored where data were missing or individuals escaped from or died during the reproductive output assay (N = 10). Virgin controls and mated controls were compared using a non-parametric Wilcoxon test, which showed significant differences between their reproductive outputs. Therefore, virgin treatment groups were compared to virgin controls, and mated treatment groups were compared to mated controls. Zero-inflated negative binomial GLMs (Zeileis, Kleiber and Jackman, 2008) were used to analyse all data after running and comparing Poisson, negative binomial, zero-inflated Poisson, and zero-inflated

negative binomial models for over- or under-dispersion and comparing goodness of fit using “performance” and “lmtest” (Zeileis and Hothorn, 2002). Models were fitted with 20-day reproductive output as the response variable and treatment group as the predictor variable. For each model, treatment effects were assessed in both the count component, representing differences in reproductive output, and the zero-inflation component, representing differences in the probability of excess zeros.

5.4 Results

5.4.1 Survival

Broadly, increasing heatwave duration and intensity resulted in reduced survival in all groups, with the worst survival being seen after a 10-hour heatwave at 50°C (Fig. 5.1). Whilst the only focal group to show survival reductions after a 2-hour heatwave were mated females subject to 50°C, by a 10-hour exposure, all focal groups showed reductions in survival at both 48°C and 50°C. Where survival reductions were seen, mated females consistently showed a more severe reaction than other focal groups. Notably, only mated females exhibited a reduction in survival in response to a 10 h, 46°C heatwave.

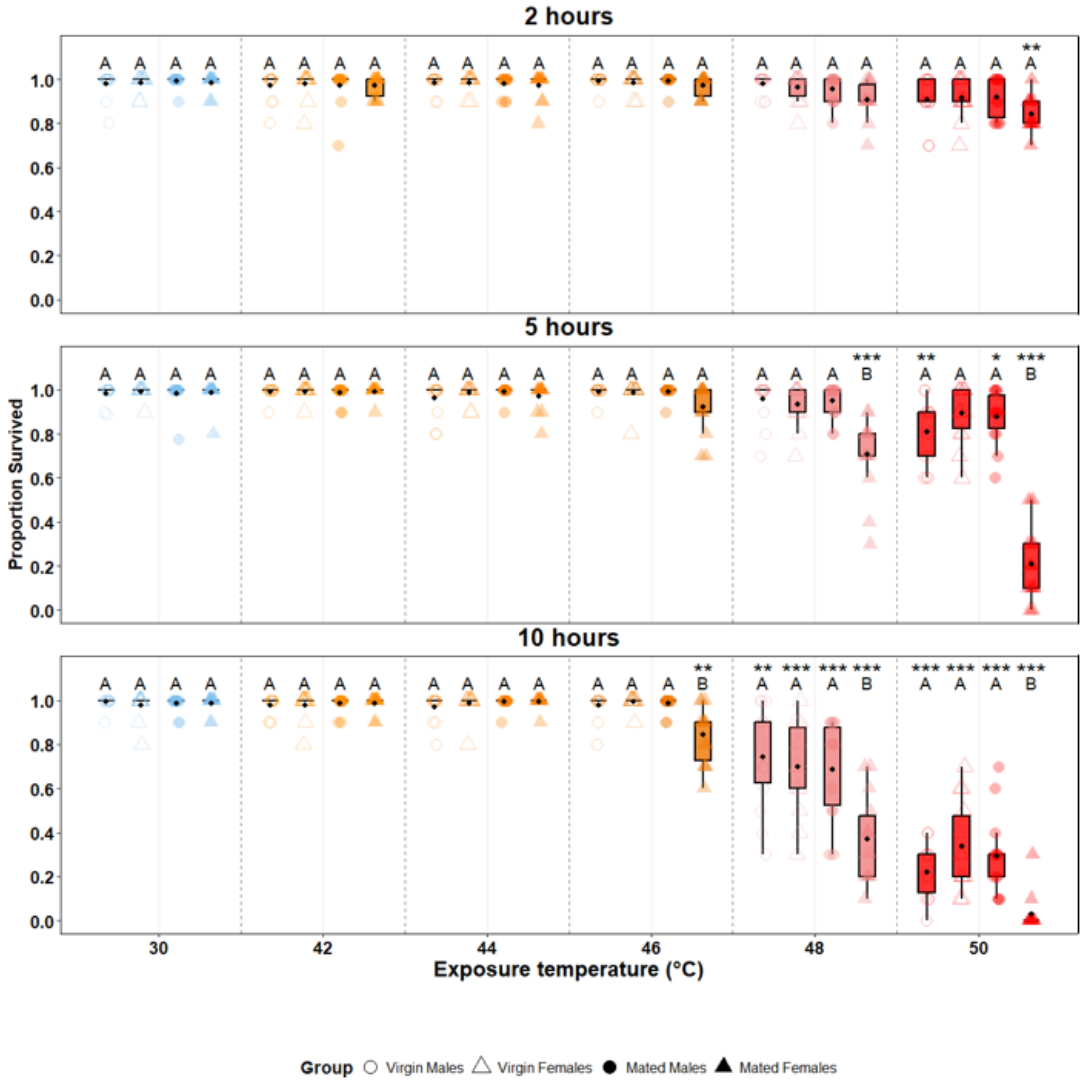

*Figure 5.1: Impact of varying heatwave conditions on survival of virgin males, virgin females, mated males and mated females. Pot level survival (representing between 8 and 10 individuals) is plotted as jittered shapes, corresponding to different focal groups. An open circle represents virgin males, an open triangle represents virgin females, a filled circle represents mated males, and a filled triangle represents mated females. Boxplots contain a median line, a mean dot, and an interquartile range box, where appropriate. Significance values represent a comparison between control conditions and the respective experimental focal group and are denoted by asterisks: * = p <0.05, ** = p <0.01, *** = p <0.001. Letters denote comparisons between focal groups within the same duration/temperature experimental group. Sample sizes for all groups are 14 pots, with 10 individuals in each pot, except for the following groups; Virgin males at 30°C for 2 hours (12 pots of 10 individuals, one pot of 9 individuals and one pot of 8 individuals); Virgin males at 30°C for 5 hours (12 pots of 10 individuals, one pot of 9 individuals and one pot of 8 individuals); Virgin males at 30°C for 10 hours (13 pots of 10 individuals, one pot of 9 individuals); Mated males at 30°C for 2 hours (12 pots of 10 individuals, two pots of 8 individuals); Mated males at 30°C for 5 hours (12 pots of 10 individuals, one pot of 9 individuals and one pot of 8 individuals); Mated males at 30°C for 10 hours (13 pots of 10 individuals, one pot of 8 individuals) and mated females at 30°C for 2 hours (13 pots of 10 individuals, one pot of 8 individuals).*

5.4.1.1 Impact of a 2 hour heatwave exposure

The overall Generalized Linear Model revealed a statistically significant main effect of temperature at 50°C (GLM: β = -1.520, SE = 0.652, z = -2.330, p = 0.0198); however, all other main and interaction effects were non-significant.

Compared to the control conditions of 30°C, being subjected to the most intense heatwave conditions tested here (50°C) resulted in significantly reduced survival for mated females, whereby survival dropped from 98.6 ± 3.6% SD to 84.3 ± 7.6% SD (Tukey: OR = 12.68, p < 0.001). No other focal groups showed a significant reduction in survival, and post-hoc comparisons between focal groups for each temperature were non-significant.

5.4.1.2 Impact of a 5 hour heatwave exposure

The overall Generalized Linear Mixed Model revealed a statistically significant main effect of temperature at 50°C (GLMM: β = −2.782, SE = 0.744, z = −3.740, p < 0.001), showing a dramatic reduction in the log-odds of survival at 50°C compared to controls. The only significant interaction effects were seen between temperature and focal group, specifically with mated females at the highest tested temperatures (GLMM: 48°C; β = −2.250, SE = 1.106, z = −2.035, p = 0.042, 50°C; β = −2.804, SE = 1.051, z = −2.668, p = 0.008).

Compared to their respective control group at 30°C, being subjected to the most intense heatwave conditions for 5 hours resulted in significantly reduced survival for all groups, except for virgin females, who showed only a trend towards significance when comparing 50°C survival to controls (Tukey: OR = 16.7, p = 0.074). In comparison, virgin male survival reduced from 98.5 ± 3.8% SD at 30°C to 80.7 ± 14.4% SD (Tukey: OR = 16.15, p = 0.002) at 30°C, mated female survival reduced from 98.6 ± 5.4% SD to 20.7 ± 15.9% SD (Tukey: OR = 266.62, p < 0.001) and mated male survival reduced from 98.4 ± 5.9% to 87.9% ± 11.9% SD (Tukey: OR = 9.33, p = 0.038). Mated females were the only group to show a significant reduction in survival at 48°C compared to controls, with survival decreasing from 98.6 ± 5.4% SD to 70.7 ± 17.3% SD (Tukey: OR = 28.67, p < 0.001).

Whilst not reaching a statistical cutoff of 0.05 during post-hoc Tukey testing, mated females subject to 46°C for 5 hours trended towards lower survival rates than the other three focal groups with a mean pot survival of 92.1 ± 11.2% SD compared to 99.3 ± 2.7% SD, 98.6 ± 5.4% SD and 99.3 ± 2.7% SD in virgin males (Tukey: OR = 11.86, p = 0.087), virgin females (Tukey: OR = 5.89, p = 0.103) and mated males

(Tukey: OR = 11.86, p = 0.087) respectively. No other focal groups showed statistical differences in survival.

This trend was made evident at 48°C, whereby mated females showed dramatically lower survival rates than the other three focal groups (mean pot survival of 70.7 + 17.3% SD compared to 95.7 ± 9.4% SD, 93.6% ± 9.3% SD and 95.0% ± 7.6% SD in virgin males (Tukey: OR = 9.28, p < 0.001), virgin females (Tukey: OR = 6.05, p < 0.001) and mated males (Tukey: OR = 7.89, p < 0.001) respectively. No other focal groups showed statistical differences in survival.

The most pronounced difference in survival between mated females and the other focal groups was observed at 50°C. Here, a large majority of mated females died (mean pot survival of 20.7 ± 15.9% SD) which compared starkly to all three other focal groups, whereby 80.7 ± 14.4% SD, 89.3 ± 12.7% SD and 87.9 ± 11.9% SD survived in the virgin male (Tukey: OR = 16.14, p < 0.001), virgin female (Tukey: OR = 32.16, p < 0.001) and mated male Tukey: (OR = 27.92, p < 0.001) groups respectively. No other focal groups showed statistical differences in survival.

5.4.1.3 Impact of a 10 hour heatwave exposure

The overall Generalized Linear Mixed Model revealed highly statistically significant main effects of temperature at 48°C (GLMM: β = −3.867, SE = 1.023, z = −3.782, p < 0.001) and 50°C (GLMM: β = −6.188, SE = 1.025, z = −6.039, p < 0.001), showing a dramatic reduction in the log-odds of survival at these temperatures compared to controls. All other main and interaction effects were non-significant.

Under control conditions of 30°C, survival was very high for all groups, with mean pot survival of 99.3 ± 2.7% SD for virgin males, 97.9 ± 5.8% SD for virgin females, 98.6 ± 3.6 % SD for mated females and 98.6 ± 3.6% SD for mated males. Compared to controls, being subjected to 46°C conditions for 10 hours resulted in a reduction in the survival of mated females to 84.3 ± 12.8% SD (Tukey: OR = 12.87, p = 0.009). Being subjected to 48°C for 10 hours resulted in significant reductions in survival for all groups when compared to controls, with virgin male survival being reduced to 74.3 ± 22.8% SD (Tukey: OR = 47.81, p = 0.002), virgin females to 70.0 ± 20.4% SD (Tukey: OR = 19.59, p < 0.001), mated females to 37.1 ± 19.4% SD (Tukey: OR = 117.05, p < 0.001), and mated males reduced to 68.6 ± 21.8% SD (OR = 31.20, p < 0.001). Being subjected to the most extreme conditions of 50°C for 10 hours produced the most severe reductions in survival compared to controls, with virgin male survival being reduced to 22.1 ± 11.9% SD (Tukey: OR = 486.63, p < 0.001), virgin females reduced to 33.6 ± 19.5% SD (Tukey: OR = 90.58, p <

0.001), mated females reduced to 2.9 ± 8.3% SD (Tukey: OR = 2354.48, p < 0.001), and mated males reduced to 29.3 ± 17.7% SD (Tukey: OR = 164.63, p < 0.001).

At 46°C, mated females showed significantly lower survival rates than the other three focal groups with a mean pot survival of 84.3 ± 12.8% SD compared to 97.9 ± 5.8% SD, 99.3% ± 2.7% SD and 96.8% ± 3.6% SD in virgin males (Tukey: OR = 8.52, p = 0.004), virgin females (Tukey: OR = 25.93, p = 0.009) and mated males (Tukey: OR = 12.87, p = 0.004) respectively. No other focal groups showed statistical differences in survival.

At 48°C, mated females continued to exhibit dramatically lower survival rates than the other three focal groups, with a mean pot survival of 37.1 ± 19.4% SD compared to 74.3 ± 22.8% SD, 70.0 ± 20.4% SD and 68.6 ± 21.8% SD in virgin males (Tukey: OR = 4.90, p < 0.001), virgin females (Tukey: OR = 3.95, p < 0.001) and mated males (Tukey: OR = 3.70, p < 0.001) respectively. No other focal groups showed statistical differences in survival.

At 50°C, the most extreme temperature/duration combination tested, mated females still showed lower survival rates than the other three focal groups, with a mean pot survival of just 2.9 ± 8.3% SD compared to 22.1 ± 11.9% SD, 33.6 ± 19.5% SD and 29.3 ± 17.7% SD in virgin males (Tukey: OR = 9.68, p < 0.001), virgin females (Tukey: OR = 17.20, p < 0.001) and mated males (Tukey: OR = 14.10, p < 0.001) respectively. No other focal groups showed statistical differences in survival.

5.4.2 Reproductive output

After exposure to a 48°C heatwave for 5 hours, virgin males, alongside mated males and mated females, all showed reductions in reproductive output compared to controls (Fig. 5.2). Both control groups showed high reproductive outputs (203.0 ± 71.9 SD and 232.0 ± 96.0 SD for virgin and mated controls, respectively). Apart from virgin females, which showed no significant drop in reproductive output (ZINB GLM; count component: z = -0.245, p = 0.807; zero-inflation component: z = 0.007, p = 0.995), all other groups saw dramatic reductions compared to controls. Virgin males saw a 39.6% drop (ZINB GLM; count component: z = -2.51, p = 0.012; zero-inflation component: z = 0.007, p = 0.994), whilst mated males and females showed a 43.6% (ZINB GLM; count component: z = -3.59, p < 0.001; zero-inflation component: z = 1.332, p = 0.183) and 42.1% (ZINB GLM; count component: z = -3.23, p = 0.0013; zero-inflation component: z = 1.259, p = 0.208) drop, respectively.

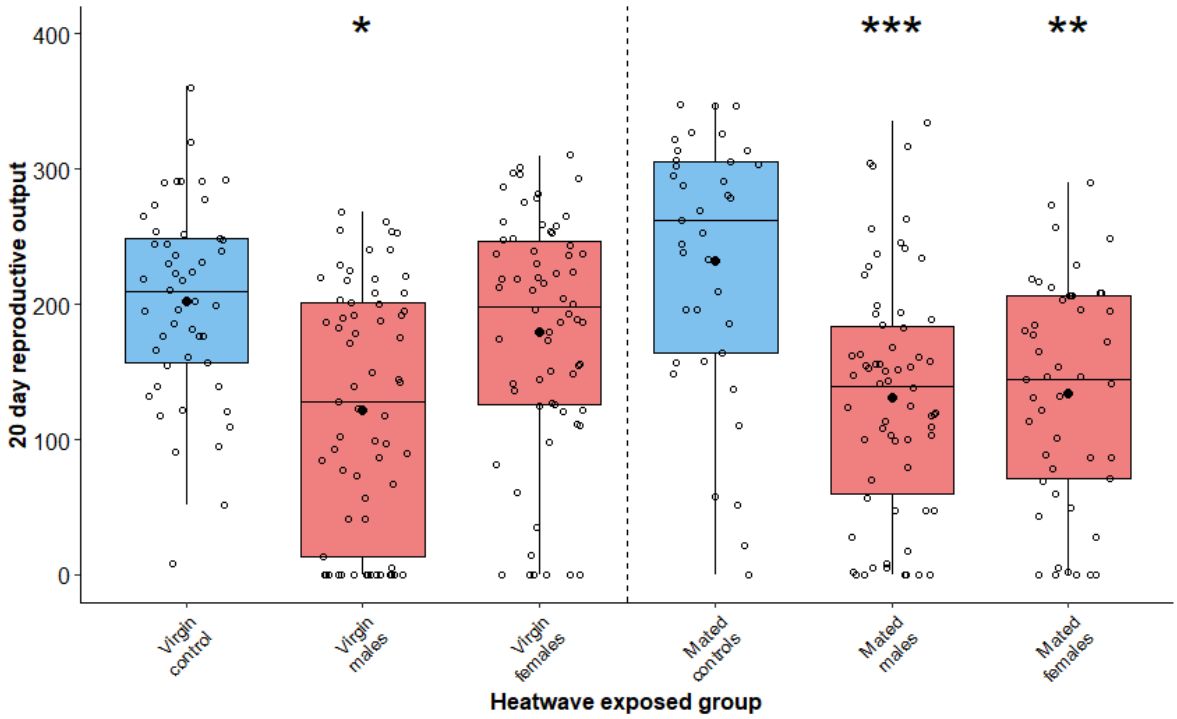

*Figure 5.2: Impact of a 48°C heatwave for 5 h on reproductive output of virgin males, virgin females, mated males and mated females. Raw data points are plotted as open jittered circles. Boxplots contain a median line, a mean dot, and an interquartile range box. Significance values representing a comparison between the respective control group and the experimental heatwave condition are denoted by stars: * = p <0.05, ** = p <0.01, *** = p <0.001. Sample sizes from left to right for each experimental group are: 49, 65, 66, 37, 66 and 49.*

5.5 Discussion

In *T. castaneum*, survival was broadly resilient across all groups across 2-h heatwaves of all intensities, with sex- and mating status-specific sensitivities becoming more pronounced at 5- and 10-h heatwaves. Interestingly, only mated females subjected to 50°C for 2 hours showed a drop in survival compared to controls, with a marked decrease of ~14% after such a short exposure, highlighting the sensitivity of this group to even very short periods of extreme heat.

In response to longer heatwave exposures, not only did mated females show significant survival reductions in response to less intense heatwaves than other groups (48°C at 5-h compared to 50°C for virgin males and mated males, and 46°C

at 10-h compared to 48°C for all other focal groups), but mated females consistently showed significantly worse survival than all other focal groups in response to the same heatwave conditions. Some of the most extreme cases of this can be observed after a 5 h, 50°C heatwave, where only ~21% of mated females survived, compared to all other groups, which displayed survival rates of over 80%. Likewise, after a 10-h, 50°C heatwave, only ~3% of mated females survived, compared to other focal groups, which all showed survival rates between ~22% and ~34%. These results indicate that mated females represent the focal group that displays the greatest sensitivity to heatwaves in terms of survival. This is greatly concerning given that females of this, and many other species, will spend the majority of their lives as mated individuals (Pai and Yan, 2003).

Potentially extreme trade-offs between survival and reproduction are highlighted by the stark differences in survival between virgin females and mated females across all temperatures which elicited a significant reduction in survival. The pronounced sensitivity of mated females suggests that the physiological requirements of reproduction impose a cost which appears to compromise their ability to resist thermal stress associated with heatwaves. Reproductive activity alone can incur energetic costs, as cellular resources are diverted from somatic cell maintenance and repair to focus on reproductive efforts (Harshman and Zera, 2007). Furthermore, evidence of trade-offs between reproduction and thermal acclimation in insects is being uncovered (Wang et al., 2024), and inhibition of reproductive genes in response to heat stress has been demonstrated (Li et al., 2024). Whilst the exact mechanisms underpinning the sensitivity of mated females shown here are not investigated in this Chapter, it appears to reflect a classic life-history trade-off but in the context of heatwaves. The molecular genetic and physiological mechanisms underpinning this response are further investigated in Chapters 6 and 7.

Preliminary assessments of future reproductive output losses showed that virgin males, mated males and mated females all showed relatively similar responses to heatwaves. This may be unsurprising, given that previous research on this species has largely highlighted sperm damage, both within males and stored in females, as the primary mechanism by which reproductive output losses are induced in this species (Sales et al., 2018). Whilst the underlying mechanisms behind reproductive output losses are not tested in this Chapter, the resilience of virgin females alone to reproductive output losses seen here aligns strongly with previous studies. While this preliminary data, examining the impacts of 48°C, 5 h

heatwaves on reproductive output, generally agrees with previous studies, it is essential to consider that different temperature/duration combinations may elicit distinct physiological responses in each focal group. A comprehensive assessment of such conditions would be insightful in future work. I further assess the reproductive response of mated females to heatwave conditions of 42°C to 50°C for 2-, 5- and 10-h in Chapter 6 to understand better this key focal group's response to such conditions, allowing for a qualitative comparison to previous work, such as the assessment of reproductive responses shown by virgin males in Chapter 4.

Considering that the vast majority of female insects possess a sperm storage organ (Pascini and Martins, 2017), and the increasing occurrence of heatwaves (Trenberth, 2018), these results showing that reproductive output can be reduced when both males or mated females are subject to heatwaves may be particularly concerning. Adverse reproductive effects may be compounded should a male be subject to a heatwave before mating with a female, who is then subject to a heatwave herself. Further research assessing the impacts of such exposure scenarios will be insightful.

When viewed holistically, I suggest that mated females represent a currently under-recognised yet key sensitivity within insects to heatwaves. Given that mated females exhibited qualitatively similar reproductive losses to virgin and mated males, yet consistently showed reduced survival at lower temperatures than all other focal groups, and greater survival losses in response to the same temperatures, this group may likely play a key role in population-level responses to heatwaves within this species.

Broadly speaking, where overlap exists, the results presented in this Chapter align well with data collected in previous Chapters, indicating replicability of results. This is positive in light of the recently highlighted 'replication crisis' in science, where many studies have shown difficulty in reproducing results across studies. For example, all survival data for 42°C across 2-h, 5-h and 10-h in Chapter 3 showed no significant reductions when compared to controls, which was replicated here. Survival trends shown in Chapter 4 for virgin males match well with the data collected here, with only a couple of minor exceptions; in this Chapter, virgin males being subject to 50°C for 5 h saw a significant, ~17% reduction, whereas in Chapter 4, a non-significant ~12% reduction was seen. However, the results were trending towards significance ($p = 0.06$). One notable exception was observed at 50°C for 10-h, where Chapter 4 reported 100% mortality among virgin males, compared to ~22% under the same conditions seen here in Chapter 5. The reason for this

difference is unclear but may reflect stochastic variation in heating equipment, and /or biological variation. The broad replicability of the results presented in this thesis is particularly interesting, given that different incubators were used (A.B. Newlife 75 Mk4 incubators in Chapter 4, Brinsea Octagon 20 Incubators here). Comparison of results to other commonly used heatwave applying mechanisms (e.g. Water baths (as in Weaving, Terblanche and English (2024)), temperature controlled rooms (as in Deschamps et al. (2025) and use of heat mats (as in Ratz et al. (2024)), would be insightful for further understanding the comparability between different studies.

In summary, this Chapter provides important insights into the survival implications of heatwave exposure in *T. castaneum*. Specifically, this work elucidates the sensitivity of mated females, a key yet under-considered insect vulnerability to heatwaves. It is demonstrated that mated females exhibit survival reductions under lower temperatures than virgin males, virgin females, and mated males, as well as showing greater survival reductions under identical heatwave conditions compared to these groups. This Chapter also provides a brief comparison of reproductive output between these focal groups under ecologically relevant heatwave conditions, showing that whilst virgin females are resilient to reproductive losses after being subject to 48°C for 5 hours, mated females, mated males, and virgin males are all equally susceptible. This work highlights that mated females display a key sensitivity to heatwaves, opening up crucial areas for future work in this thesis. An assessment of the implications of a wider array of heatwaves on mated females is required, alongside an investigation into the physiological basis behind this sensitivity.

6. Ejaculate receipt reduces mated female survival under ecologically relevant heatwave exposures in *Tribolium castaneum*

6.1 Abstract

Mated females are central to population persistence, yet their vulnerability to heatwaves remains poorly understood when compared to the more established sensitivities shown in male reproduction. Here, I assess mated female responses to ecologically relevant heatwaves of 42–50°C for 2, 5 or 10 h, measuring both survival and reproductive output. Then, I assess whether heatwave-associated mortality was linked to ejaculate receipt by experimentally preventing insemination while retaining exposure to males and attempted mating. I show that mated females were highly vulnerable to heatwave exposure, with survival dropping sharply after 5 h and 10 h exposures at temperatures of 46°C and above. After 10 h of exposure, survival was reduced to 47.1% at 46°C, 41.4% at 48°C and 1.5% at 50°C, compared to 98% in controls. Reproductive output was also reduced after the most intense heatwave conditions, including after a 2 h exposure at 50°C, a 5 h exposure at 50°C, and 10 h exposures at 48°C and 50°C. Importantly, females prevented from receiving an ejaculate survived a 48°C, 5 h heatwave substantially better than unsealed females, with survival of 83.8% compared to 38.5%, while sealing had no survival effect under control conditions. These findings show that ejaculate receipt, rather than harassment or attempted mating alone, contributes to mated female heatwave vulnerability.

6.2 Contributors

Ramakrishnan Vasudeva and Natasha Brown assisted with sexing experimental individuals. Natasha Brown assisted with ‘sealing’ individuals with super glue.

6.3 Introduction

Under anthropogenic climate change, short-duration heatwaves are becoming more common, and their impacts on the environment are intensifying (Trenberth, 2018). The severity of such extreme climatic events is increasing more rapidly than mean temperatures (Seneviratne et al., 2021). Therefore, the impacts of short-duration heatwaves on biological systems are likely to be dramatic in the future, with

implications for both natural and anthropogenic ecosystems; yet research on this topic remains limited (Perkins-Kirkpatrick and Lewis, 2020; Dougherty et al., 2024).

Ectothermic taxa such as the insects, may be particularly susceptible, as their limited capacity to buffer against extreme heat through physiological mechanisms has been linked to elevated mortality (González-Tokman et al., 2020). Exposure to a wide array of heatwave exposures not only leads to survival costs but also to sub-lethal impacts on various aspects of physiology, affecting key life-history traits such as reproduction (Walsh et al., 2019). The implications of such sub-lethal exposures can have a wide-reaching effect on populations, as sterilisation can alter reproductively active sex ratios and reduce effective population sizes (Bressac et al., 2023).

Within insects, reproduction is often sensitive at sub-lethal limits, and heat stress has been extensively shown to reduce fertility in this taxonomic group (Iossa, 2019; Walsh et al., 2019). Research assessing the impacts of heatwaves on insects has placed a heavy emphasis on male insects as a key reproductively sensitive group with many studies showing severe reductions in sperm production or viability as a key driver for reduced insect fertility in response to heat (Vasudeva, Deeming and Eady, 2014; Uy et al., 2015; Porcelli et al., 2016, Sales et al., 2018; Canal Domenech and Fricke, 2023; Sales et al., 2024). In contrast, females – and oogenesis – have broadly been identified as comparatively resilient to heat (Sales et al., 2018; Meena et al., 2024). Whilst the mechanisms behind this suggested resilience are still unclear (Iossa, 2019), it may be attributed to a comparative robustness of eggs, potentially linked to unequal investment in gametes between sexes.

While an emerging trend of male sensitivity is evident, a large proportion of research assessing sex-specific sensitivities to heat in insects has focused on Drosophila, suggesting that the potential for variability in sex-specific vulnerabilities across taxa need to be considered. Likewise, much of the work assessing sex-specific reproductive susceptibility to heatwaves focuses on subjecting females to heat stress as virgins and then measuring their subsequent reproduction. Yet, females of many insect species will only remain virgins for a small fraction of their reproductively active life (Pai and Yan, 2003), and individuals would be expected to be mated at the onset of heatwave exposures they may experience, making mated females a particularly important group to study.

Mating can carry substantial costs for females through several mechanisms. Repeated courtship, male exposure and harassment can impose energetic and behavioural costs, as females may spend more time avoiding males, resisting mating attempts or recovering from copulation, reducing the time and energy available for feeding, oviposition and other fitness-related behaviours (Rowe et al., 1994; Arnqvist and Nilsson, 2000). In some species, mating attempts and copulation can also cause direct physical damage, meaning that the costs associated with male harassment and mating can be substantial and may exceed the costs of mating itself (Rowe et al., 1994). Whilst mating initially increases a female's lifetime fitness, optimal mating rates exist, and when exceeded, result in a reduction in a female's lifetime fitness (Arnqvist and Nilsson, 2000). Along with the direct costs of mating above, this reduction may be partly driven by indirect costs whereby mating can increase fatigue, reduce feeding opportunities, and time available for oviposition or other fitness-related behaviours, which may decrease lifetime fitness (Arnqvist and Nilsson, 2000). Furthermore, both the receipt of ejaculate components and storage of received sperm are known to induce a wide range of physiological changes in females post-mating, which are often dramatic and alter many life-history traits, including stress responses, immune function and reproductive behaviour (Baer, Sophie and Boomsma, 2006; Nanfack-Minkeu and Sirot, 2022). Such impacts may have negative consequences for female survival and reproductive investment. The degree to which receipt of an ejaculate may modulate females' susceptibility to heatwaves is largely unclear, representing a critical knowledge gap that must be filled to better understand the implications of heatwaves for insect persistence under climate change.

In Chapter 5, I demonstrated that within *Tribolium castaneum*, mated females exhibited the greatest mortality under a wide range of short heatwave exposures (42-50°C for 2, 5, or 10 h) when compared to other groups (virgin males, virgin females and mated males). Mated females also exhibited significant survival drops in response to less intense heatwaves than these other groups. Following this, I demonstrated that under a specific exposure (48 °C for 5 h), virgin female reproductive output was unaffected, whereas mated females exhibited reductions comparable to those observed in virgin and mated males. When the survival and reproductive data are taken together, they broadly conflict with the emerging paradigm of female resilience previously discussed, and even contrasts with previous experiments on this species assessing chronic heat stress whereby continuous exposure to 42°C for 5 d resulted in a ~50% reduction in reproductive

output for virgin males, but only a ~33% reduction for mated females (Sales et al., 2018).

Here, I aim to replicate my assessment of survival of mated females under a wide array of short heatwave exposures (42-50°C for 2, 5 or 10 h), before also assessing the reproductive implications of these heatwave exposures as well, allowing for a qualitative comparison to the virgin male results presented in Chapter 4. I then investigate the mechanisms behind survival sensitivity of mated female to heatwaves by controlling ejaculate receipt through selective sealing of the female genital opening. This approach distinguishes the effects of mating-induced physiology and offspring production from those of male harassment and attempted copulation. Using a split design that exposes females to harassment/attempted copulation while either permitting or preventing ejaculate transfer, I disentangle the processes driving the observed survival sensitivity.

In this Chapter, I ask whether the mated female vulnerability identified in Chapter 5 is repeatable and whether this vulnerability is linked to ejaculate receipt. I predict that mated female survival and reproductive output will decline under longer duration and more intense heatwave exposures. I also predict that individuals who are prevented from receiving an ejaculate will show higher survival than normally mated females under identical heatwave exposures.

6.4 Materials and methods

6.4.1 Line maintenance and experimental individuals

T. castaneum used in these experiments were from the Krakow Super Strain (KSS) outbred stock line, which was maintained as described in Chapter 2. Experimental individuals were sexed at the pupal stage by visual identification of sexually dimorphic genital papillae (on day 18 of development) and then kept in single-sex groups of 20 individuals for a further 10 days to allow for development to sexual maturity. Groups were kept in 6cm Petri dishes filled with 3 grams (g) of fodder and topped with an even layer of oats. Once matured, females were identified with a dot of Uni Posca non-toxic marker (Uni-ball, Tokyo, Japan) on the dorsal thorax.

6.4.2 Experimental heatwave treatments

Heatwave conditions were applied using an Octagon 20 Eco incubator (Brinsea Incubation Specialists, Somerset, UK). For both experiments, female beetles were exposed as individuals in 6cm Petri dishes filled with 3g of ambient temperature fodder and topped with a layer of oats.

To assess the reproductive output of mated females, beetles were exposed to heatwave conditions (42°C, 44°C, 46°C, 48°C or 50 °C ± 1°C, ± 10 % RH) for one of the following durations: 2 h, 5 h or 10 h, with controls (30 ± 1°C, 60 ± 10 % RH) run in parallel.

To assess the impact of 'sealing' the female genital opening to prevent receipt of an ejaculate on survival, beetles were exposed to heatwave conditions of 48°C ± 1°C and 60 ± 10% RH for 5 h, with controls (30 ± 1°C, 60 ± 10% RH) run in parallel.

6.4.3 Survival and reproductive output assay

48 h after eclosion, virgin females were moved into a 7 ml mating vial (filled with 2.4 g of fodder and topped with an even layer of oats) with an age-matched member of the opposite sex from a KSS stock for 48 h at 30 ± 1 °C. After this, individual females were placed into a 6cm Petri dish (filled with 3g of fodder and a layer of oats) and were subject to either heatwave conditions or control conditions as described above. After this, all individuals underwent a 24 h rest period at 30 ± 1 °C. Individuals were then visually assessed for survival at this time, and surviving females were placed into a reproductive output assay. They were moved into individual 6cm Petri dishes (filled with 3g of fodder and topped with an even layer of oats) and allowed to oviposit. Oviposition occurred across two separate ten-day blocks (twenty days in total) to reduce cannibalism associated with overlapping offspring developmental life stages (Park et al., 1965). After twenty days, the females were removed, and the oviposited eggs were allowed to develop until maturity for an additional 35 days under standard developmental conditions (30 ± 1°C, 60 ± 10 % RH). The reproductive output of each pair was then assessed as the number of mature adults produced from twenty days of oviposition (equating to ~ 51% of a female's lifetime reproductive output; pg. 31, Dickinson, 2018).

6.4.4 Female ejaculate receipt experiment

To assess whether receipt of an ejaculate influences female beetles' susceptibility to heatwave-induced mortality, a fully factorial experiment was conducted in which all females were presented with a mating opportunity with an age-matched male. A subset of these females had their genital opening sealed by the application of non-toxic super glue to prevent ejaculate transfer. In contrast, the remaining individuals had an identically sized dot of superglue placed on their thorax to control for any potential effects of the glue itself (e.g. via olfactory cues).

48 h after eclosion, virgin females either had superglue placed over their genital opening or on their thorax (as above). After the glue had dried, a visual assessment was made to ensure there were no impacts on mobility. Females were moved into a 7 ml mating vial (filled with 2.4 g of fodder and topped with an even layer of oats) with an age-matched member of the opposite sex from a KSS stock for 48 h at 30 ± 1 °C. After this, individual females were placed into a 6cm Petri dish (filled with 3g of fodder and a layer of oats) and were subject to either heatwave conditions or control conditions as described above. After this, all individuals underwent a 24 h rest period at 30 ± 1 °C. Individuals were then visually assessed for survival at this time. Data were censored where individuals who should have been sealed produced offspring (N = 4). Because sealed and unsealed females were both exposed to males before heatwave treatment, this design allowed survival effects associated with ejaculate receipt to be separated from effects associated with male exposure, harassment, attempted copulation or glue application.

6.4.5 Statistical analysis

All data were analysed using R version 4.4.1 (R Core team, 2024) in RStudio Version 2024.04.2+764 (Posit team, 2024). Plots were created using “ggplot2” (Wickham, 2016). Data handling was done using “tidyverse” (Wickham et al., 2019).

The impact of heatwave temperature on survival was analysed separately for each heatwave exposure duration, with survival status as the binomial response variable and heatwave temperature treatment as the predictor variable. Binomial GLMs were initially fitted for each exposure duration. Firth's penalised logistic regression from the brglm2 package was used where near-separation or complete separation issues caused model convergence issues (Kosmidis, 2023). Fit of the models were then assessed using “performance” (Lüdecke et al., 2021) and residuals were visually evaluated. Post-hoc comparisons between temperatures within the same duration were made using “emmeans” (Lenth, 2025).

Reproductive output data were first censored where data were missing, individuals escaped from or died during the reproductive output assay, or pots were too mouldy to count offspring from (N = 93). Zero-inflated negative binomial GLMs (Zeileis, Kleiber and Jackman, 2008) were used to analyse all data after running and comparing Poisson, negative binomial, zero-inflated and zero-inflated negative binomial models for over- or under-dispersion and comparing goodness of fit using “performance” and “lmtest” (Zeileis and Hothorn, 2002). Models were fitted separately for each heatwave exposure duration, with 20-day reproductive output as

the response variable and heatwave temperature treatment as the predictor variable. For each model, treatment effects were assessed in both the count component, representing differences in reproductive output, and the zero-inflation component, representing differences in the probability of excess zeros.

The impacts of altering the ability of females to receive an ejaculate on heatwave survival were assessed using a binomial GLM, with survival as the binomial response variable and experimental treatment group as the predictor variable. Post-hoc comparisons within groups were made using “emmeans” (Lenth, 2025). Fit of the model were assessed using “performance” (Lüdecke et al., 2021) and residuals were visually evaluated.

6.5 Results

6.5.1 Survival

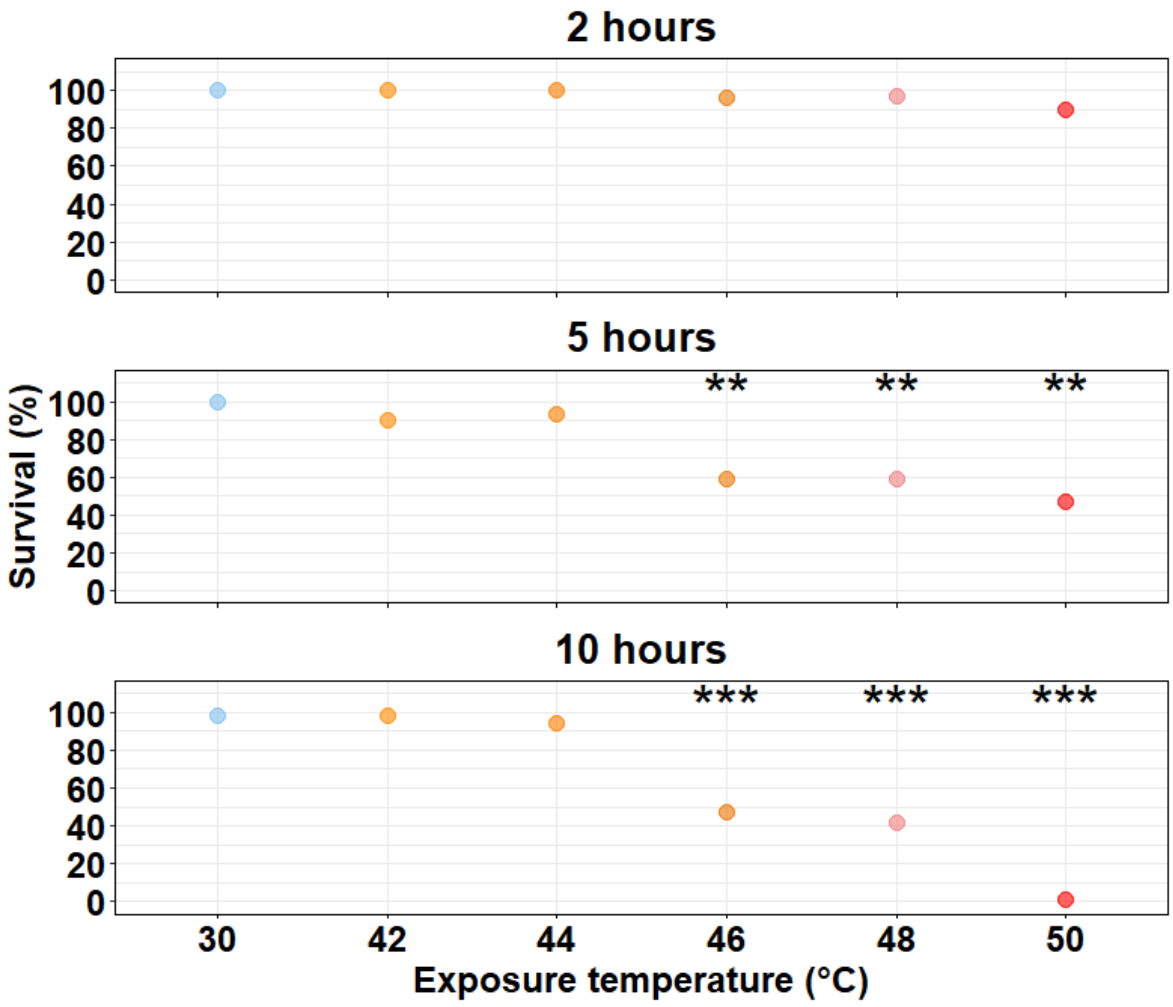

Figure 6.1: Impact of varying heatwave exposures on survival of mated females. Plotted points represent a point estimate of survival, based on groups of individual binary survival scores. The sample sizes from left to right for each exposure duration are as follows: 2 hours: 51, 50, 60, 50, 65,

*70; 5 hours: 50, 50, 59, 59, 68, 70; 10 hours: 50, 50, 50, 70, 70, 68. Significance values representing a comparison between 30°C and the experimental heatwave condition are denoted by asterisks: ** = p < 0.01, *** = p < 0.001.*

6.5.1.1 Impact of a 2 hour heatwave exposure

Survival rates for groups exposed to all temperatures for two hours were generally high, with controls as well as groups exposed to 42°C or 44°C heatwaves showing 100% survival (Fig. 6.1). A trend towards a reduction in survival was seen after a 50°C heatwave exposure (Firth's penalised regression: z = -1.681, p = 0.09), where survival was 90%. However, no significant decreases in survival were observed in any group when compared to controls.

6.5.1.2 Impact of a 5 hour heatwave exposure

Exposure to 42°C or 44°C heatwave exposures did not significantly impact survival (Fig. 6.1). This changed dramatically at more intense exposures, whereby being subject to a 46°C heatwave exposure reduced survival to 59.3% compared to 100% in the controls (Firth's penalised regression: z = -2.908, p = 0.003). Similarly, being subject to a 48°C heatwave exposure reduced survival to 58.8% (Firth's penalised regression: z = -2.928, p = 0.003), and being subject to a 50°C heatwave exposure reduced survival to 47.1% (Firth's penalised regression: z = -3.249, p = 0.0011). Survival did not differ significantly among 46°C, 48°C and 50°C (Tukey: p > 0.7 for all pairwise comparisons).

6.5.1.3 Impact of a 10 hour heatwave exposure

Exposure to 42°C or 44°C heatwave exposures did not significantly impact survival. This changed dramatically at more intense exposures, whereby being subject to a 46°C heatwave exposure reduced survival to 47.1% compared to 98% in the controls (Firth's penalised regression: z = -4.146, p < 0.001). Similarly, being subject to a 48°C heatwave exposure reduced survival to 41.4% (Firth's penalised regression: z = -4.404, p < 0.001). Being subject to a 50°C heatwave exposure reduced survival the most dramatically when compared to controls, with only one individual out of 68 surviving (Firth's penalised regression: z = -6.190, p < 0.001). Whilst survival did not differ significantly between groups subject to 46°C and 48°C exposures (Tukey: OR = 1.26, p = 0.985), a 50°C exposure produced a significant difference to both 46°C and 48°C (Tukey: 46°C; OR = 40.2, p < 0.001, 48°C; OR = 31.99, p < 0.001).

6.5.2 Reproductive output

As exposure duration and intensity increased, increasingly negative impacts on mated female reproductive output were observed. Heatwave exposure at 50°C for 2 h reduced female reproductive output by 23.7% compared to controls (ZINB GLM: count component; $z = –2.021$, $p = 0.043$; zero-inflation component: $z = 0.706$, $p = 0.480$; Fig. 6.2). For 5 h exposures, heatwave exposure at 50°C produced a significant reduction (–46.6%; ZINB GLM: count component; $z = –4.093$, $p < 0.001$; zero-inflation component: $z = 1.348$, $p = 0.178$; Fig. 6.2), whereas 42–48°C produced no significant effects. By contrast, for 10 h exposures, significant reductions in reproductive output were observed at both 48 °C (–29.2%; ZINB GLM: count component; $z = –2.557$, $p = 0.011$; zero-inflation component: $z = 0.000$, $p = 1.000$) and 50°C (–69.3%; ZINB GLM: count component; $z = –2.378$, $p = 0.017$; zero-inflation component: $z = 0.000$, $p = 1.000$; Fig. 6.2). Due to extreme numbers of deaths in the 50°C, 10 h treatment, reproductive output was only collected for one individual.

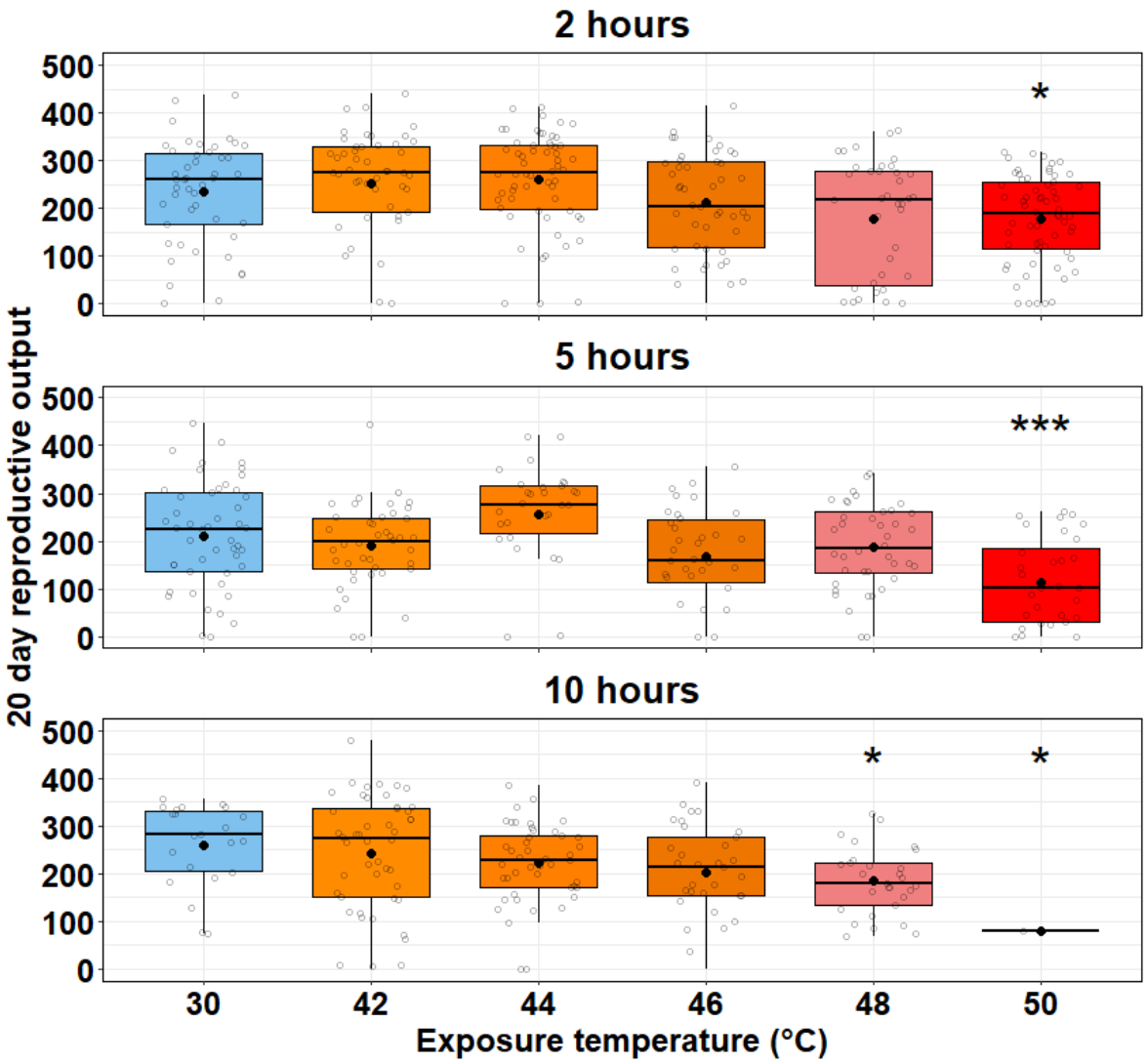

*Figure 6.2: Impact of varying heatwave exposures on reproductive output of mated females. Raw data points are plotted as open jittered circles. Boxplots contain a median line, a mean dot and an interquartile range box. The sample sizes from left to right for each exposure duration are as follows: 2 hours: 46, 49, 60, 47, 39, 62; 5 hours: 49, 42, 30, 35, 40, 32; 10 hours: 22, 48, 46, 33, 29, 1. Significance values representing a comparison between 30°C and the experimental heatwave condition are denoted by asterisks: * = $p < 0.05$, *** = $p < 0.001$.*

6.5.3 Female ejaculate receipt impact on survival

At 30°C, survival was very high in both groups of females that were sealed and those that weren't (95.1% for unsealed females vs 97.4% for sealed; Fig. 6.3). There was no statistical difference between their survival, showing that sealing had no direct impact on survival (Tukey: OR = 1.48, p = 0.96) under control conditions. When compared to these controls, subjecting unsealed females to heatwave conditions of 48°C significantly reduced their survival to 38.5% (Tukey: OR = 8.27, $p < 0.001$). In contrast, subjecting sealed females to heatwave conditions of 48°C did

not significantly reduce their survival (Tukey: 83.8%, OR = 0.14, p = 0.28). This was further demonstrated by a direct comparison at 48°C, which showed that sealed females survived significantly better than unsealed females (Tukey: OR = 8.27, p < 0.001), highlighting the protective effect of sealing.

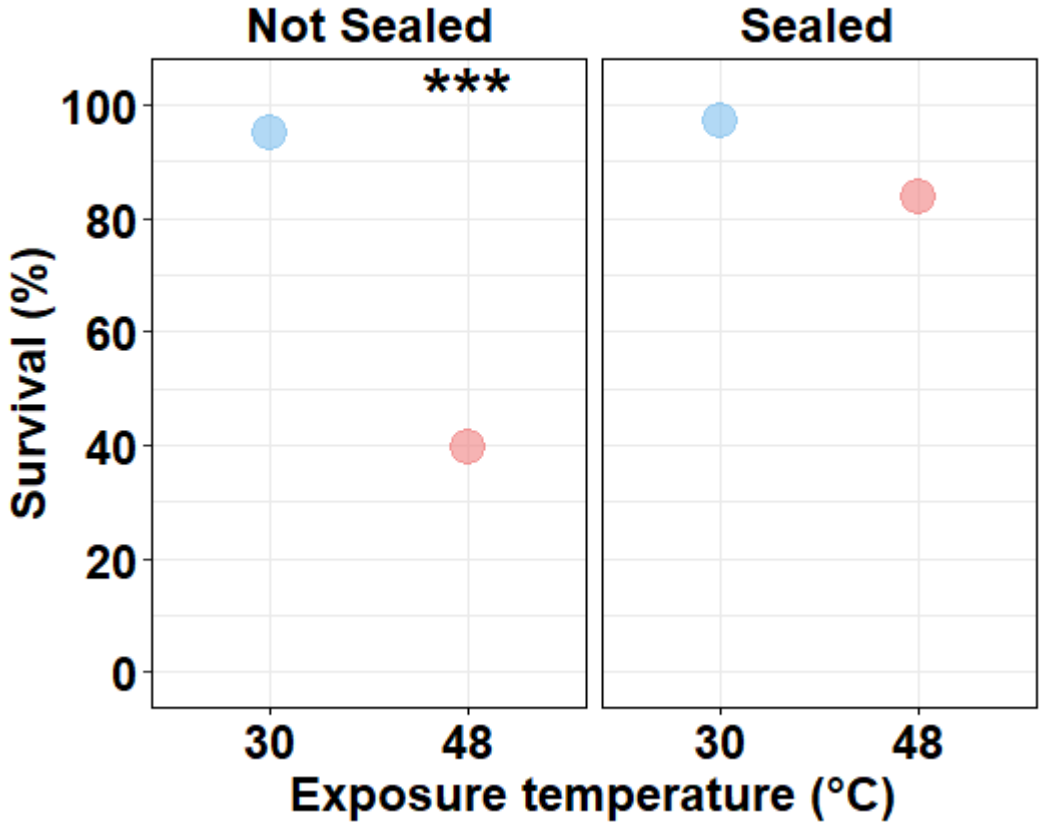

*Figure 6.3: Impact of females' ability to receive an ejaculate on their survival susceptibility to heatwaves. Plotted points represent a point estimate of survival, based on groups of individual binary survival scores. The sample sizes from left to right for each panel are as follows: Not Sealed: 40, 38; Sealed: 38, 37. Significance values representing a comparison between 30°C and the experimental heatwave condition are denoted by asterisks: *** = p < 0.001.*

6.6 Discussion

In *T. castaneum,* mated females demonstrate susceptibility to short-duration heatwave exposures, with survival dropping drastically in response to both 5-h and 10-h heatwave exposures at temperatures of ≥ 46°C. Broadly speaking, this replicates the survival results observed previously in Chapter 5. Mated females also displayed some level of sub-lethal reproductive losses, as seen after a 2-h exposure to 50°C. Interestingly, at 5-h and 10-h exposures, however, mated females showed reproductive losses at temperatures above those at which drastic survival

reductions occurred. Importantly, the survival vulnerability is closely linked to receipt of an ejaculate and associated post-mating physiological changes, rather than to stress related to male harassment and mating attempts. This provides a new insight into mechanisms underpinning sex- and mating status-specific responses to heatwaves in this species.

In the previous Chapter, I showed that mated females consistently displayed reduced survival in response to identical heatwaves when compared to other focal groups (virgin males or females and mated males). Here, I broadly replicated the responses shown by mated females to a wide range of heatwave exposures (42–50 °C for 2-h, 5-h, or 10-h), providing further evidence of the vulnerabilities of mated females in this species. Furthermore, I show that mated females display a qualitatively similar drop in reproduction in response to the most intense heatwaves to those shown in virgin males in Chapter 4. There, I showed that after exposure to a heatwave of 50°C for 2-h and 5-h, virgin males displayed a ~33% and ~50% reduction in reproductive output, respectively, compared to ~34% and ~47% in mated females shown here. Remarkably comparable decreases were also seen after an exposure of 48°C for 10 h (virgin males; ~66%, mated females; ~69%). The only exception to this was where virgin males showed a ~23% reduction in response to 48°C exposure for 5 h, whereas mated females showed no significant reduction here.

When taken together, these results show that mated females exhibit reduced survival at temperatures lower than those of other focal groups, as well as identical reproductive losses to groups such as virgin males, who have classically been assumed to be the group with the greatest sensitivity to heatwaves. These findings challenge the prevailing paradigm that female reproductive output is more resilient to heat than males (Sales et al., 2018; Meena et al., 2024).

Females whose genital opening was sealed with glue – thereby preventing ejaculate transfer – showed dramatically higher survival at 48°C than females that could receive an ejaculate. Therefore, it is the physiological costs associated with ejaculate receipt or storage of sperm that is a key driver of reduced survival in mated females, rather than being related to purely to male harassment and resulting damage and/or fatigue. Whilst a link between reproduction and survival trade-offs is generally well-known (Baer, Sophie and Boomsma, 2006; Yu et al., 2021), and some work has assessed trade-offs between responses to heat and reproduction (e.g. Wang et al., 2024, Li et al., 2024), a focus on mated females has broadly been overlooked in previous research, with little work assessing male versus female

contributions to reproductive fertility loss (Dougherty et al., 2024). Classically, where these sex-specific contributions have been evaluated, research has focused mainly on the impacts of heat applied to virgin females, who are subsequently mated and their reproduction assessed. A focus on the survival and reproductive responses of mated female insects to direct heat represents a key area for future research to understand better insect vulnerabilities to heat and their implications for population and species persistence.

Within the current literature, species vulnerability to heatwaves is often framed in the context of thermal limits, whether this be in the context of survival or reproduction. Many insect species currently exist close to their upper thermal limits, particularly in regions such as the tropics and other areas with high insect biodiversity (Deutsch et al., 2008; Novotny and Miller, 2014; Stork, 2018; Sánchez-Bayo and Wyckhuys, 2019). These results suggest that mating status may also narrow such thermal limits for females, in contrast to males, who have been the focus of much of the seminal research highlighting male fertility limits as the key driver behind persistence and distribution in studies on *Drosophila* (Parratt et al., 2021; van Heerwaarden and Sgrò, 2021).

The reproductive declines shown in mated females here, coupled with the extensive literature showing male-focused reproductive losses, suggest a potential for complementary negative impacts of heatwaves on populations. This sensitivity to heatwaves in both sexes highlights a key future research need to assess the implications of heatwaves under more ecologically representative scenarios, where both sexes are simultaneously exposed to heatwave conditions and may experience multiple heatwaves over time. On a population level, such repeated heatwave exposures may also first damage sperm in males, who then mate with females, who, in response to a subsequent heatwave, may i) suffer mating status-related heatwave mortality and ii) receive degraded quality sperm. Such compounding impacts of thermal stress may lead to greater issues for populations than has been previously assumed.

While the results here clearly show that being mated causes females to suffer a survival cost in response to heatwaves, the proximate mechanisms underpinning such losses remain unclear. This may involve a wide array of mechanisms, including shifts in resource allocation or the inactivation of stress-response pathways. In Chapter 7, I aim to address this knowledge gap by undertaking transcriptomic analysis of focal groups to examine the key pathways and mechanisms altered by mating and in response to heatwaves.

In summary, this Chapter reinforces the survival and reproductive vulnerability of mated females to heatwaves in *T. castaneum*. I demonstrate that heatwave exposures as low as 46°C for 5 h can produce drastic survival impacts, and that heatwave exposures as low as 50°C for 2 h can produce reproductive output losses, which broadly align with the trends previously shown in virgin males. Moreover, the survival sensitivity of mated females is linked specifically to ejaculate receipt rather than to harassment or attempted mating *per se*, raising critical questions about the proximate mechanisms driving these vulnerabilities.

7. Sex-, mating status- and body part-specific transcriptomic responses to ecologically relevant heatwave exposures in *Tribolium castaneum*

7.1 Abstract

Heatwave exposure induces transcriptional stress responses that can protect organisms from cellular damage, yet these responses may be altered by sex, mating status and tissue function. Previous chapters identified mated female *Tribolium castaneum* as unusually vulnerable to short, intense heatwaves, but the molecular mechanisms underlying this sensitivity remain unclear. Here, I used RNA sequencing to assess sex-, mating status- and body-part-specific transcriptional responses to a 48°C, 5 h heatwave exposure. Gene expression was analysed separately in the head, thorax and abdomen of virgin and mated males and females, using principal component analysis, differential gene expression and functional enrichment analysis. I show that virgin males and females mounted broad transcriptional responses to heatwave exposure, including enrichment of terms associated with protein folding, protein refolding, unfolded protein binding and heat shock protein binding across body parts. However, this canonical heat stress response was strongly altered by mating status. Mated males retained a restricted heat stress response, particularly in the thorax, whereas mated females showed no enrichment of these heat stress terms in any body part. These findings suggest that mating suppresses or redirects the female molecular heat stress response, providing a potential mechanism for the elevated heatwave mortality observed in mated females.

7.2 Contributors

Joshua Cole assisted with the methodology for and execution of the bioinformatic pipeline, as well as the production of volcano and PCA plots.

7.3 Introduction

Heatwaves are becoming more severe and frequent under human-driven climate change (Perkins-Kirkpatrick and Lewis, 2020). The rate of change in extreme weather events, such as heatwaves, exceeds the change in mean global temperatures, and thus potentially poses a greater threat to biodiversity (Ruthrof et al., 2018). This may be particularly true for insects, which are vulnerable to such

extreme heat due to being ectotherms and possessing only a limited ability to behaviourally thermoregulate. This couples poorly with the fact that many insects are living close to their thermal limits, particularly in areas such as the tropics, where they are highly concentrated, meaning that even short durations of extreme heat exposure may push a vast number of insect species beyond their thermal limits (Deutsch et al., 2008; Novotny and Miller, 2014).

Heat stress can induce a wide range of responses in insects, from the more striking impacts on mortality and behaviour to more subtle changes in immune responses and reproductive processes (González-Tokman et al., 2020; Ma, Ma and Pincebourde, 2020). Increasingly, sublethal impacts affecting reproduction are being recognised as key drivers of insect declines (Walsh et al., 2019); yet, mortality remains the most extreme outcome that individuals must evade by responding to extreme heat. At a physiological level, responses to heat stress can be mediated through rapid changes in gene expression (González-Tokman et al., 2020).

The importance of such physiological responses to heat stress is well understood, with extensive literature demonstrating that the induction of heat stress responses, and more specifically the upregulation in the expression of heat shock proteins (HSPs), represents an essential and highly conserved response in insects (Nguyen, Gotelli, and Helms Cahan, 2016). Experimental knockdown of a wide variety of HSPs can dramatically reduce the ability of insects to respond to heat stress, for example, RNAi suppression of HSP70 has been linked to reduced survival, longevity and reproduction in the Adonis ladybird under heat stress (Yang and Lu, 2024) and RNAi knockdown of HSP20.8 increased mortality during heat stress in the celery leafminer (Zhang et al., 2024).

Experimental work has shown that species are capable of evolving an enhanced heat shock response through increased HSP expression (e.g. Bettencourt, Feder and Cavicchi, 1999), highlighting the potential for long-term adaptation of insects under scenarios with increasingly severe and prevalent heatwaves. However, this potential assumes such adaptive processes are not overwhelmed by the transient and unpredictable nature of heatwaves in natural environments (van de Pol et al., 2017; Harvey et al., 2020).

Whilst the heat shock response may be inducible and adaptable, its expression is not solely determined by exposure to heat stress. A wide variety of additional biotic and abiotic stressors, including nutrition, immune function, and age, can also modulate the expression of heat shock response genes, including HSPs

(Sørensen, Kristensen, and Loeschcke, 2003; Banfi et al., 2025). Importantly, it is well known in insects that investment in reproduction may induce trade-offs with other physiological mechanisms, such as immunity, metabolism, and chemosensation, mediated by gene expression (Nanfack-Minkeu and Sirot, 2022). For example, in *Cephalcia chuxiongica*, RNA sequencing experiments showed that mating induces downregulation of genes associated with general stress responses and longevity (Yu et al., 2021). Such trade-offs provide a potential mechanistic explanation for why mated females may be disproportionately vulnerable to heatwaves, highlighting transcriptomics as a crucial step in identifying and understanding the underlying causes of vulnerabilities to heatwaves within insects. This understanding can help determine whether mating status alters the ability of females to mount an appropriate response to thermal stress.

Transcriptomic responses to stressors are unlikely to show uniform trends across an individual's body as different body parts contain tissues with distinct makeups, physiological roles and baseline expression levels. This is particularly relevant in *Tribolium castaneum*, where BeetleAtlas, a transcriptomics resource, has been curated and demonstrates tissue and life-stage-specific gene expression (Leader, Naseem and Halberg, 2024). In the context of Insects broadly, body parts may be expected to display different transcriptional responses based on their biological functions, with prior studies focusing on three main body parts: the head, thorax and abdomen (Chauhan et al., 2014). The head is likely to capture transcriptional changes associated with sensory perception, neural signalling, behaviour and neuroendocrine regulation, all of which may respond to either thermal stress or mating state (Dalton et al., 2010). In contrast, the thorax is dominated by locomotor musculature and may therefore provide insight into energetically demanding processes associated with movement, metabolism and physiological stress (Bretscher and O'Connor, 2020). The abdomen is particularly relevant to reproduction and resource allocation, as it contains reproductive tissues, the gut, and the fat body, thereby linking it to sperm storage, oviposition, reproductive investment, metabolism, and immune regulation (Arrese and Soulages, 2010; Nanfack-Minkeu and Sirot, 2022). Separating these body parts, therefore, allows heatwave- and mating-associated transcriptional responses to be interpreted in relation to their likely functional context, rather than treating gene expression as a single whole-body response. Some work on Insect transcriptional responses to heat alone has been undertaken. For example, short-term heat stress in adult honeybees induced transcriptomic changes associated with protein folding and unfolded protein

binding, while tissue-specific validation showed that several heat-responsive genes were highly expressed in the thorax compared with the head and abdomen (Zhang et al., 2023). Similarly, heat-shock protein expression differs among adult body parts in *Monochamus alternatus*, with Hsp70 and Hsp60 showing particularly high expression in the abdomen (Cai et al., 2017).

It is currently unclear how mating status affects transcriptional responses to short heatwave exposures in insects, with most studies focusing on the impacts on factors such as fertility or immune pathways under benign conditions. Given my previous findings that mated female *Tribolium castaneum* beetles exhibit increased susceptibility to death under heatwave conditions, this represents a significant knowledge gap in understanding the underpinning mechanisms behind insect vulnerabilities to heatwaves. Furthermore, there is a need to explore whether any such transcriptional changes are linked exclusively to one sex or both sexes, and whether there is an interaction between sex, mating status and heatwave exposure, as changes in reproductive physiology in response to mating likely differ between sexes.

Here, I aim to fill this knowledge gap by assessing the impacts of heatwaves on transcriptional profiles of male and female *Tribolium castaneum* beetles in a full factorial design: i) as virgins under control conditions ii) as virgins after a heatwave exposure iii) mated animals under control conditions and iv) mated animals after a heatwave exposure. This design enables a clear analysis of the effects of both heatwaves and mating on transcriptional profiles, while also investigating interactive effects in the context of the survival costs previously detected. Within each beetle, transcriptomics was performed on the head, thorax, and abdomen to assess potential body part-specific responses, which may be particularly relevant in the context of reproductive trade-offs. These changes are examined in the context of ecologically relevant heatwave exposures of 48°C ± 1°C for 5 hours, which represent conditions experienced within the natural range of *Tribolium castaneum* (Campbell et al., 2022; Christidis et al., 2023) and also represent conditions previously shown to induce sex-specific survival costs (see previous Chapters).

In this chapter, I ask whether transcriptional responses to heatwave exposure differ according to sex, mating status and body part in *Tribolium castaneum*. I predict that heatwave exposure will induce molecular stress responses, but that these responses will be dampened in mated females in comparison to other focal groups, potentially providing a molecular mechanism underlying their elevated vulnerability to heatwave exposures.

7.4 Materials and methods

7.4.1 Line maintenance and experimental individuals

T. castaneum used in these experiments were from the Krakow Super Strain (KSS) outbred stock line, which was maintained as described in Chapter 2. Experimental individuals were sexed at the pupal stage by visual identification of sexually dimorphic genital papillae (on day 18 of development) and then kept in single-sex groups of 20 individuals for a further 10 days to allow for development to sexual maturity. Groups were kept in 6cm Petri dishes filled with 3 grams (g) of fodder and topped with an even layer of oats. Once matured, females were identified with a dot of Uni Posca non-toxic marker (Uni-ball, Tokyo, Japan) on the dorsal thorax.

7.4.2 Experimental treatment groups

The sex-specific impacts of heatwaves on gene expression were investigated by selectively exposing combinations of focal individuals to various mating statuses and heatwave conditions. A fully factorial design was implemented, with the following categories: sex (male or female), mating status (virgin or mated), and heatwave exposure (yes or no). This created eight different focal groups. This factorial structure allowed gene expression responses to be compared between males and females, between virgin and mated individuals, and between control and heatwave-exposed beetles.

7.4.3 Experimental heatwave treatments

Heatwave conditions were applied using an Octagon 20 Eco incubator (Brinsea Incubation Specialists, Somerset, UK). Beetles were exposed as individuals in 6cm Petri dishes filled with 3g of ambient temperature fodder and topped with a layer of oats. Beetles were exposed to heatwave conditions of 48°C ± 1°C and 60 ± 10% RH for 5 h, with controls (30 ± 1°C, 60 ± 10% RH) run in parallel. A single 48°C, 5 h heatwave exposure was used so that transcriptomic responses could be compared across all focal groups under the same thermal stress, under conditions previously shown to produce clear sex- and mating-status-specific differences in survival and reproductive output.

7.4.4 Virgin treatment groups

48 h after eclosion, individual virgin males and females were subject to either heatwave conditions or control conditions as described above.

7.4.5 Mated treatment groups

48 h after eclosion, a single virgin female and an age-matched virgin male were moved into a 7 ml mating vial (filled with 2.4 g of fodder and topped with an even layer of oats) for 48 h at 30 ± 1 °C. After this, individuals were placed into a 6cm Petri dish (filled with 3g of fodder and a layer of oats) and were subject to either heatwave conditions or control conditions as described above.

7.4.6 Sample preparation

Immediately following the treatment period, surviving individuals were moved into an Eppendorf tube and placed in liquid nitrogen. Following this, individuals were stored in a -80°C freezer. Individuals were then removed from the -80°C freezer and maintained on a sterilised petri dish, held within a Styrofoam plate in a tray of liquid nitrogen. Using a sterilised surgical scalpel, individuals were sectioned into the head, thorax and abdomen. Six individuals from each focal group were sectioned and used for analysis. Head, thorax and abdomen samples were processed separately so that gene expression responses could be assessed independently across body parts with different behavioural, physiological and reproductive functions.

7.4.7 Library preparation and sequencing

RNA was extracted from each sample using a Quick-RNA™ Miniprep Kit (Zymo Research, Irvine, USA), following the manufacturer's protocol. Homogenisation was performed using ZR BashingBead™ Lysis Tubes (Zymo Research), which were used to ensure complete tissue disruption. The extraction protocol included DNase I digestion to remove genomic DNA. A Nanodrop spectrophotometer was used to initially investigate quality, before the sample was sent for sequencing. RNA samples were submitted to Novogene (UK) Company Limited (Cambridge, UK) for library preparation and sequencing. All samples underwent initial QC and were assessed for their RIN score, total volume, concentration and baseline characteristics. Where samples failed QC, they were not utilised further for library preparation (N = 12). mRNA libraries were prepared for the remaining 132 samples using poly(A) enrichment, and sequencing was performed using an Illumina platform to generate paired-end 150-bp reads. A minimum of 6 GB of raw data was produced per sample.

7.4.8 Read processing and quality control

Raw paired-end Illumina reads were quality- and adapter-trimmed using Cutadapt v4.8 (Martin, 2011). Specifically, reads shorter than 40 bp were discarded, and the

remaining reads were trimmed by 7 bp. Low-quality bases were filtered with a quality cutoff of Q15. Trimmed reads were assessed using FastQC v0.12.1 (Andrews, 2010) and MultiQC v1.13 (Ewels et al., 2016) to validate post-trimmed read quality.

7.4.9 Read alignment and quantification

Trimmed reads were aligned to the *Tribolium castaneum* reference genome (Tcas5.2 assembly, obtained from the NCBI genome database: https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_000002335.3/) using Hisat2 v2.2.0 (Kim et al., 2019). Alignment was performed with default settings and by providing a reference splice site annotation with the -known-splicesite-infile option. HISAT2 summary reports were assessed for alignment quality. SAM alignment files were converted to BAM format and sorted by coordinate using SAMtools v1.18. Subsequently, gene-level read counts were obtained with HTSeq v2.0.3 (Putri et al., 2022), with counting performed in a stranded manner.

7.4.10 Differential gene expression analysis and functional analysis

Differential gene expression and exploratory transcriptomic analyses were performed separately for each sex and body part to allow sex- and body-part-specific responses to be independently assessed. Principal component analysis (PCA) was used as an exploratory approach to visualise broad patterns of transcriptomic variation among samples. PCA plots were used to visually assess whether samples clustered according to heatwave exposure, mating status or their combination, and to identify whether the major axes of transcriptomic variation differed between the head, thorax and abdomen. PCA was performed on variance-stabilised transformed count data to reduce the influence of differences in sequencing depth and mean-dependent variance.

Differential gene expression (DGE) analysis was performed using the DESeq2 R package v1.36.0 (Love et al., 2014). For each sex and body part, pairwise contrasts were used to compare transcriptional responses associated with mating, heatwave exposure, and the combined effect of mating followed by heatwave exposure. Differentially expressed genes were identified based on log2 fold change cutoffs of 1 and an FDR cutoff of 0.05. Volcano plots were produced for each comparison to visualise both the magnitude and statistical significance of gene expression change. In these plots, log2 fold change was used to indicate the magnitude of expression differences, while statistical significance was used to identify genes with evidence of differential expression. Significantly upregulated and downregulated genes were

highlighted separately to allow treatment-, sex- and body-part-specific patterns of expression to be compared.

To identify biological pathways and functions enriched in the differentially expressed genes, a functional enrichment analysis was performed using gProfiler (Kolberg et al., 2023). Upregulated and downregulated gene lists were analysed separately, allowing biological processes associated with increased and decreased expression to be independently interpreted. A custom background gene list was created for each tissue and sex combination by selecting the list of all genes included in the corresponding DESeq2 analysis, ensuring that enrichment analysis was restricted to genes detectable in the relevant sample set. Enriched GO and KEGG terms were then visualised using bubble plots, with the 35 terms with the highest gene ratio scores plotted for each group. These plots were used to identify the dominant biological processes associated with mating, heatwave exposure and their combined effects across sex- and body-part-specific comparisons.

7.5 Results

7.5.1 Principal component analysis

Principal component analysis of RNA-seq data clearly revealed both body part- and sex-specific responses to different treatments. In females, all body parts displayed distinct clustering of individuals who had been maintained under control conditions versus those who had been subject to heatwave exposure (Fig. 7.1). Within this distinct clustering, separation between virgin females and mated females was also visually clear in both the head and the abdomen, whereas the thorax lacked such separation. In males, a strong effect of exposure temperature was also seen, with all body parts showing clear clustering of individuals who had been maintained under control conditions versus those who had been subject to heatwave exposure (Fig. 7.2). Males also broadly showed clustering by mating status, with distinct separation of virgin and mated individuals evident under heatwave conditions in both the head and the abdomen. In contrast, the thorax did not show such separation.

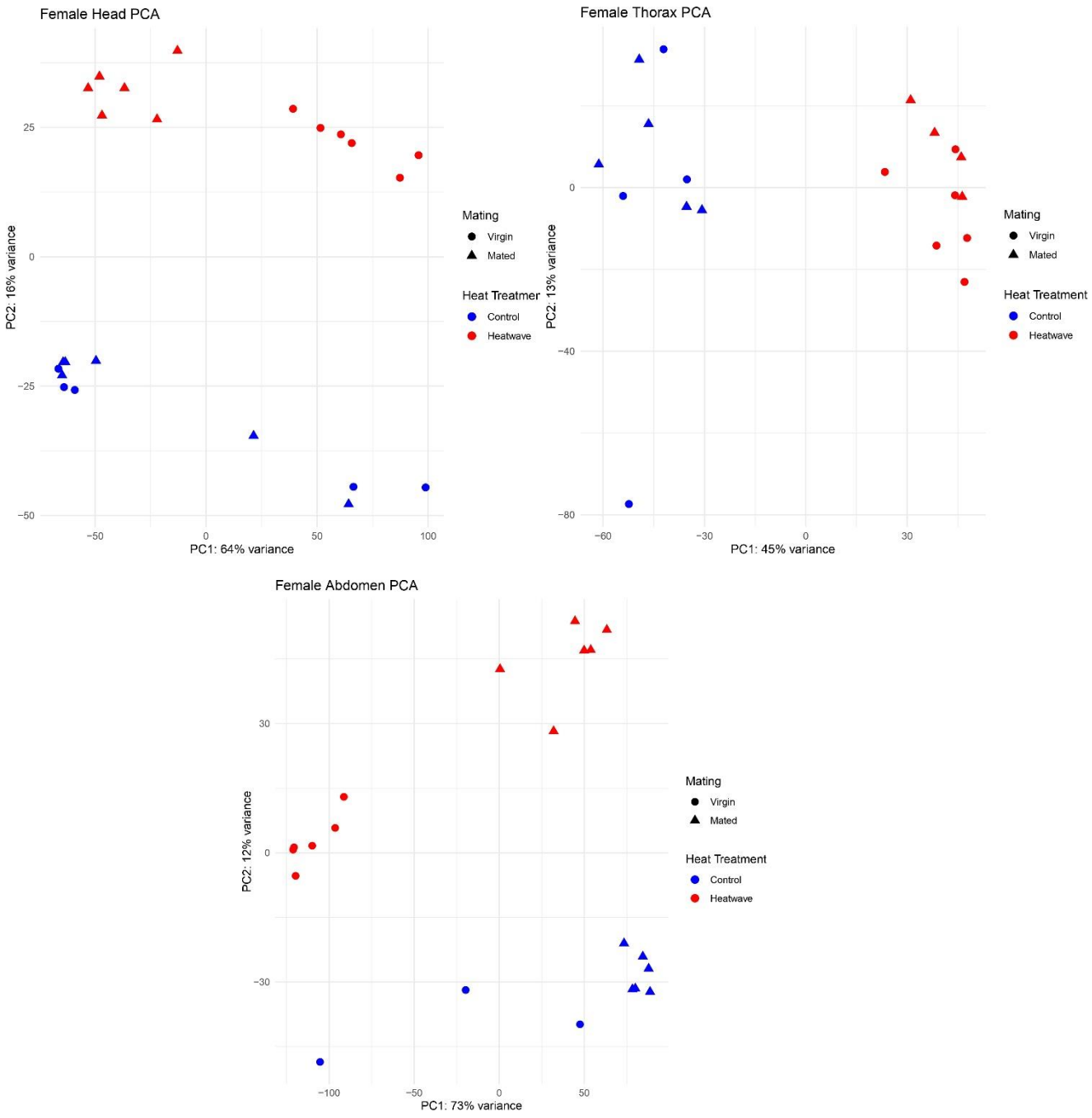

Figure 7.1: Principal Component Analysis of RNA-seq samples illustrating gene expression profiles of female Tribolium castaneum heads, thoraxes and abdomens under the following combinations of conditions: Virgin individuals maintained under control conditions, virgin individuals after heatwave exposure, mated individuals maintained under control conditions and mated individuals after heatwave exposure.

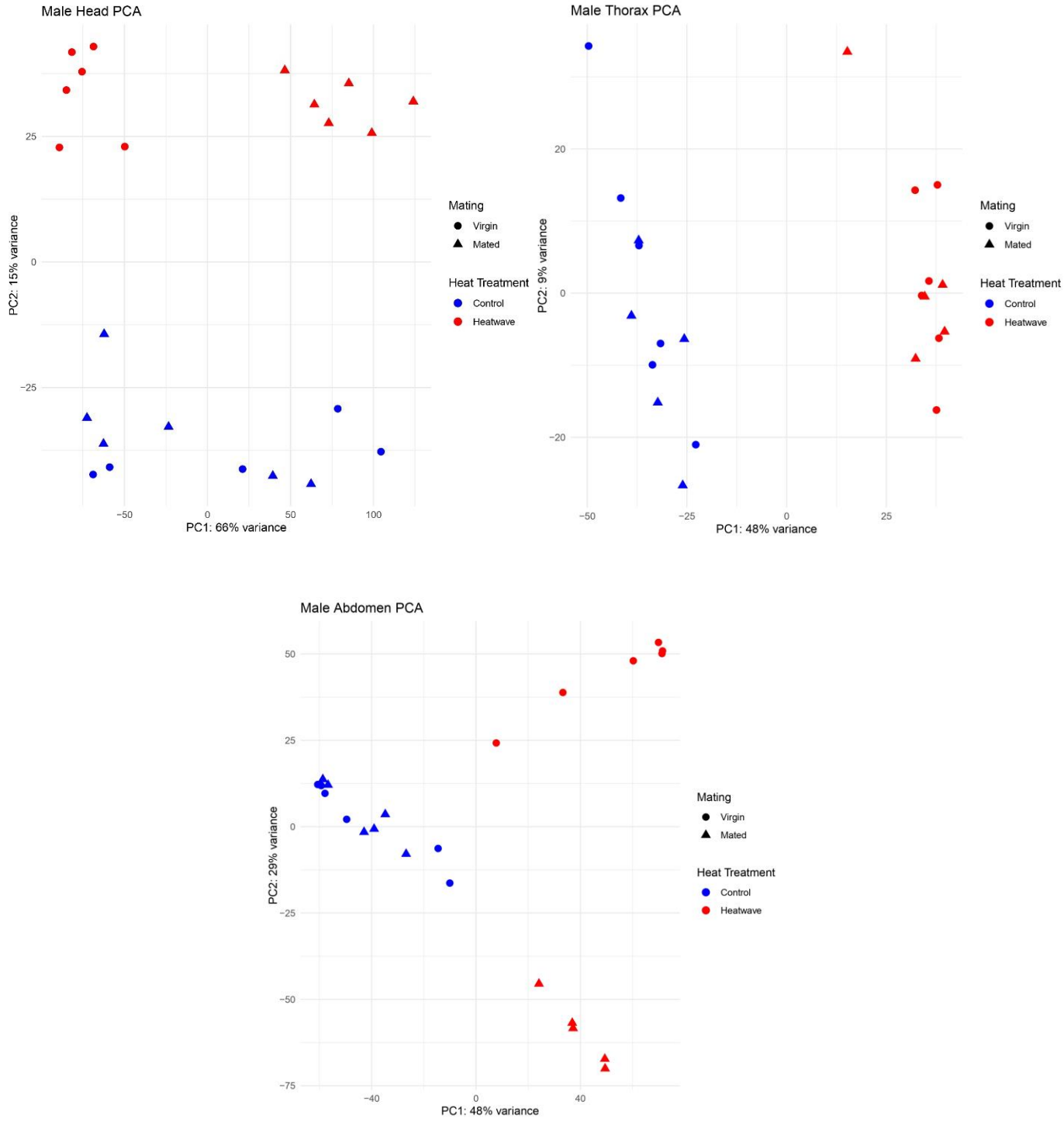

Figure 7.2: Principal Component Analysis of RNA-seq samples illustrating gene expression profiles of male Tribolium castaneum heads, thoraxes and abdomens under the following combinations of conditions: Virgin individuals maintained under control conditions, virgin individuals after heatwave exposure, mated individuals maintained under control conditions and mated individuals after heatwave exposure.

7.5.2 Volcano plots

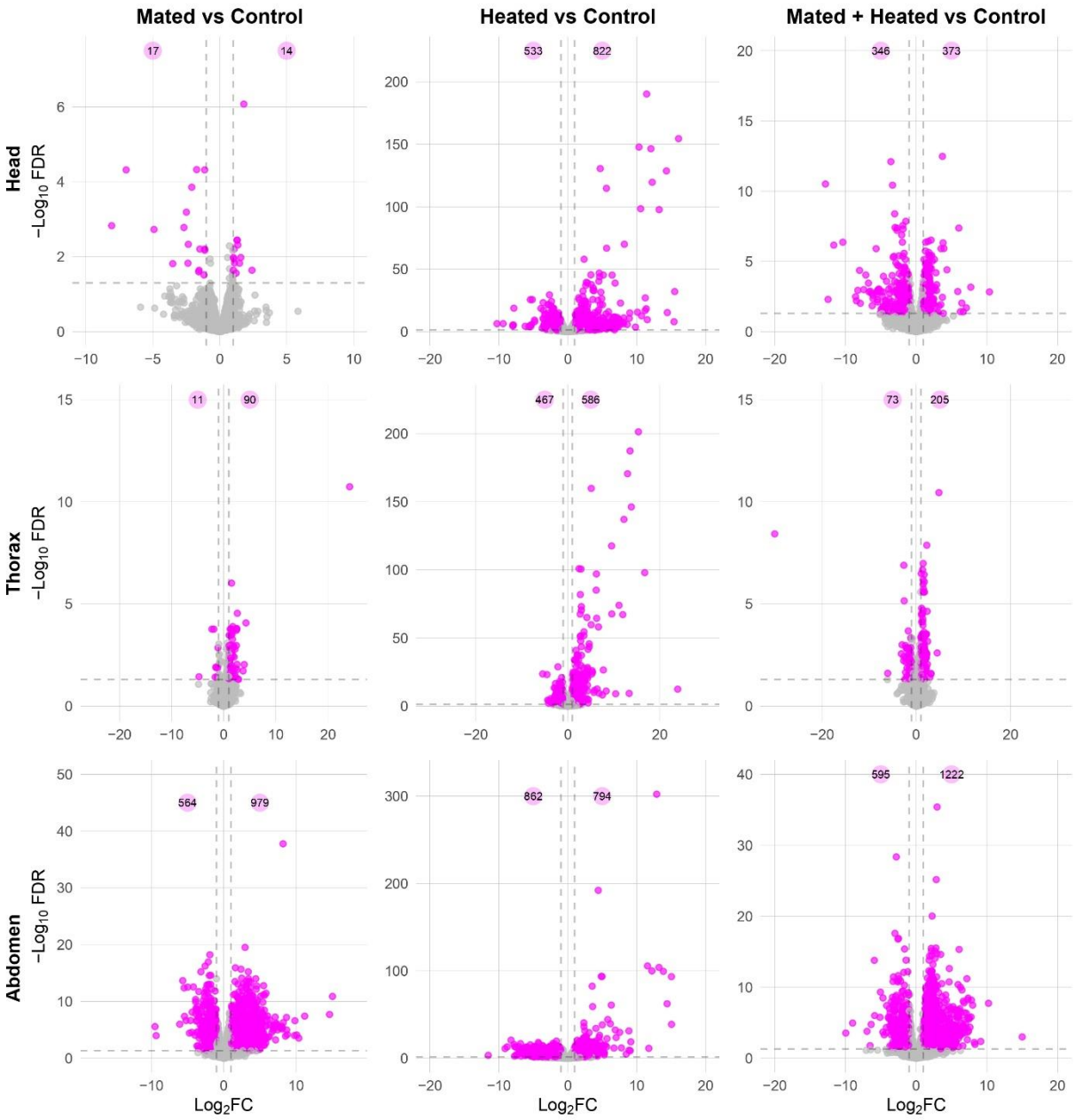

Figure 7.3: The impact of combinations of mating status and heatwave exposure on female Tribolium castaneum differential gene expression in the head, thorax and abdomen. Significantly upregulated and downregulated genes (FDR < 0.05 and $log_2FC > 1$) are represented as coloured circles, whereas non-significantly expressed genes are represented as grey circles. The total number of differentially expressed genes is indicated in the coloured circle at the top of each graph (downregulated to the left of the centre line and upregulated to the right).

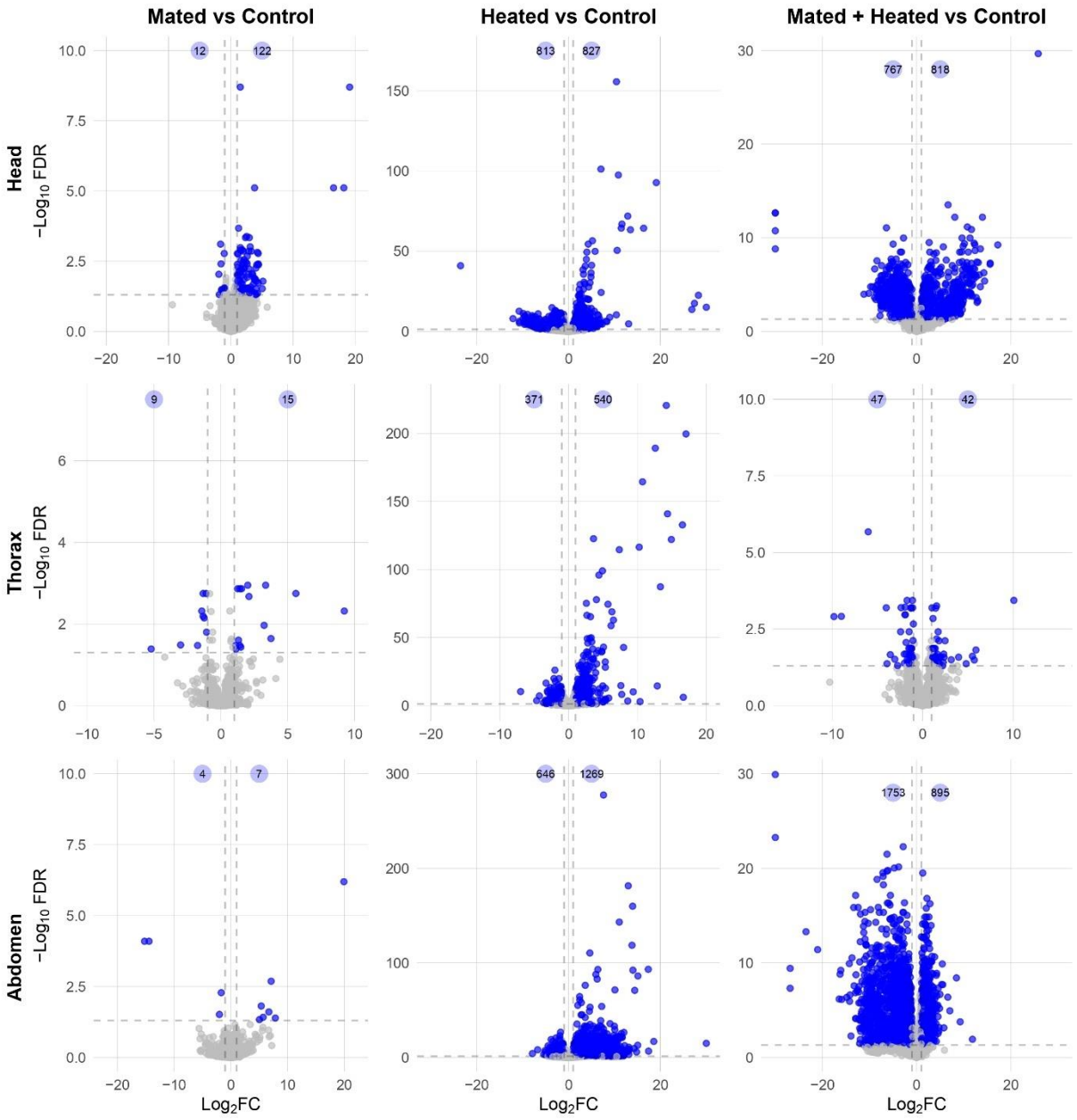

Figure 7.4: The impact of combinations of mating status and heatwave exposure on male Tribolium castaneum differential gene expression in the head, thorax and abdomen. Significantly upregulated and downregulated genes (FDR < 0.05 and $log_2FC > 1$) are represented as coloured circles, whereas non-significantly expressed genes are represented as grey circles. The total number of differentially expressed genes is indicated in the coloured circle at the top of each graph (downregulated to the left of the centre line and upregulated to the right).

Female *Tribolium castaneum* beetles showed tissue-specific differential gene expression in response to heatwave exposure, mating or a combination of both. Across all three treatments, the abdomen consistently showed a markedly higher

number of differentially expressed genes (DEGs) compared to the head and thorax (Fig. 7.3). In response to mating, the head exhibited only 31 DEGs, while the thorax showed 101. In contrast, the abdomen had 1,543 DEGs in response to mating. This trend persisted across both the heatwave-only treatment and the mating and heatwave treatment. However, the magnitude to which the abdomen had a greater number of DEGs was diminished in both of these treatment groups compared to mating alone. In the heatwave exposure group, abdomens had 1,646 DEGs compared to 1,355 in the head and 1,352 in the thorax. After mating and exposure to a heatwave, the abdomen had 1,817 DEGs compared to 718 in the head and 278 in the thorax.

Within body parts, the strongest transcriptional responses (in terms of fold change in gene expression) were consistently observed in the group subject to only heatwave exposure. All three body parts contained genes with extremely high expression changes, with multiple genes in each case (>9 genes for all body parts) showing a maximum fold change of greater than 1000 times that of the control group ($\log_2$FC > ~10). By contrast, such extreme fold changes in DEGs were rare in the mating-only group (none in the head, one in the thorax, and five in the abdomen) and in the combined treatment (one in the head, none in the thorax, and two in the abdomen).

Similar to females, male *Tribolium castaneum* beetles showed body part-specific differential gene expression in response to heatwave exposure, mating or a combination of both (Fig. 7.4). In the mating-only group, few DEGs were detected across all body parts. Furthermore, in contrast to females, the abdomen did not display a markedly increased number of DEGs in response to mating when compared with other body parts, with 134 DEGs seen in the head, 24 in the thorax and 11 in the abdomen. In contrast to the low number of DEGs in response to mating, the heatwave-only treatment resulted in 1,640 DEGs in the head, 911 in the thorax and 1,915 in the abdomen. Similarly high numbers of DEGs were also seen in the head and abdomen after mating and heatwave exposure (1,585 and 2,648, respectively), yet only 89 DEGs were detected in the thorax.

7.5.3 Functional enrichment analysis

7.5.3.1 Females

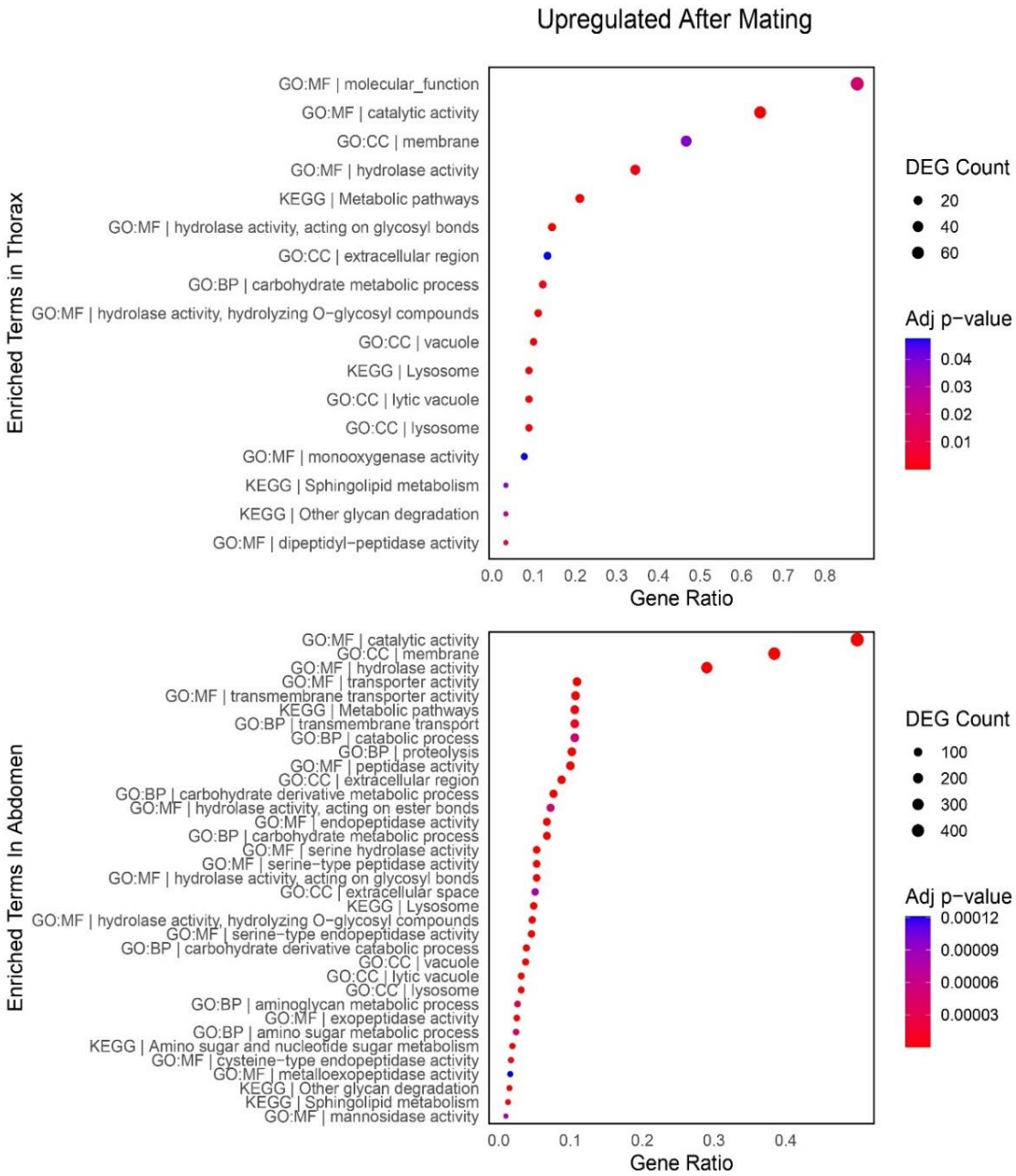

Figure 7.5: Functional enrichment of upregulated genes in female Tribolium castaneum after mating. Bubble plots show enriched GO terms and KEGG pathways for the thorax and abdomen (top to bottom). Dot size reflects the number of DEGs in each term, and the colour scale indicates adjusted p-values. The gene ratio represents the proportion of DEGs mapping to each pathway relative to the query set size.

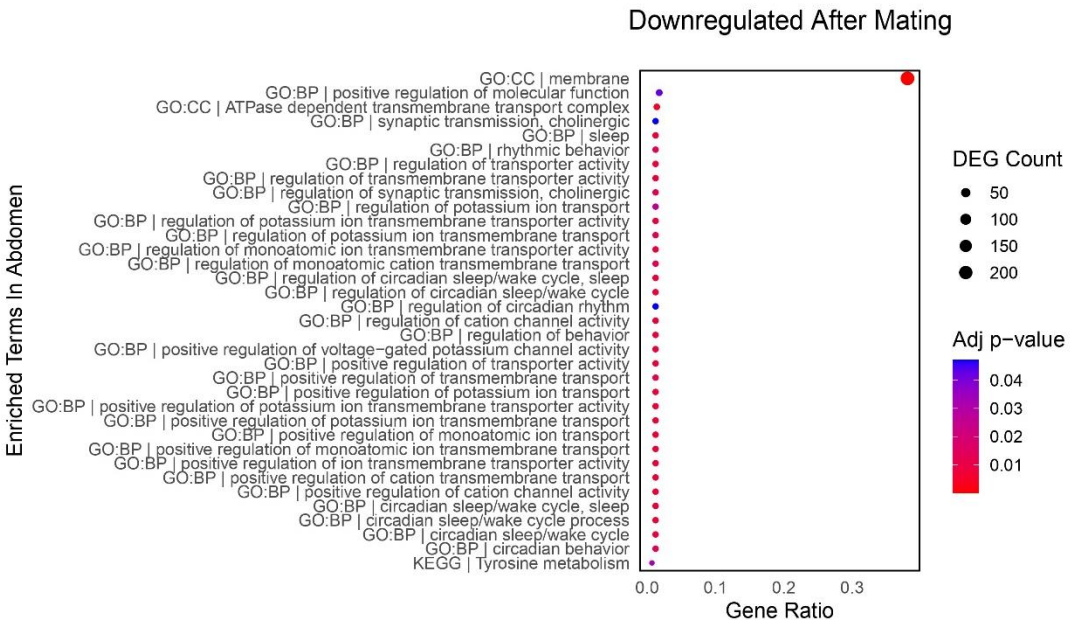

Figure 7.6: Functional enrichment of downregulated genes in female Tribolium castaneum after mating. Bubble plots show enriched GO terms and KEGG pathways for the abdomen. Dot size reflects the number of DEGs in each term, and the colour scale indicates adjusted p-values. The gene ratio represents the proportion of DEGs mapping to each pathway relative to the query set size.

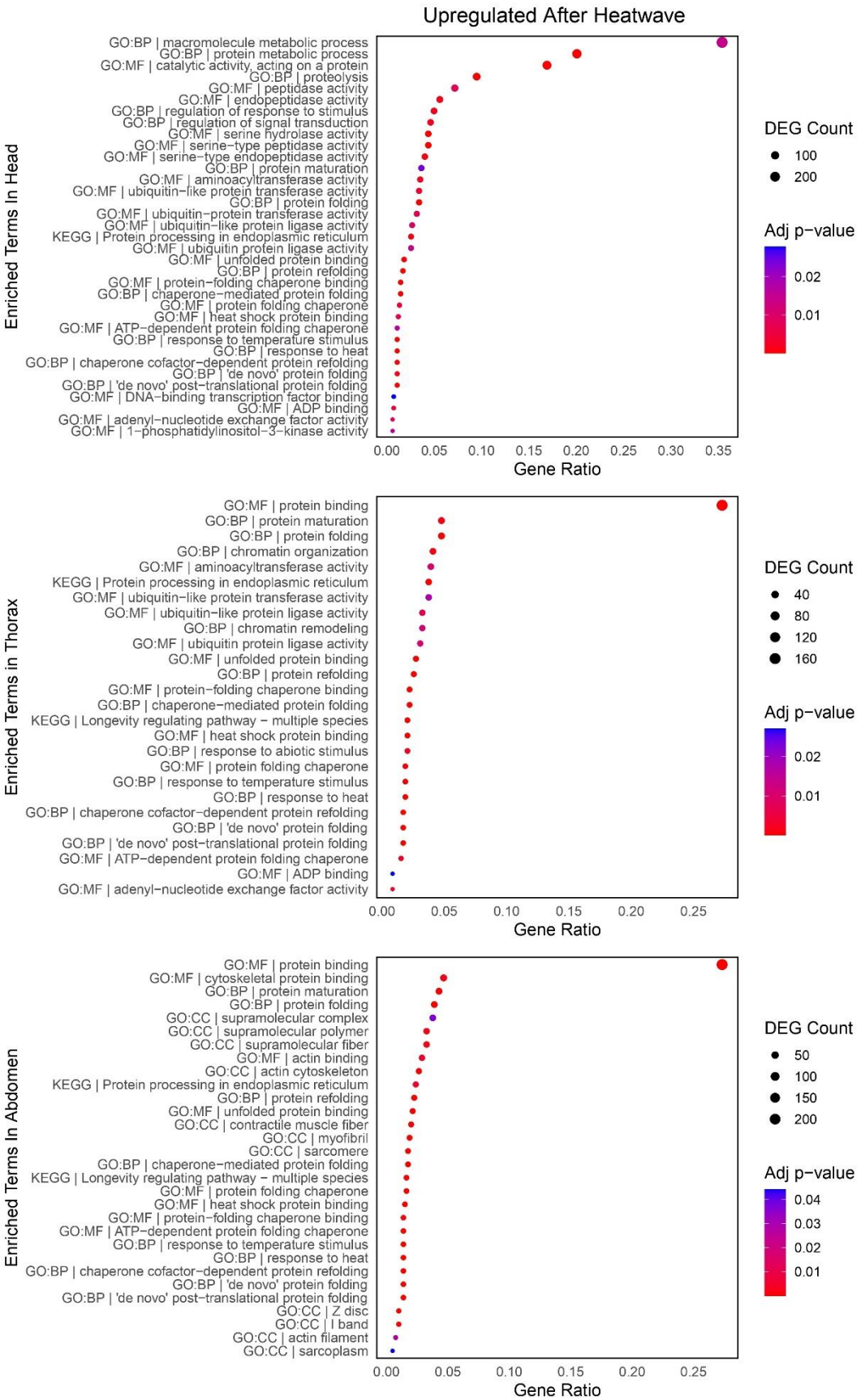

Figure 7.7: Functional enrichment of upregulated genes in female Tribolium castaneum after heatwave exposure. Bubble plots show enriched GO terms and KEGG pathways for the head, thorax, and abdomen (top to bottom). Dot size reflects the number of DEGs in each term, and the colour scale indicates adjusted p-values. The gene ratio represents the proportion of DEGs mapping to each pathway relative to the query set size.

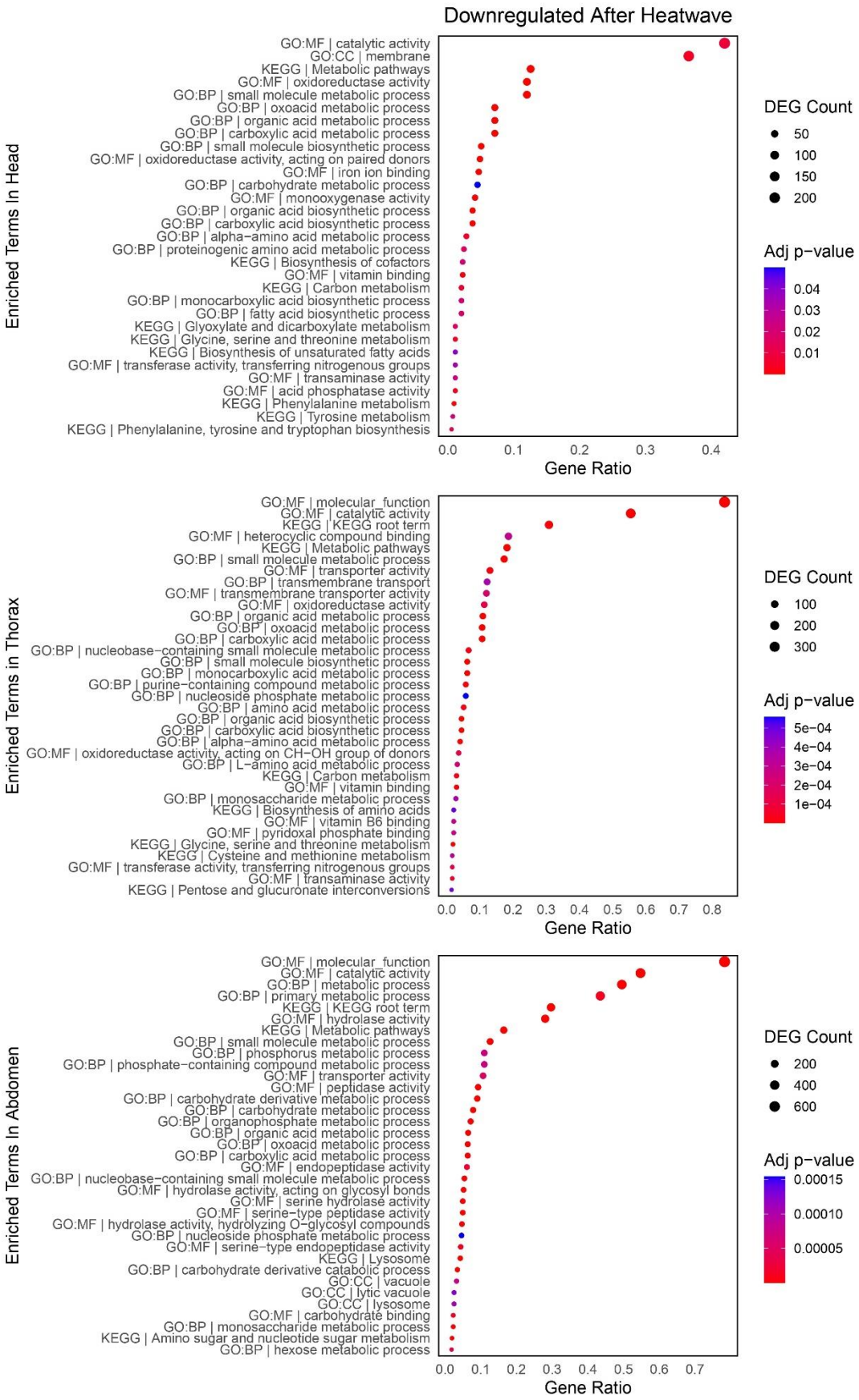

Figure 7.8: Functional enrichment of downregulated genes in female Tribolium castaneum after heatwave exposure. Bubble plots show enriched GO terms and KEGG pathways for the head, thorax, and abdomen (top to bottom). Dot size reflects the number of DEGs in each term, and the colour scale indicates adjusted p-values. The gene ratio represents the proportion of DEGs mapping to each pathway relative to the query set size.

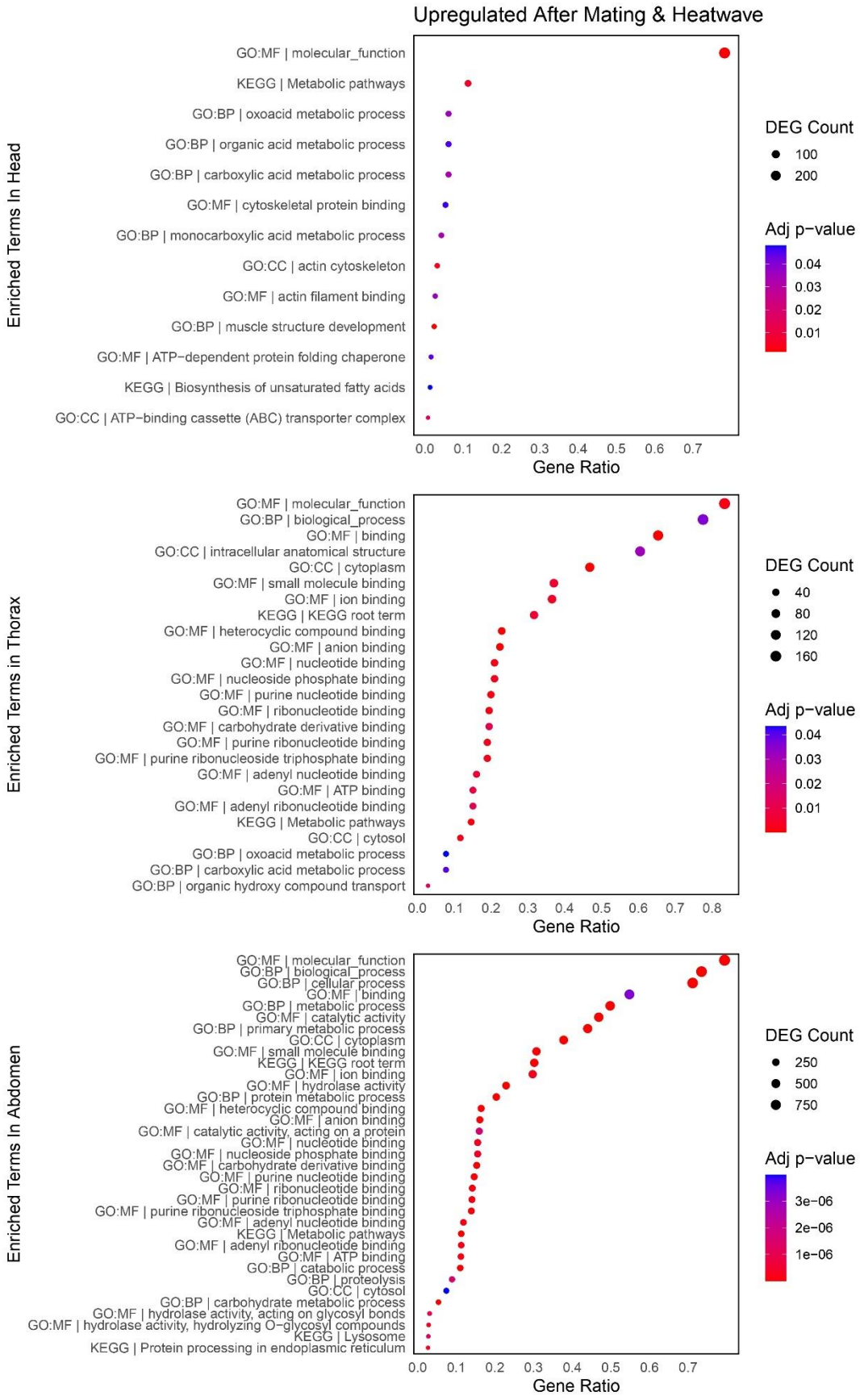

Figure 7.9: Functional enrichment of upregulated genes in female Tribolium castaneum after mating and subsequent heatwave exposure. Bubble plots show enriched GO terms and KEGG pathways for the head, thorax, and abdomen (top to bottom). Dot size reflects the number of DEGs in each term, and the colour scale indicates adjusted p-values. The gene ratio represents the proportion of DEGs mapping to each pathway relative to the query set size.

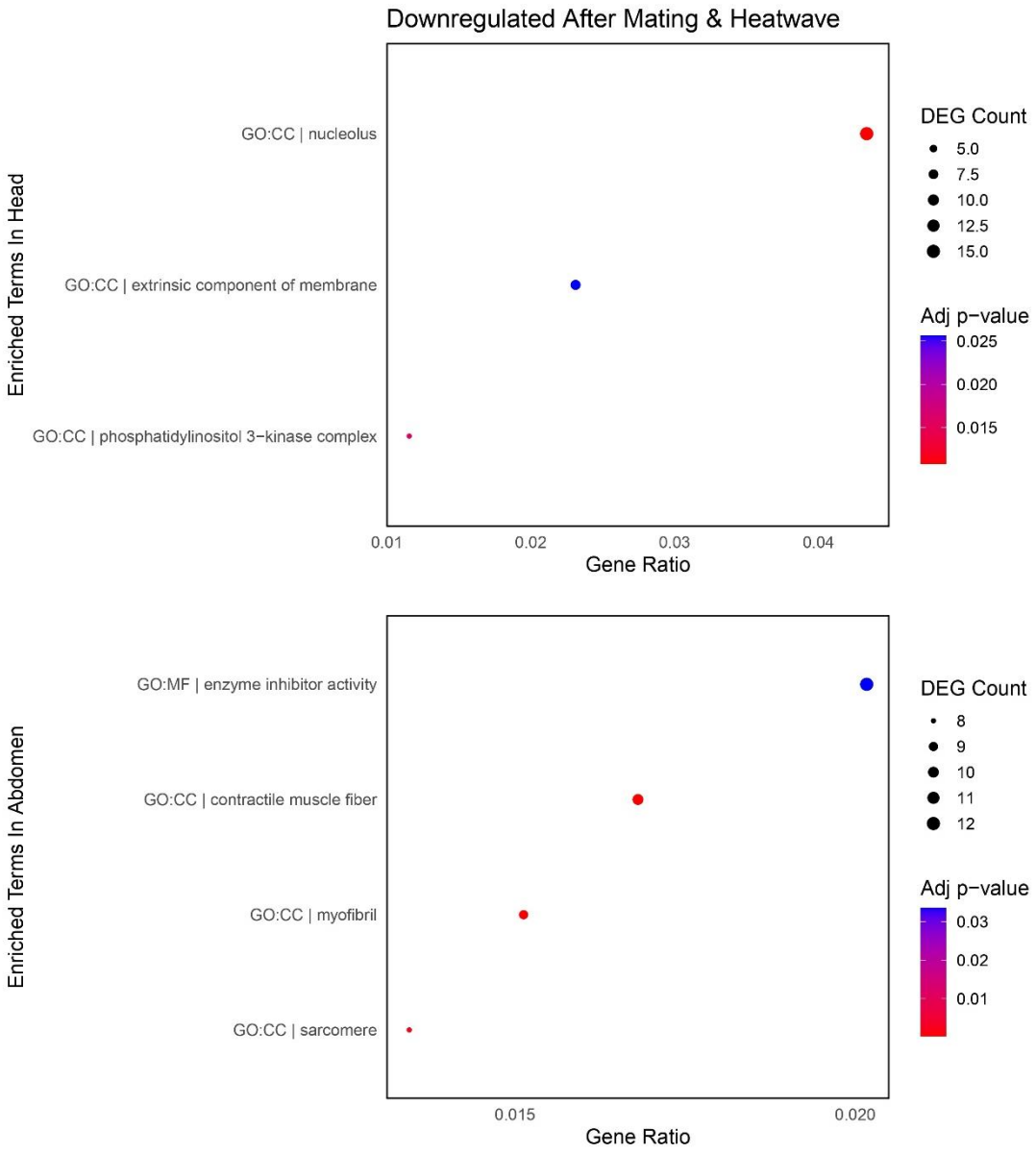

Figure 7.10: Functional enrichment of downregulated genes in female Tribolium castaneum after mating and subsequent heatwave exposure. Bubble plots show enriched GO terms and KEGG pathways for the head and abdomen (top to bottom). Dot size reflects the number of DEGs in each term, and the colour scale indicates adjusted p-values. The gene ratio represents the proportion of DEGs mapping to each pathway relative to the query set size.

Males:

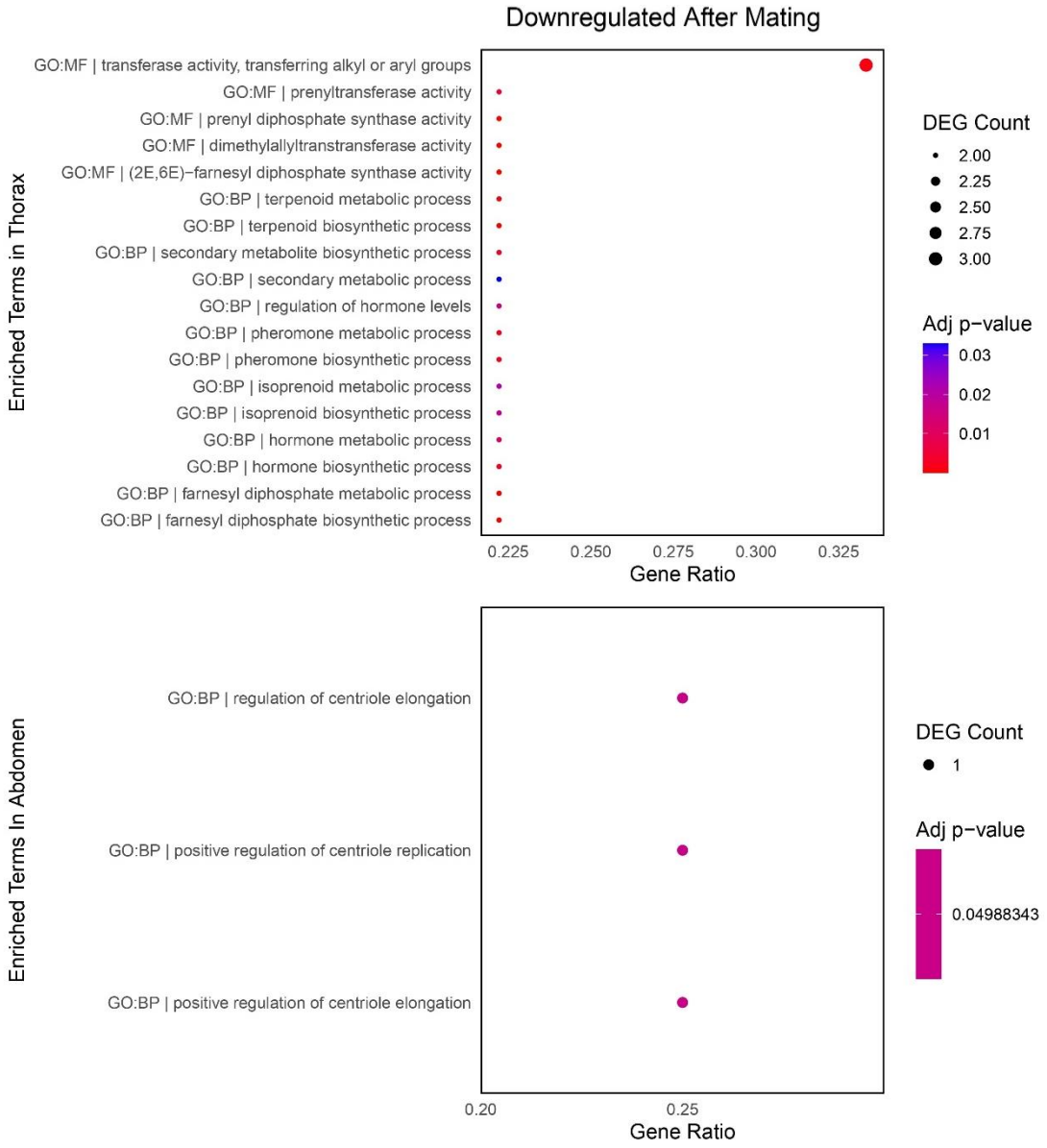

Figure 7.11: Functional enrichment of downregulated genes in male Tribolium castaneum after mating. Bubble plots show enriched GO terms and KEGG pathways for the thorax and abdomen (top to bottom). Dot size reflects the number of DEGs in each term, and the colour scale indicates adjusted p-values. The gene ratio represents the proportion of DEGs mapping to each pathway relative to the query set size.

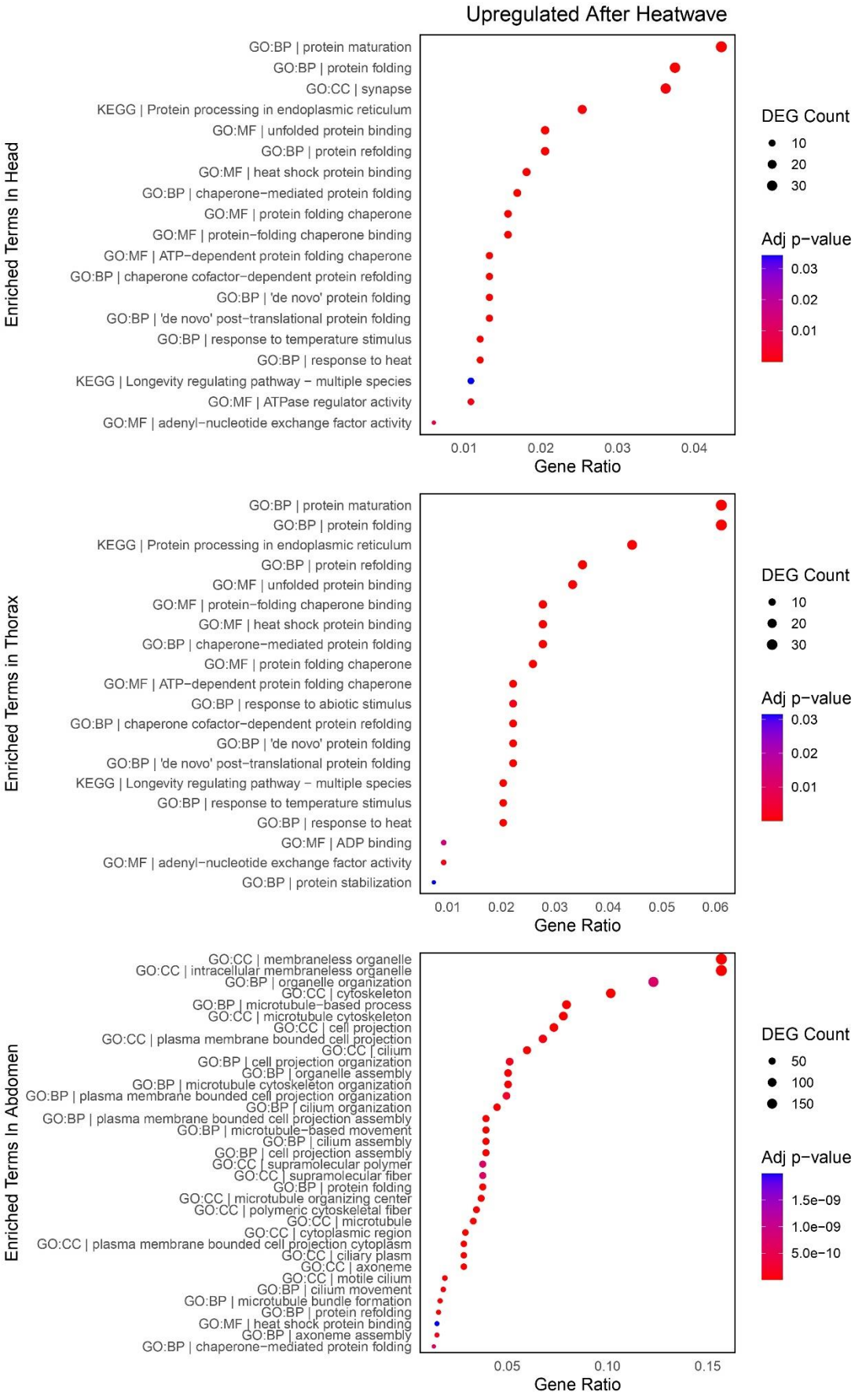

Figure 7.12: Functional enrichment of upregulated genes in male Tribolium castaneum after heatwave exposure. Bubble plots show enriched GO terms and KEGG pathways for the head, thorax, and abdomen (top to bottom). Dot size reflects the number of DEGs in each term, and the colour scale indicates adjusted p-values. The gene ratio represents the proportion of DEGs mapping to each pathway relative to the query set size.

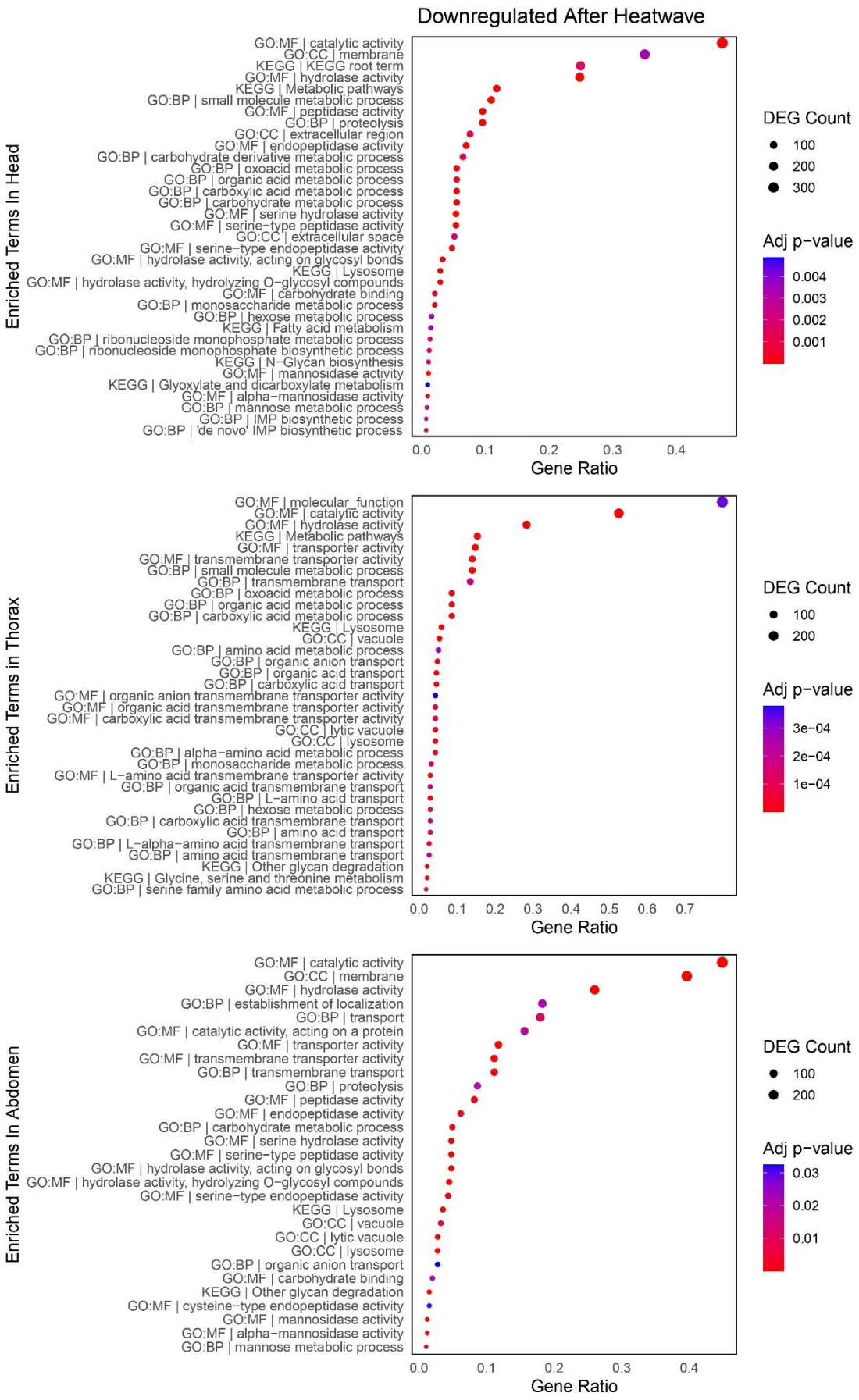

Figure 7.13: Functional enrichment of downregulated genes in male Tribolium castaneum after heatwave exposure. Bubble plots show enriched GO terms and KEGG pathways for the head, thorax, and abdomen (top to bottom). Dot size reflects the number of DEGs in each term, and the colour scale indicates adjusted p-values. The gene ratio represents the proportion of DEGs mapping to each pathway relative to the query set size.

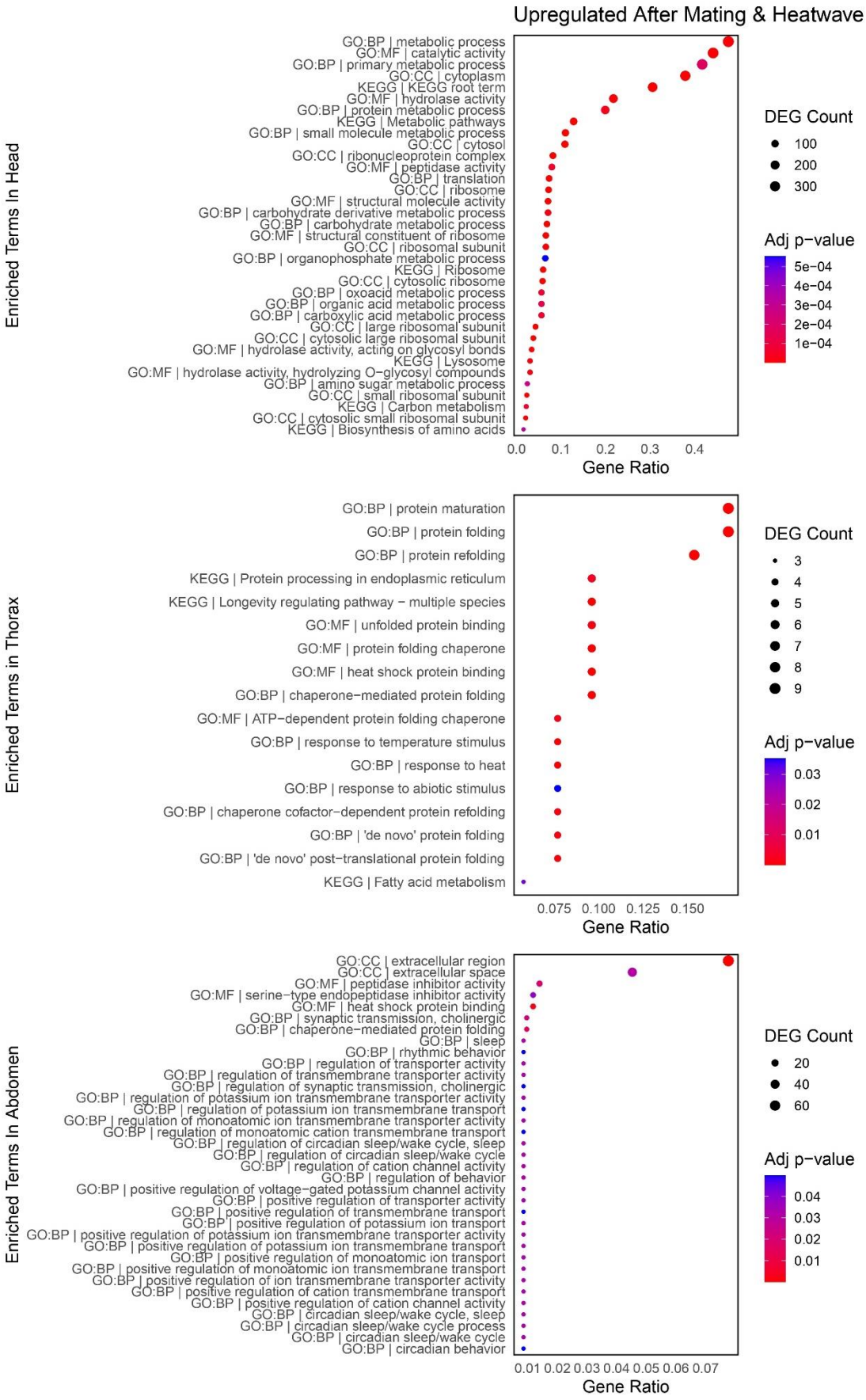

Figure 7.14: Functional enrichment of upregulated genes in male Tribolium castaneum after mating and subsequent heatwave exposure. Bubble plots show enriched GO terms and KEGG pathways for the head, thorax, and abdomen (top to bottom). Dot size reflects the number of DEGs in each term, and the colour scale indicates adjusted p-values. The gene ratio represents the proportion of DEGs mapping to each pathway relative to the query set size.

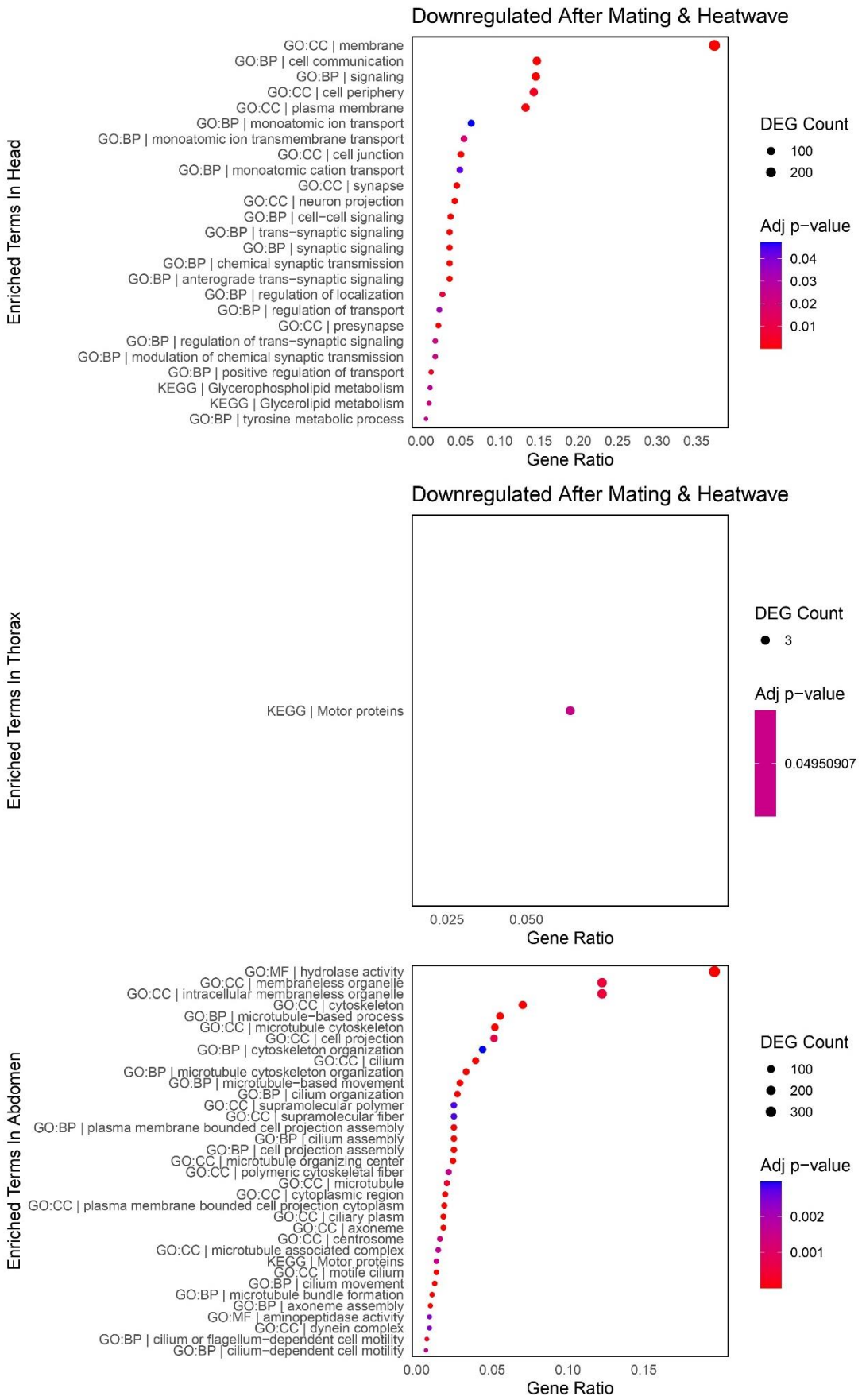

Figure 7.15: Functional enrichment of downregulated genes in male Tribolium castaneum after mating and subsequent heatwave exposure. Bubble plots show enriched GO terms and KEGG pathways for the head, thorax, and abdomen (top to bottom). Dot size reflects the number of DEGs in each term, and the colour scale indicates adjusted p-values. The gene ratio represents the proportion of DEGs mapping to each pathway relative to the query set size.

Functional enrichment analysis showed that several treatment-tissue combinations produced no enrichment of GO terms. In females, no upregulated or downregulated terms were observed in the head after mating, and no downregulated terms were observed in the thorax after mating or after mating and heatwave exposure. In males, no upregulated terms were observed in the head, thorax or abdomen after mating, and no downregulated terms were observed in the head after mating.

Functional enrichment analysis shows both sex-specific and sex-shared responses to both mating and heat stress. In response to just heatwave exposure, both virgin males and females showed a clear and widespread upregulated enrichment of terms associated with responses to heat (females: Fig. 7.7; males: Fig. 7.12), such as protein folding (GO:0006457), protein refolding (GO:0042026), unfolded protein binding (GO:0051082), heat shock protein binding (GO:0031072), response to heat (GO:0009408) and protein refolding (GO:0042026). This was seen consistently across all body parts.

Whilst the above terms were seen nearly ubiquitously in both males and females after just heatwave exposure, the presence of these terms was muted in response to mating and heatwave exposure, especially in females. Mated females did not show upregulation of any of these terms canonically associated with the heat stress response in any body part (Fig. 7.9). Whilst males also showed no presence of such terms in the head, they were all present in the thorax, and heat shock protein binding was also associated with the abdomen (Fig. 7.14).

Alongside the enrichment terms directly associated with heat shock responses, functional enrichment analysis also highlighted several other trends in the presence of enrichment terms. Within females, unique patterns of upregulation and downregulation were observed under different treatments for lysosomal and membrane remodelling processes, with enrichment of terms such as lysosome (GO:0005764), lytic vacuole (GO:0000323), and sphingolipid metabolic process (GO:0006665). Furthermore, these are associated with terms such as transmembrane transporter activity (GO:0022857). Such terms are likely associated with both lipid and protein trafficking within the female abdominal section, linked to reproductive investment and remodelling. Consistently, such terms were upregulated in mated females in the thorax and abdomen, and downregulated in the same body parts of females who were exposed to a heatwave. In contrast to both of these, such terms were not present in the mated and heatwave female group, with the one exception being the term ‘lysosome’, which was upregulated in the abdomen.

7.6 Discussion

Functional enrichment analysis has revealed that *Tribolium castaneum* beetles exhibit both sex- and body-part-specific transcriptional changes in response to mating, heatwave exposure, and a combination of mating followed by heatwave exposure, providing cues to potential mechanisms to explain the previously observed sensitivities in mated females. The different analytical approaches used here provide complementary levels of interpretation. The PCA plots indicate that heatwave exposure was a major driver of overall transcriptomic variation, with heatwave-exposed and control individuals separating clearly across both sexes and multiple body parts (Figs 7.1 and 7.2). This suggests that exposure to heatwave conditions of 48°C for 5 h induced broad genome-wide shifts in gene expression, rather than changes restricted to a small number of genes or pathways. However, the PCA plots also show that this response was modified by mating status in a body-part-specific manner. In females, separation between virgin and mated individuals was most evident in the head and abdomen, whereas the thorax showed clearer separation by heatwave exposure than by mating status (Fig. 7.1). Similarly, in males, mating-associated separation was most apparent in the head and abdomen under heatwave conditions, while the thorax again showed weaker separation by mating status (Fig. 7.2). These patterns support a central premise that transcriptional responses to heatwave exposure are shaped not only by thermal stress, but also by sex, mating status and body part.

The volcano plots further clarify the scale, direction and body-part distribution of these transcriptional responses. In females, the abdomen consistently showed the greatest number of differentially expressed genes across mating, heatwave exposure, and the combined mating and heatwave treatment (Fig. 7.3). This is consistent with the abdomen containing reproductive tissues, the gut and fat body, and therefore being closely linked to post-mating reproductive investment, metabolism and immune regulation (Arrese and Soulages, 2010; Nanfack-Minkeu and Sirot, 2022). However, the nature of this abdominal dominance differed between treatments. Mating alone produced a strongly abdomen-biased response, whereas heatwave exposure alone produced large numbers of differentially expressed genes across all three body parts, indicating a broader, more systemic transcriptional response to acute thermal stress. Across both sexes, the strongest fold-change responses were also observed after heatwave exposure alone, consistent with the expectation that acute heat stress can induce large transcriptional shifts in stress-associated genes (Feder and Hofmann, 1999;

Sørensen, Kristensen and Loeschcke, 2003). By contrast, the combined mating and heatwave treatment produced a different pattern, with a stronger abdominal response but fewer differentially expressed genes in the head and thorax than under heatwave exposure alone. This suggests that mating did not simply add to the heatwave response but altered both the magnitude and distribution of transcriptional responses across body parts. In males, mating alone produced relatively few differentially expressed genes, whereas heatwave exposure produced large transcriptional responses, particularly in the head and abdomen (Fig. 7.4). Interestingly, this head- and abdomen-biased response was retained after mating and heatwave exposure, while the thorax showed very few differentially expressed genes under the combined treatment. Given that the thorax is closely associated with locomotor musculature, energetic demand and metabolic regulation, this suggests that mating may alter the body-part distribution of the male heatwave response, rather than uniformly reducing transcriptional responses across the whole body (Bretscher and O'Connor, 2020). Together, Figs 7.3 and 7.4 show that heatwave exposure was a major driver of differential gene expression, but that the scale and body-part distribution of this response depended strongly on sex and mating status.

Together, the functional enrichment shows that the biological meaning of differential gene expression differed strongly between treatments, with mating-associated responses, heatwave-associated responses and combined mating–heatwave responses producing distinct patterns of upregulated and downregulated functional terms across sexes and body parts (Figs 7.5–7.15). A wide array of cellular processes were differentially enriched in the mating, heatwave exposure, and mating and heatwave exposure groups, highlighting the broad and sometimes conflicting transcriptional consequences of both thermal and reproductive stress. Among such conflicting enrichment terms were those typically associated with lysosomal and membrane remodelling processes, including lysosome, lytic vacuole, and sphingolipid metabolism. These processes are well known to play key roles in cellular degradation and lipid signalling (Gault, Obeid and Hannun, 2010; Settembre et al., 2013). These terms were all upregulated in the thorax and abdomen of mated females, yet downregulated in the abdomen of mated females exposed to heatwaves, suggesting that mating and/or reproduction drive upregulation of some aspects of membrane trafficking and lipid metabolism, whereas heat exposure suppresses them. Curiously, the terms lytic vacuole and sphingolipid metabolism showed no enrichment in response to mating followed by heatwave exposure in

females, suggesting that these pathways are either suppressed under heatwave stress despite mating or deprioritised in favour of other cellular responses. Contrastingly, the term lysosome remained upregulated in females who were mated and exposed to a heatwave. This likely reflects the complex role of lysosomal activity, which is associated with both stress responses and reproduction (Li et al., 2022), and may therefore remain upregulated even when other membrane remodelling pathways are suppressed after heatwave exposure.

Perhaps the most important result of the enrichment analysis was in relation to the heat stress response. Following heatwave exposure, virgin beetles showed consistent upregulation of terms associated with responses to heat stress, such as protein folding, protein refolding, unfolded protein binding, heat shock protein binding, response to heat and protein refolding. These terms were enriched strongly in the head, thorax, and abdomen of both males and females and represent commonly seen and expected upregulated terms in response to heat stress (Li et al., 2020; Krog Noer et al., 2023). Such terms were not observed within the mating-only group, and may potentially explain some of the distinct clustering of the heatwave-exposed and control groups in principal components analyses. More broadly, these terms represent a well-described response to heat exposure, whereby the upregulation of heat shock proteins and associated pathways protects against the denaturation, accumulation, and aggregation of proteins (Feder and Hofmann, 1999).

In contrast, the presence of such enrichment terms was not so consistent in the groups that were mated before being subject to heatwave exposure. Whilst mated males maintained upregulated enrichment of all the heat response terms discussed above, this was limited only to the thorax, with heat shock protein binding being the only of these terms upregulated within the abdomen. Consistent with this more restricted transcriptional response, mated males also experienced reduced survival under heatwave exposure. However, this was much less pronounced than in mated females, who showed none of these terms being differentially enriched in any body part. Here, it is clearly demonstrated that females exhibited a strongly reduced transcriptional response to a heatwave exposure after mating. The transcriptomic samples were collected after a heatwave exposure of 48°C for 5 hours, representing a temperature at which mated females showed a significantly lower survival rate than virgin males, virgin females and mated males. In females, it appears that reproduction dampens the heat stress response, providing a potential mechanism explaining the susceptibility and increased mortality observed in

previous Chapters. This aligns with broader reproductive-associated trade-offs, such as those involving immunity, metabolism, and lifespan (Yu et al., 2021; Nanfack-Minkeu and Sirot, 2022).

This male ejaculate-mediated trade-off between reproduction and the heat-stress response may be the driving mechanism behind the mortality susceptibility of mated females previously observed. It therefore raises profound questions about the factors affecting insect populations' reaction to current and future heatwaves and general climate change. Furthermore, this result challenges the current model of insect vulnerability to heatwaves, as the majority of studies remain primarily focused on male-driven susceptibility, mainly due to the apparent reproductive sensitivity of males and sperm (Iossa, 2019; Dougherty et al., 2024). By revealing molecular sex-specific trade-offs, I emphasise the need for a greater focus on understanding female insect responses to climate change, with a requirement for a more holistic approach to thermal vulnerabilities that factors in both survival and reproductive costs. By showing that female mating status impacts heat tolerance and, consequently, survival, I suggest that population resilience to heatwaves may be constrained by female mortality as well as male reproductive limits.

8. General discussion

8.1 Chapter outline

In this Chapter, I summarise and present the key findings of this thesis before discussing these results within the context of the current research base. Then, I propose ideas for future research, building on the novel findings presented in this thesis and considering the broader requirements of the field.

8.2 Aims and scope

Broadly, this thesis has aimed to enhance the understanding of the impacts of short-duration heatwave exposure on insect reproduction and survival, using *Tribolium castaneum*, a model insect in evolution and ecology (Pointer, Gage, and Spurgin, 2021; Campbell et al., 2022). This research is essential and timely, given that heatwaves are becoming more common and intense due to anthropogenic climate change, with short duration heatwaves expected to display a particularly dramatic increase in frequency (Meehl and Tebaldi, 2004; Trenberth, 2018; Perkins-Kirkpatrick and Lewis, 2020).

Within this thesis, three main areas of findings have been covered: i) the impact of short-duration heatwave exposures on survival, ii) the impact of short-duration heatwave exposures on reproductive output and iii) the impact of short-duration heatwaves on gene expression, focusing on the molecular mechanisms underpinning the phenotypic effects.

8.3 Summary of findings

8.3.1 Survival

Across Chapters, I assessed the survival of *T. castaneum* across a wide range of heatwave exposures, representing ecologically relevant heatwave conditions for this species, which are expected to become increasingly common across *T. castaneum's* range (Campbell et al., 2022; Christidis et al., 2023). *T. castaneum* beetles were shown to be broadly resilient to a lower range of exposure temperatures, with all focal groups (virgin males, virgin females, mated males, and mated females) exhibiting resilience against exposure temperatures of 42°C and 44°C. Within 42°C exposures, this resilience was demonstrated not only over single-day exposures but also over five consecutive days of exposure, highlighting these temperature conditions as broadly benign in this system.

As exposure intensity increased above these benign temperatures, mortality rates generally increased, although not uniformly across focal groups. In Chapter 5, I show that at temperatures of 46°C, all focal groups exhibited resilience to heatwaves of durations shorter than 5 hours. However, mated females stand out as they displayed a drop in survival in response to a 10-hour exposure, resulting in a ~15% reduction in survival compared to controls. This represents a key finding, whereby mated females display significant survival reductions in response to less intense heatwave exposures than other groups. As exposure temperatures increased to 48°C and 50°C, all focal groups showed reductions in survival. This was well replicated where groups were assessed multiple times under identical conditions.

Under these more extreme exposures, mated females consistently showed significantly worse survival than all other focal groups in response to the same heatwave conditions. These differences were often stark; for example I found female survival rate of just ~20% in response to a 5-hour, 50°C heatwave exposure, in contrast to a greater than 80% survival in all other focal groups.

Experimental manipulations reinforced this mating status-mediated effect: sealing females' genital openings so they could participate in mating behaviours but not receive an ejaculate increased survival under a 5-hour, 48°C heatwave, relative to females with unsealed openings that could therefore receive an ejaculate. These results clearly demonstrate that the reduced survival of mated females, as extensively shown in this thesis, is caused by male ejaculate and is not simply a result of male harassment. I thus show that male ejaculate alters female physiology and mediates the trade-off between reproductive investment and thermal tolerance.

Generally, studies to date have failed to find clear patterns of sex-biased thermal limits, and meta-analyses have indicated that there is no sex difference in the ability to acclimate to thermal limits (Pottier et al., 2021). Additionally, there is no systematic sex difference in phenotypic plasticity under thermal manipulations (Hangartner et al., 2022). For some time, research has pointed towards a female-biased mortality resilience to heat (e.g. Cui et al., 2008; Roux et al., 2010; Chen et al., 2018); however, this has focused on comparing virgin females and males. The findings presented in this thesis extend such work by showing that mating status can fundamentally alter female survival under thermal stress, revealing a previously under-tested and unrecognised vulnerability.

In summary, this thesis has demonstrated that *T. castaneum* beetle survival is resilient to milder, short-duration heatwave exposures but becomes susceptible to exposures of 46 °C and above, depending on the exposure duration and focal group. The consistent vulnerability of mated females highlights a crucial and heretofore unrecognised interaction between mating status and heatwave exposure, with important implications for population-level responses of ectotherms to a warming world.

8.3.2 Reproduction

Across Chapters, I also examined how short-duration heatwaves impact reproduction in *T. castaneum*. As with survival, reproductive output was resilient to milder heatwave exposures, with reproductive output being maintained in response to heatwave conditions of 42°C and 44°C.

At increasing temperatures, sex-specific reductions in reproductive output became apparent. Comparing qualitatively between the results in Chapters 4 and 6, mated females subjected to heatwave exposures of 46°C maintained reproductive output, whereas virgin males exhibited clear reductions in reproductive output. This highlights that at intermediate heatwave intensities, male reproduction may be more sensitive to heat stress than female reproduction, in line with some of the recent work in this field (e.g. Sales et al. 2018; Zwoinska et al., 2020; Meena et al., 2024), contrasting with the trends seen for survival.

Results in Chapter 5 show that reproductive output was consistently and dramatically reduced across virgin males, mated males, and mated females after a 5-hour, 48°C heatwave exposure. Interestingly, virgin females showed resilience against these conditions, highlighting sperm damage (whether in the male or stored post-mating in the female) as the avenue by which reproductive losses are incurred in response to heatwave exposure. These interpretations align strongly with the growing body of research showing that male reproductive traits are the key drivers of reproductive losses in response to thermal stress (Porcelli et al., 2016; Sales et al., 2018; Walsh et al., 2019; Canal Domenech and Fricke, 2023; Gandara and Drummond-Barbosa, 2023). This interpretation was further strengthened by morphological investigations reported in Chapter 4, which demonstrated that heatwave exposures as low as 42°C for 2 hours resulted in reduced sperm length, and 42°C for 10 hours resulted in nearly a halving of testes volume. However, these impacts did not translate into fitness consequences at these lower temperature exposures.

In summary, this thesis has demonstrated that reproductive output and reproductive morphology are key vulnerabilities to short-duration heatwave exposure in *T. castaneum*. Male reproductive morphology is particularly susceptible, with sperm and testes being impacted by the least intense heatwave exposures tested. Reproductive output was maintained under milder short-duration heatwave exposures, but began to decline rapidly after 46°C, with an initial male-biased susceptibility.

8.3.3 Gene expression

In Chapter 7, I presented results from the investigation of the transcriptional responses to a 48°C, 5-hour heatwave exposure in *T. castaneum*. Using functional enrichment analysis, I showed widespread upregulation of enrichment terms associated with the heat stress response in virgin beetles of both sexes, including protein folding and heat-shock protein binding. This response was consistent across all body parts and represents a well-known response to thermal stress (Li et al., 2020; Krog Noer et al., 2023). Crucially, however, this response was mediated by mating status and sex. In mated males, enrichment of heat stress terms was found only within the thorax, except for one term that was also enriched in the abdomen. Even more dramatically, in mated females, there was no enrichment of any terms associated with the heat stress response in any body part. This is particularly salient given that such heatwave conditions had previously been shown to induce mortality in mated females, whereas other focal groups maintained high levels of survival. It is well established that mating can induce changes in gene expression in mated female insects (McGraw et al., 2004). In some instances, mating may also suppress stress and immune responses, as well as reduce longevity (Yu et al., 2021; Nanfack-Minkeu and Sirot, 2022). This inability to mount a molecular heat stress response following mating therefore provides a potential mechanism underlying the vulnerability of mated females to heatwaves, as demonstrated in this thesis. More broadly, it suggests that mating can directly reduce the capacity to induce a protective transcriptional response to heatwave exposure in insects. This could be a result of an increased allocation of limited resources to reproduction in mated females, but this question requires further investigation.

8.4 Future work

The primary outcome of this thesis is the concept that female mating status significantly alters thermal tolerance to short-duration heatwave exposure, increasing mortality risk. This has clear and vital implications for population

persistence under climate change scenarios, whereby such heatwaves are becoming more common and intense (Meehl and Tebaldi, 2004; Trenberth, 2018; Perkins-Kirkpatrick and Lewis, 2020). Understanding the broader significance of these findings is clearly necessary in light of such results. A key research priority should therefore be to test sex- and mating-specific vulnerabilities to heatwaves in a diverse range of insects, to assess whether this trend persists across the whole group or just in select taxa.

Social insects are a particularly critical group for assessing such vulnerabilities. In these species, a single queen (or a small number of queens) is responsible for founding and maintaining a colony, meaning their survival in the early period of colony life is solely responsible for colony survival. Many social insect species undergo nuptial flights before founding their own colony and, therefore, would be exposed to any ongoing heatwaves at this time. This represents a crucial period during which females may be subject to extreme temperatures while in a mated state. Species such as *Lasius niger* have well-studied cues which induce nuptial flights, with 'flying ants' being observed on all days with mean daily temperatures > 25°C across summer months in the United Kingdom (Hart et al., 2017). Therefore, the potential for such species to be subject to extreme heat is relatively high and may represent a key driver of population and species declines during periods when heatwaves occur, particularly under future climate change.

Alongside the mortality vulnerabilities of mated females, colony persistence could also be hindered due to sperm sensitivities to heat, which are comparatively well studied in many insects, including social insects (e.g., Porcelli et al., 2016; Sales et al., 2018; McAfee et al., 2020), and are also covered extensively in Chapter 4 of this thesis.

The ability for female and male-specific sensitivities to combine to induce population and species losses should be considered holistically in future studies. Within a natural heatwave scenario, populations exposed to the heat will comprise individuals with varying sexes, mating statuses, and ages, rather than single, isolated focal groups. These overlapping vulnerabilities, as shown in this thesis, may amplify population-level consequences, particularly if survival costs in mated females coincide with reductions in reproductive output resulting from mating with reproductively impaired males. Conversely, compensatory mechanisms may buffer some of these vulnerabilities. Previous research on *T. castaneum* has shown that remating can restore female reproductive output after initially mating with a heatwave-exposed male (Vasudeva et al., 2021). The degree to which these

vulnerabilities and potential compensatory mechanisms may interact at a population level is currently unknown and represents a key next step in understanding responses of insect populations in natural settings.

Future work should also aim to more closely replicate natural heatwave conditions. Laboratory studies often impose simplified, thermally homogeneous environmental conditions, which may limit the behavioural strategies that mitigate thermal impacts (Ma and Ma, 2012). In natural settings, insects may seek more favourable microclimates or employ behavioural thermoregulation, which may be impossible under experimental setups such as the commonly used incubators or water baths. Furthermore, the thermal profile of natural heatwaves will display fluctuations which may produce periods of relatively high stress and periods of thermal relief. Such fluctuations may alter the outcomes of heatwave exposure, enhancing the negative impacts of peaks and allowing for potential recovery during cooler periods, as has been observed on wider time scales (Colinet et al., 2015; Bai et al., 2019). By incorporating these environmental complexities into future studies, resilience or further vulnerabilities to heatwaves may be identified.

In the same vein, future work should also assess the potential for the evolution of compensatory mechanisms to counter the survival and reproductive impacts documented throughout this thesis. Whilst research has shown that the evolutionary potential of upper thermal limits is limited within insects (Kellermann and van Heerwaarden, 2019), it is unclear to what extent species can to evolve to withstand the specific impacts of heatwaves, especially given their transient and unpredictable nature (van de Pol et al., 2017).

9. References

Abram, P.K., Boivin, G., Moiroux, J. and Brodeur, J. (2016). Behavioural effects of temperature on ectothermic animals: unifying thermal physiology and behavioural plasticity. *Biological Reviews*, 92(4), pp.1859–1876.

Anderson, T.R., Hawkins, E. and Jones, P.D. (2016). CO2, the greenhouse effect and global warming: from the pioneering work of Arrhenius and Callendar to today's Earth System Models. *Endeavour*, [online] 40(3), pp.178–187.

Andrews S. (2010). FastQC: a quality control tool for high throughput sequence data. Available online at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc

Appell, R.A., Evans, P.R. and Blandy, J.P. (1977). The Effect of Temperature on the Motility and Viability of Sperm. *British Journal of Urology*, 49(7), pp.751–756.

Argov, N., Moallem, U. and Sklan, D. (2005). Summer heat stress alters the mRNA expression of selective-uptake and endocytotic receptors in bovine ovarian cells. *Theriogenology*, 64(7), pp.1475–1489.

Arora, R. and Sandhu, S. (2017). *Breeding Insect Resistant Crops for Sustainable Agriculture*. Singapore Springer Singapore, pp.45–66.

Bader, C. and Williams, C. (2012). Mating, ovariole number and sperm production of the dengue vector mosquitoAedes aegypti(L.) in Australia: broad thermal optima provide the capacity for survival in a changing climate. *Physiological Entomology*, 37(2), pp.136–144.

Baer, B., Sophie and Boomsma, J.J. (2006). Sperm storage induces an immunity cost in ants. *Nature*, 441(7095), pp.872–875. doi:https://doi.org/10.1038/nature04698.

Bai, C.-M., Ma, G., Cai, W.-Z. and Ma, C.-S. (2019). Independent and combined effects of daytime heat stress and nighttime recovery determine thermal performance. *Biology Open*, 8(3). doi:https://doi.org/10.1242/bio.038141.

Banfi, D., Bianchi, T., Maristella Mastore and Maurizio Francesco Brivio (2025). The Role of Heat Shock Proteins in Insect Stress Response, Immunity, and Climate Adaptation. *Insects*, 16(7), pp.741–741. doi:https://doi.org/10.3390/insects16070741.

Basso, A.L., Forneris, N.S., Filiberti, A., Argaraña, C.E., Rabossi, A. and Quesada-Allué, L.A. (2011). Metamorphosis and Gonad Maturation in the Horn Fly*Haematobia irritans*. *Journal of Insect Science*, 11(174), pp.1–10. doi:https://doi.org/10.1673/031.011.17401.

Bauerfeind, S.S., Sørensen, J.G., Loeschcke, V., Berger, D., Broder, E.D., Geiger, M., Ferrari, M. and Blanckenhorn, W.U. (2018). Geographic variation in responses of European yellow dung flies to thermal stress. *Journal of Thermal Biology*, 73, pp.41–49.

Berger, D., Walters, R. and Gotthard, K. (2008). What limits insect fecundity? Body size- and temperature-dependent egg maturation and oviposition in a butterfly. *Functional Ecology*, 22(3), pp.523–529.

Best, A.R., Lewis, Z., Hurst, G.D.D. and Lizé, A. (2012). Thermal environment during and outside courtship jointly determine female remating rate in Drosophila melanogaster. *Animal Behaviour*, 83(6), pp.1483–1490.

Bettencourt, B.R., Feder, M.E. and Cavicchi, S. (1999). Experimental Evolution of Hsp70 Expression and Thermotolerance in Drosophila melanogaster. *Evolution*, 53(2), p.484. doi:https://doi.org/10.2307/2640784.

Bilinski, S.M., Jaglarz, M.K., Halajian, A. and Tworzydlo, W. (2018). Unusual morphological adaptations and processes associated with viviparity in an epizoic dermapteran. *PLOS ONE*, 13(4), p.e0195647.

Blanckenhorn, W.U., Gautier, R., Nick, M., Puniamoorthy, N. and Schäfer, M.A. (2014). Stage- and sex-specific heat tolerance in the yellow dung fly Scathophaga stercoraria. *Journal of Thermal Biology*, 46, pp.1–9.

Bressac, C., El Sabrout, A., Kifouche, F., Anne, M., Capdevielle-Dulac, C., Mougel, F. and Kaiser, L. (2023). Hot and cold waves decrease sperm production and bias sex ratio in the parasitoid wasp Cotesia typhae (Hymenoptera, Braconidae). *Journal of insect physiology*, [online] 149, p.104553. doi:https://doi.org/10.1016/j.jinsphys.2023.104553.

Cally, J.G., Stuart-Fox, D. and Holman, L. (2019). Meta-analytic evidence that sexual selection improves population fitness. *Nature Communications*, [online] 10(1).

Campbell, J.F., Athanassiou, C.G., Hagstrum, D.W. and Zhu, K.Y. (2022). Tribolium castaneum: A Model Insect for Fundamental and Applied Research. *Annual Review of Entomology*, 67(1), pp.347–365. doi:https://doi.org/10.1146/annurev-ento-080921-075157.

Campion, C., Rajamohan, A. and Dillon, M.E. (2023). Sperm can't take the heat: Short-term temperature exposures compromise fertility of male bumble bees (Bombus impatiens). *Journal of Insect Physiology*, [online] 146, p.104491. doi:https://doi.org/10.1016/j.jinsphys.2023.104491.

Canal Domenech, B. and Fricke, C. (2023). Developmental heat stress interrupts spermatogenesis inducing early male sterility in Drosophila melanogaster. *Journal of Thermal Biology*, [online] 114, p.103589. doi:https://doi.org/10.1016/j.jtherbio.2023.103589.

CaraDonna, P.J., Cunningham, J.L. and Iler, A.M. (2018). Experimental warming in the field delays phenology and reduces body mass, fat content and survival: Implications for the persistence of a pollinator under climate change. *Functional Ecology*, 32(10), pp.2345–2356.

Ceballos, G., Ehrlich, P.R., Barnosky, A.D., García, A., Pringle, R.M. and Palmer, T.M. (2015). Accelerated Modern Human–induced Species losses: Entering the Sixth Mass Extinction. *Science Advances*, 1(5).

Chakir, M., Chafik, A., Moreteau, B., Gilbert, P. and David, J. (2002). Male sterility thermal thresholds in Drosophila: D. simulans appears more cold-adapted than its sibling D. melanogaster. *Genetica*, 114, pp.195–205.

Chapman, T., Liddle, L.F., Kalb, J.M., Wolfner, M.F. and Partridge, L. (1995). Cost of mating in Drosophila melanogaster females is mediated by male accessory gland products. *Nature*, 373(6511), pp.241–244. doi:https://doi.org/10.1038/373241a0.

Chen, H., Zheng, X., Luo, M., Guo, J., Solangi, G.S., Wan, F. and Zhou, Z. (2018). Effect of short-term high-temperature exposure on the life history parameters of Ophraella communa. *Scientific Reports*, 8(1). doi:https://doi.org/10.1038/s41598-018-32262-z.

Chen, I-Ching., Hill, J.K., Shiu, H.-J., Holloway, J.D., Benedick, S., Chey, V.K., Barlow, H.S. and Thomas, C.D. (2010). Asymmetric boundary shifts of tropical montane Lepidoptera over four decades of climate warming. *Global Ecology and Biogeography*, 20(1), pp.34–45.

Cheng, J., Su, Q., Jiao, X., Shi, C., Yang, Y., Han, H., Xie, W., Guo, Z., Wu, Q., Xu, B., Wang, S. and Zhang, Y. (2017). Effects of Heat Shock on the Bradysia odoriphaga (Diptera: Sciaridae). *Journal of Economic Entomology*, 110(4), pp.1630–1638.

Chevin, L.-M. and Hoffmann, A.A. (2017). Evolution of phenotypic plasticity in extreme environments. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 372(1723), p.20160138.

Chirault, M., Lucas, C., Goubault, M., Chevrier, C., Bressac, C. and Lécureuil, C. (2015). A Combined Approach to Heat Stress Effect on Male Fertility in Nasonia vitripennis: From the Physiological Consequences on Spermatogenesis to the Reproductive Adjustment of Females Mated with Stressed Males. *PLOS ONE*, 10(3), p.e0120656.

Christidis, N., Mitchell, D. and Stott, P.A. (2023). Rapidly increasing likelihood of exceeding 50 °C in parts of the Mediterranean and the Middle East due to human influence. *npj Climate and Atmospheric Science*, [online] 6(1), pp.1–12. doi:https://doi.org/10.1038/s41612-023-00377-4.

Cinto Mejía, E. and Wetzel, W.C. (2023). The ecological consequences of the timing of extreme climate events. *Ecology and Evolution*, 13(1). doi:https://doi.org/10.1002/ece3.9661.

Cocroft, R. and Rodriguez, R. (2005). The Behavioral Ecology of Insect Vibrational Communication. *BioScience*, 55(4), p.323.

Colinet, H., Sinclair, B.J., Vernon, P. and Renault, D. (2015). Insects in Fluctuating Thermal Environments. *Annual Review of Entomology*, 60(1), pp.123–140. doi:https://doi.org/10.1146/annurev-ento-010814-021017.

Conrad, T., Stöcker, C. and Ayasse, M. (2017). The effect of temperature on male mating signals and female choice in the red mason bee, Osmia bicornis (L.). *Ecology and Evolution*, 7(21), pp.8966–8975.

Crozier, L.G., Hendry, A.P., Lawson, P.W., Quinn, T.P., Mantua, N.J., Battin, J., Shaw, R.G. and Huey, R.B. (2008). Potential responses to climate change in organisms with complex life histories: evolution and plasticity in Pacific salmon. *Evolutionary Applications*, 1(2), pp.252–270.

Cui, X., Wan, F., Xie, M. and Liu, T. (2008). Effects of heat shock on survival and

reproduction of two whitefly species, Trialeurodes vaporariorum and Bemisia tabaci biotype B. *Journal of Insect Science*, 8(1).

Dada, R. (2017). Sperm DNA damage diagnostics: when and why. *Translational Andrology and Urology*, 6(S4), pp.S691–S694.

David, J.R., Araripe, L.O., Chakir, M., Legout, H., Lemos, B., Petavy, G., Rohmer, C., Jolu, D. and Moreteau, B. (2005). Male sterility at extreme temperatures: a significant but neglected phenomenon for understanding Drosophila climatic adaptations. *Journal of Evolutionary Biology*, 18(4), pp.838–846.

Davies, Z., Wilson, R., Coles, S. and Thomas, C. (2006). Changing habitat associations of a thermally constrained species, the silver-spotted skipper butterfly, in response to climate warming. *Journal of Animal Ecology*, 75(1), pp.247–256.

Dawson, P.S. (1977). Life History Strategy and Evolutionary History of Tribolium Flour Beetles. *Evolution*, 31(1), p.226. doi:https://doi.org/10.2307/2407562.

Delisle, J., Bernier-Cardou, M. and Laroche, G. (2016). Reproductive performance of the hemlock looper,Lambdina fiscellaria, as a function of temperature and population origin. *Entomologia Experimentalis et Applicata*, 161(3), pp.219–231.

Denis, D., van Baaren, J., Pierre, J.-S. and Wajnberg, E. (2013). Evolution of a physiological trade-off in a parasitoid wasp: how best to manage lipid reserves in a warming environment. *Entomologia Experimentalis et Applicata*, 148(1), pp.27–38.

Deschamps, M., Giménez, L., Astley, C., Boersma, M. and Torres, G. (2025). Heatwave duration, intensity and timing as drivers of performance in larvae of a marine invertebrate. *Scientific Reports*, [online] 15(1). doi:https://doi.org/10.1038/s41598-025-98259-7.

Deutsch, C.A., Tewksbury, J.J., Huey, R.B., Sheldon, K.S., Ghalambor, C.K., Haak, D.C. and Martin, P.R. (2008). Impacts of climate warming on terrestrial ectotherms across latitude. *Proceedings of the National Academy of Sciences*, 105(18), pp.6668–6672.

Dick, C.A., Rank, N.E., McCarthy, M., McWeeney, S., Hollis, D. and Dahlhoff, E.P. (2013). Effects of Temperature Variation on Male Behavior and Mating Success in a Montane Beetle. *Physiological and Biochemical Zoology*, 86(4), pp.432–440.

Dickinson, M. (2018). *The impacts of heat-wave conditions on reproduction in a*

model insect, Tribolium castaneum. Doctoral thesis.

Dougherty, L.R., Frost, F., Maenpaa, M.I., Rowe, M., Cole, B.J., Vasudeva, R., Pottier, P., Schultner, E., Macartney, E.L., Lindenbaum, I., Smith, J.L., Carazo, P., Graziano, M., Weaving, H., Canal Domenech, B., Berger, D., Meena, A., Tom Rhys Bishop, Daniel and Simões, P. (2024). A systematic map of studies testing the relationship between temperature and animal reproduction. *Ecological solutions and evidence*, 5(1). doi:https://doi.org/10.1002/2688-8319.12303.

Du, S.S., Ashok Agarwal and Sabanegh, E.S. (2014). *Male infertility a complete guide to lifestyle and environmental factors*. New York, Ny Springer, pp.105–125.

Dumser, J.B. (1980). The Regulation of Spermatogenesis in Insects. *Annual Review of Entomology*, 25(1), pp.341–369. doi:https://doi.org/10.1146/annurev.en.25.010180.002013.

Dunn, R. (2005). Modern Insect Extinctions, the Neglected Majority. *Conservation Biology*, 19(4), pp.1030–1036.

Evans, J.P., Lymbery, R.A., Wiid, K.S., Rahman, Md.M. and Gasparini, C. (2017). Sperm as moderators of environmentally induced paternal effects in a livebearing fish. *Biology Letters*, 13(4), p.20170087.

Ewels P, Magnusson M, Lundin S, Käller M. MultiQC: summarize analysis results for multiple tools and samples in a single report. Bioinformatics. 2016; 32(19): 3047-3048.

Fabian, L. and Brill, J.A. (2012). Drosophila spermiogenesis. *Spermatogenesis*, 2(3), pp.197–212. doi:https://doi.org/10.4161/spmg.21798.

Feder, M.E. and Hofmann, G.E. (1999). HEAT-SHOCK PROTEINS, MOLECULAR CHAPERONES, AND THE STRESS RESPONSE: Evolutionary and Ecological Physiology. *Annual Review of Physiology*, 61(1), pp.243–282. doi:https://doi.org/10.1146/annurev.physiol.61.1.243.

Forister, M.L., Halsch, C.A., Nice, C.C., Fordyce, J.A., Dilts, T.E., Oliver, J.C., Prudic, K.L., Shapiro, A.M., Wilson, J.K. and Glassberg, J. (2021). Fewer butterflies seen by community scientists across the warming and drying landscapes of the American West. *Science*, 371(6533), pp.1042–1045.

Franke, K., Heitmann, N., Tobner, A. and Fischer, K. (2014). Fitness costs

associated with different frequencies and magnitudes of temperature change in the butterfly Bicyclus anynana. *Journal of Thermal Biology*, 41, pp.88–94.

Frankham, R. (2005). Genetics and extinction. *Biological Conservation*, 126(2), pp.131–140.

Frich, P., Alexander, L., Della-Marta, P., Gleason, B., Haylock, M., Klein Tank, A. and Peterson, T. (2002). Observed coherent changes in climatic extremes during the second half of the twentieth century. *Climate Research*, 19(1), pp.193–212.

Friedländer, M. (1997). Control of the eupyrene–apyrene sperm dimorphism in Lepidoptera. *Journal of Insect Physiology*, 43(12), pp.1085–1092.

Frölicher, T.L., Fischer, E.M. and Gruber, N. (2018). Marine heatwaves under global warming. *Nature*, 560(7718), pp.360–364.

Galil, K.A.A. and Setchell, B.P. (1988). Effects of local heating of the testis on testicular blood flow and testosterone secretion in the rat. *International Journal of Andrology*, 11(1), pp.73–85.

Gallai, N. and Vaiisiere, B.E. (2009). *Guidelines for the economic valuation of pollination services at a national scale.* Rome: FAO.

Gallinat, A.S., Primack, R.B. and Wagner, D.L. (2015). Autumn, the neglected season in climate change research. *Trends in Ecology & Evolution*, 30(3), pp.169–176.

Gandara, A. and Drummond-Barbosa, D. (2023). Chronic exposure to warm temperature causes low sperm abundance and quality in Drosophila melanogaster. *Scientific Reports*, 13(1). doi:https://doi.org/10.1038/s41598-023-39360-7.

García-Robledo, C. and Baer, C.S. (2021). Positive genetic covariance and limited thermal tolerance constrain tropical insect responses to global warming. *Journal of Evolutionary Biology*, 34(9), pp.1432–1446. doi:https://doi.org/10.1111/jeb.13905.

Garnett, T. (2009). Livestock-related greenhouse gas emissions: impacts and options for policy makers. *Environmental Science & Policy*, [online] 12(4), pp.491–503.

Gasparini, C., Lu, C., Dingemanse, N.J. and Tuni, C. (2017). Paternal-effects in a terrestrial ectotherm are temperature dependent but no evidence for adaptive

effects. *Functional Ecology*, 32(4), pp.1011–1021.

Gault, C.R., Obeid, L.M. and Hannun, Y.A. (2010). An overview of sphingolipid metabolism: from synthesis to breakdown. *Advances in experimental medicine and biology*, [online] 688, pp.1–23. doi:https://doi.org/10.1007/978-1-4419-6741-1_1.

Gilbert, K.J., Peischl, S. and Excoffier, L. (2018). Mutation load dynamics during environmentally-driven range shifts. *PLOS Genetics*, 14(9), p.e1007450.

Glatz, J., du Plessis, H. and Van den Berg, J. (2016). The effect of temperature on the development and reproduction of Busseola fusca (Lepidoptera: Noctuidae). *Bulletin of Entomological Research*, 107(1), pp.39–48.

Godfray, H.C.J. and Garnett, T. (2014). Food security and sustainable intensification. *Philosophical Transactions of the Royal Society B: Biological Sciences*, [online] 369(1639), pp.20120273–20120273.

Gong, Y., Guo, H., Zhang, Z., Zhou, H., Zhao, R. and He, B. (2017). Heat Stress Reduces Sperm Motility via Activation of Glycogen Synthase Kinase-3α and Inhibition of Mitochondrial Protein Import. *Frontiers in Physiology*, 8.

González-Tokman, D., Córdoba-Aguilar, A., Dáttilo, W., Lira-Noriega, A., Sánchez-Guillén, R.A. and Villalobos, F. (2020). Insect responses to heat: physiological mechanisms, evolution and ecological implications in a warming world. *Biological Reviews*, 95(3), pp.802–821. doi:https://doi.org/10.1111/brv.12588.

Grandela, A., Antunes, M.A., Santos, M.A., Matos, M., Rodrigues, L.R. and Simões, P. (2023). Detrimental impact of a heatwave on male reproductive behaviour and fertility. *Acta Ethologica*, 27. doi:https://doi.org/10.1007/s10211-023-00431-7.

Grazer, V.M. and Martin, O.Y. (2011). Elevated temperature changes female costs and benefits of reproduction. *Evolutionary Ecology*, 26(3), pp.625–637.

Grimaldi, D.A. and Engel, M.S. (2005). *Evolution of the insects*. Cambridge U.K. ; New York: Cambridge University Press.

Hallmann, C.A., Sorg, M., Jongejans, E., Siepel, H., Hofland, N., Schwan, H., Stenmans, W., Müller, A., Sumser, H., Hörren, T., Goulson, D. and de Kroon, H. (2017). More than 75 percent decline over 27 years in total flying insect biomass in protected areas. *PLOS ONE*, [online] 12(10), p.e0185809.

Hamilton, T.R. dos S., Mendes, C.M., Castro, L.S. de, Assis, P.M. de, Siqueira, A.F.P., Delgado, J. de C., Goissis, M.D., Muiño-Blanco, T., Cebrián-Pérez, J.Á., Nichi, M., Visintin, J.A. and Assumpção, M.E.O.D. (2016). Evaluation of Lasting Effects of Heat Stress on Sperm Profile and Oxidative Status of Ram Semen and Epididymal Sperm. *Oxidative Medicine and Cellular Longevity*, 2016, pp.1–12.

Hangartner, S., Sgrò, C.M., Connallon, T. and Booksmythe, I. (2022). Sexual dimorphism in phenotypic plasticity and persistence under environmental change: An extension of theory and meta-analysis of current data. *Ecology Letters*. doi:https://doi.org/10.1111/ele.14005.

Hansen, P.J. (2009). Effects of heat stress on mammalian reproduction. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 364(1534), pp.3341–3350.

Harada, T., Takenaka, S., Maihara, S., Ito, K. and Tamura, T. (2011). Changes in life-history traits of the water strider Aquarius paludum in accordance with global warming. *Physiological Entomology*, 36(4), pp.309–316.

Harshman, L.G. and Zera, A.J. (2007). The cost of reproduction: the devil in the details. *Trends in Ecology & Evolution*, [online] 22(2), pp.80–86. doi:https://doi.org/10.1016/j.tree.2006.10.008.

Hart, A.G., Hesselberg, T., Nesbit, R. and Goodenough, A.E. (2017). The spatial distribution and environmental triggers of ant mating flights: using citizen-science data to reveal national patterns. *Ecography*, 41(6), pp.877–888. doi:https://doi.org/10.1111/ecog.03140.

Harvey, J.A., Heinen, R., Gols, R. and Thakur, M.P. (2020). Climate change-mediated temperature extremes and insects: from outbreaks to breakdowns. *Global Change Biology*, 26(12). doi:https://doi.org/10.1111/gcb.15377.

Hayes, T. and López-Martínez, G. (2021). Resistance and survival to extreme heat shows circadian and sex-specific patterns in A cavity nesting bee. *Current Research in Insect Science*, 1, p.100020. doi:https://doi.org/10.1016/j.cris.2021.100020.

Hayward, A. and Gillooly, J.F. (2011). The Cost of Sex: Quantifying Energetic Investment in Gamete Production by Males and Females. *PLoS ONE*, [online] 6(1), p.e16557.

Hegland, S.J., Nielsen, A., Lázaro, A., Bjerknes, A.-L. and Totland, Ø. (2009). How

does climate warming affect plant-pollinator interactions? *Ecology Letters*, 12(2), pp.184–195.

Herndon, N., Shelton, J., Gerischer, L., Ioannidis, P., Ninova, M., Dönitz, J., Waterhouse, R.M., Liang, C., Damm, C., Siemanowski, J., Kitzmann, P., Ulrich, J., Dippel, S., Oberhofer, G., Hu, Y., Schwirz, J., Schacht, M., Lehmann, S., Montino, A. and Posnien, N. (2020). Enhanced genome assembly and a new official gene set for Tribolium castaneum. *BMC Genomics*, 21(1). doi:https://doi.org/10.1186/s12864-019-6394-6.

Hill, J.K., Griffiths, H.M. and Thomas, C.D. (2011). Climate Change and Evolutionary Adaptations at Species' Range Margins. *Annual Review of Entomology*, 56(1), pp.143–159.

Hill, J.K., Thomas, C.D., Fox, R., Telfer, M.G., Willis, S.G., Asher, J. and Huntley, B. (2002). Responses of butterflies to twentieth century climate warming: implications for future ranges. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, 269(1505), pp.2163–2171.

Hinton, H.E. (1948). A Synopsis of the Genus *Tribolium* Macleay, with some Remarks on the Evolution of its Species-groups (Coleoptera, Tenebrionidae). *Bulletin of Entomological Research*, 39(1), pp.13–55. doi:https://doi.org/10.1017/s0007485300024287.

Hoffmann, A.A. (2010). Physiological climatic limits in Drosophila: patterns and implications. *Journal of Experimental Biology*, 213(6), pp.870–880.

Holmes, T.R.H., Crow, W.T., Hain, C., Anderson, M.C. and Kustas, W.P. (2015). Diurnal temperature cycle as observed by thermal infrared and microwave radiometers. *Remote Sensing of Environment*, 158, pp.110–125. doi:https://doi.org/10.1016/j.rse.2014.10.031.

Hu, J., Medison, R.G., Zhang, S., Ma, P. and Shi, C. (2022). Impacts of Non-Lethal High-Temperature Stress on the Development and Reproductive Organs of Bradysia odoriphaga. *Insects*, 13(1), p.74. doi:https://doi.org/10.3390/insects13010074.

Huey, R.B., Kearney, M.R., Krockenberger, A., Holtum, J.A.M., Jess, M. and Williams, S.E. (2012). Predicting organismal vulnerability to climate warming: roles of behaviour, physiology and adaptation. *Philosophical Transactions of the Royal*

Society B: Biological Sciences, [online] 367(1596), pp.1665–1679.

Humphries, S. (2013). A physical explanation of the temperature dependence of physiological processes mediated by cilia and flagella. *Proceedings of the National Academy of Sciences*, 110(36), pp.14693–14698.

Iltis, C., Moreau, J., Pecharová, K., Thiéry, D. and Louâpre, P. (2020). Reproductive performance of the European grapevine moth Lobesia botrana (Tortricidae) is adversely affected by warming scenario. *Journal of Pest Science*, 93(2), pp.679–689.

Iossa, G. (2019). Sex-Specific Differences in Thermal Fertility Limits. *Trends in Ecology & Evolution*, 34(6), pp.490–492. doi:https://doi.org/10.1016/j.tree.2019.02.016.

Iossa, G., Maury, C., Fletcher, R.M. and Eady, P.E. (2019). Temperature-induced developmental plasticity inPlodia interpunctella: Reproductive behaviour and sperm length. *Journal of Evolutionary Biology*.

IPCC (2013). Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change [Stocker, T.F., D. Qin, G.-K. Plattner, M. Tignor, S.K. Allen, J. Boschung, A. Nauels, Y. Xia, V. Bex and P.M. Midgley (eds.)]. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA, 1535 pp.

IPCC (2014) Climate Change 2014: Synthesis Report. Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change [Core Writing Team, R.K. Pachauri and L.A. Meyer (eds.)]. IPCC, Geneva, Switzerland, 151 pp.

IPCC (2023) Climate Change 2023: Synthesis Report. Contribution of Working Groups I, II and III to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change [Core Writing Team, H. Lee and J. Romero (eds.)]. IPCC, Geneva, Switzerland, pp. 1-34.

Jepsen, J.U., Hagen, S.B., Ims, R.A. and Yoccoz, N.G. (2008). Climate change and outbreaks of the geometrids Operophtera brumata and Epirrita autumnata in subarctic birch forest: evidence of a recent outbreak range expansion. *Journal of Animal Ecology*, 77(2), pp.257–264.

Jerbi-Elayed, M., Lebdi-Grissa, K., Foray, V., Muratori, F. and Hance, T. (2015). Using multiple traits to estimate the effects of heat shock on the fitness ofAphidius colemani. *Entomologia Experimentalis et Applicata*, 155(1), pp.18–27.

Jia, D., Yuan, X., Liu, Y., Xu, C., Wang, Y., Gao, L. and Ma, R. (2018). Heat sensitivity of eggs attributes to the reduction in Agasicles hygrophila population. *Insect Science*, 27(1), pp.159–169.

Jocson, D.M.I., Smeester, M.E., Leith, N.T., Macchiano, A. and Fowler-Finn, K.D. (2019). Temperature coupling of mate attraction signals and female mate preferences in four populations of Enchenopa treehopper (Hemiptera: Membracidae). *Journal of Evolutionary Biology*, 32(10), pp.1046–1056.

Johnson, C.A., Coutinho, R.M., Berlin, E., Dolphin, K.E., Heyer, J., Kim, B., Leung, A., Sabellon, J.L. and Amarasekare, P. (2015). Effects of temperature and resource variation on insect population dynamics: the bordered plant bug as a case study. *Functional Ecology*, 30(7), pp.1122–1131.

Jørgensen, K.T., Sørensen, J.G. and Bundgaard, J. (2006). Heat tolerance and the effect of mild heat stress on reproductive characters in Drosophila buzzatii males. *Journal of Thermal Biology*, 31(3), pp.280–286.

Jørgensen, L.B., Malte, H. and Overgaard, J. (2019). How to assess Drosophila heat tolerance: Unifying static and dynamic tolerance assays to predict heat distribution limits. *Functional Ecology*, 33(4), pp.629–642. doi:https://doi.org/10.1111/1365-2435.13279.

Jung, J.-M., Byeon, D., Kim, S.-H., Jung, S. and Lee, W.-H. (2020). Estimating economic damage to cocoa bean production with changes in the spatial distribution of Tribolium castaneum (Herbst) (Coleoptera: Tenebrionidae) in response to climate change. *Journal of Stored Products Research*, 89, p.101681.

Kaspari, M., Clay, N.A., Lucas, J., Yanoviak, S.P. and Kay, A. (2014). Thermal adaptation generates a diversity of thermal limits in a rainforest ant community. *Global Change Biology*, 21(3), pp.1092–1102.

Keena, M.A. (2006). Effects of Temperature onAnoplophora glabripennis(Coleoptera: Cerambycidae) Adult Survival, Reproduction, and Egg Hatch. *Environmental Entomology*, 35(4), pp.912–921.

Keller, L. and Chapuisat, M. (2017). Eusociality and Cooperation. *eLS*, pp.1–7. doi:https://doi.org/10.1002/9780470015902.a0003670.pub3.

Kellermann, V. and van Heerwaarden, B. (2019). Terrestrial insects and climate change: adaptive responses in key traits. *Physiological Entomology*, 44(2), pp.99–115.

Kellermann, V., Overgaard, J., Hoffmann, A.A., Flojgaard, C., Svenning, J.-C. . and Loeschcke, V. (2012). Upper thermal limits of Drosophila are linked to species distributions and strongly constrained phylogenetically. *Proceedings of the National Academy of Sciences*, 109(40), pp.16228–16233.

Keret, N.M., Mutanen, M.J., Orell, M.I., Itämies, J.H. and Välimäki, P.M. (2020). Climate change-driven elevational changes among boreal nocturnal moths. *Oecologia*, 192(4), pp.1085–1098.

Kerr, J.T., Pindar, A., Galpern, P., Packer, L., Potts, S.G., Roberts, S.M., Rasmont, P., Schweiger, O., Colla, S.R., Richardson, L.L., Wagner, D.L., Gall, L.F., Sikes, D.S. and Pantoja, A. (2015). Climate change impacts on bumblebees converge across continents. *Science*, [online] 349(6244), pp.177–180.

Khan, S. and Mishra, R.K. (2024). Multigenerational Effect of Heat Stress on the *Drosophila melanogaster* Sperm Proteome. *Journal of Proteome Research*, [online] 23(6), pp.2265–2278. doi:https://doi.org/10.1021/acs.jproteome.4c00205.

Kim D, Paggi JM, Park C, Bennett C, Salzberg SL. Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype. Nature Biotechnology. 2019; 37: 907-915.

Kim, K.E., Jang, T. and Lee, K.P. (2020). Combined effects of temperature and macronutrient balance on life-history traits in Drosophila melanogaster: implications for life-history trade-offs and fundamental niche. *Oecologia*, 193.

Kingsolver, J.G., Diamond, S.E. and Buckley, L.B. (2013). Heat stress and the fitness consequences of climate change for terrestrial ectotherms. *Functional Ecology*, 27(6), pp.1415–1423.

Kirkpatrick, M. and Ryan, M.J. (1991). The evolution of mating preferences and the paradox of the lek. *Nature*, 350(6313), pp.33–38.

Klein, A.-M., Vaissière, B.E., Cane, J.H., Steffan-Dewenter, I., Cunningham, S.A.,

Kremen, C. and Tscharntke, T. (2007). Importance of pollinators in changing landscapes for world crops. *Proceedings of the Royal Society B: Biological Sciences*, [online] 274(1608), pp.303–313.

Kolanowska, M. (2025). Climate change will cause the spatial mismatch between sexually deceptive beetle daisy (Gorteria diffusa, Asteraceae) and its pollinator. *Scientific Reports*, 15(1). doi:https://doi.org/10.1038/s41598-025-06976-w.

Kolberg L, Raudvere U, Kuzmin I, Alder P, Vilo J, Peterson H. g:Profiler—interoperable web service for functional enrichment analysis and gene identifier mapping (2023 update). Nucleic Acids Research. 2023; 51(1): 207-212.

Kollberg, I., Bylund, H., Schmidt, A., Gershenzon, J. and Bjorkman, C. (2013). Multiple effects of temperature, photoperiod and food quality on the performance of a pine sawfly. *Ecological Entomology*, 38(2), pp.201–208.

Kosmidis I (2023). _brglm2: Bias Reduction in Generalized Linear Models_. R package version 0.9.2, <https://CRAN.R-project.org/package=brglm2>.

Krog Noer, N., Lehmann Nielsen, K., Sverrisdóttir, E., Nygaard Kristensen, T. and Bahrndorff, S. (2023). Temporal regulation of temperature tolerances and gene expression in an arctic insect. *Journal of experimental biology*, 226(11). doi:https://doi.org/10.1242/jeb.245097.

Leader, D.P., Naseem, M.T. and Halberg, K.V. (2024). BeetleAtlas: An Ontogenetic and Tissue-specific Transcriptomic Atlas of the Red Flour Beetle Tribolium castaneum. *Journal of Molecular Biology*, 436(17), p.168520. doi:https://doi.org/10.1016/j.jmb.2024.168520.

Lehmann, P., Ammunét, T., Barton, M., Battisti, A., Eigenbrode, S.D., Jepsen, J.U., Kalinkat, G., Neuvonen, S., Niemelä, P., Terblanche, J.S., Økland, B. and Björkman, C. (2020). Complex responses of global insect pests to climate warming. *Frontiers in Ecology and the Environment*, 18(3), pp.141–150.

Leith, N.T., Miller, E.A. and Fowler-Finn, K.D. (2024). Thermoregulation enhances survival but not reproduction in a plant-feeding insect. *Functional ecology*, 38(6), pp.1344–1356. doi:https://doi.org/10.1111/1365-2435.14546.

Lenth R (2025). emmeans: Estimated Marginal Means, aka Least-Squares Means. R package version 1.11.2-80003, https://rvlenth.github.io/emmeans/.

Lewis, R., Pointer, M.D., Friend, L., Gage, M. and Spurgin, L.G. (2024). Tests of evolutionary and genetic rescue using flour beetles, *Tribolium castaneum*, experimentally evolved to thermal conditions. *Ecology and Evolution*, [online] 14(5). doi:https://doi.org/10.1002/ece3.11313.

Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R. The Sequence Alignment/Map format and SAMtools. Bioinformatics. 2009; 25(16): 2078-2079.

Li, H., Li, S., Chen, J., Dai, L., Chen, R., Ye, J. and Hao, D. (2022). A heat shock 70kDa protein MaltHSP70-2 contributes to thermal resistance in Monochamus alternatus (Coleoptera: Cerambycidae): quantification, localization, and functional analysis. *BMC Genomics*, 23(1). doi:https://doi.org/10.1186/s12864-022-08858-1.

Li, H., Li, S., Yang, H., Tan, Y., Zhao, P., Ye, J. and Hao, D. (2024). The fertility recovering from heat stress and interactions of heat shock protein 20 with reproduction-related proteins in *Monochamus alternatus*. *Insect Science*. doi:https://doi.org/10.1111/1744-7917.13470.

Li, H., Zhao, X., Qiao, H., He, X., Tan, J. and Hao, D. (2020). Comparative Transcriptome Analysis of the Heat Stress Response in Monochamus alternatus Hope (Coleoptera: Cerambycidae). *Frontiers in Physiology*, 10. doi:https://doi.org/10.3389/fphys.2019.01568.

Li, R., Xiao, Y., Li, K. and Tian, L. (2022). Transcription and Post-translational Regulation of Autophagy in Insects. *Frontiers in Physiology*, 13. doi:https://doi.org/10.3389/fphys.2022.825202.

Lister, B.C. and Garcia, A. (2018). Climate-driven declines in arthropod abundance restructure a rainforest food web. *Proceedings of the National Academy of Sciences*, 115(44), pp.E10397–E10406.

Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biology. 2014; 5(12): 550.

Lüdecke et al., (2021). performance: An R Package for Assessment, Comparison and Testing of Statistical Models. Journal of Open Source Software, 6(60), 3139. https://doi.org/10.21105/joss.03139

Lue, Y.-H., Sinha Hikim, A.P., Swerdloff, R.S., Im, P., Taing, K.S., Bui, T., Leung, A. and Wang, C. (1999). Single Exposure to Heat Induces Stage-Specific Germ Cell

Apoptosis in Rats: Role of Intratesticular Testosterone on Stage Specificity. *Endocrinology*, 140(4), pp.1709–1717.

Lv, W., Jiang, C., Wang, F., Liu, H., Stanley, D. and Zhang, L. (2024). Experimental heatwaves impair male fertility in oriental armyworm, *Mythimna separata*. *Entomologia Generalis*, 44(4). doi:https://doi.org/10.1127/entomologia/2024/2476.

Lymbery, R.A., Evans, J.P. and Kennington, W.J. (2020). Post-ejaculation thermal stress causes changes to the RNA profile of sperm in an external fertilizer. *Proceedings of the Royal Society B: Biological Sciences*, 287(1938), p.20202147.

Ma, C.-S., Ma, G. and Pincebourde, S. (2020). Survive a Warming Climate: Insect Responses to Extreme High Temperatures. *Annual Review of Entomology*, 66(1). doi:https://doi.org/10.1146/annurev-ento-041520-074454.

Ma, G. and Ma, C.-S. (2012). Climate warming may increase aphids' dropping probabilities in response to high temperatures. *Journal of Insect Physiology*, 58(11), pp.1456–1462.

Ma, G., Bai, C.-M., Wang, X.-J., Majeed, M.Z. and Ma, C.-S. (2018). Behavioural thermoregulation alters microhabitat utilization and demographic rates in ectothermic invertebrates. *Animal Behaviour*, 142, pp.49–57.

Maldonado, M.B., Aranibar, J.N., Serrano, A.M., Chacoff, N.P. and Vázquez, D.P. (2019). Dung beetles and nutrient cycling in a dryland environment. *CATENA*, 179, pp.66–73.

Martin, M. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet.Journal. 2011;17: 10–12

Martinet, B., Zambra, E., Przybyla, K., Lecocq, T., Anselmo, A., Nonclercq, D., Rasmont, P., Michez, D. and Hennebert, E. (2020). Mating under climate change: Impact of simulated heatwaves on the reproduction of model pollinators. *Functional Ecology*, 35(3), pp.739–752. doi:https://doi.org/10.1111/1365-2435.13738.

McAfee, A., Chapman, A., Higo, H., Underwood, R., Milone, J., Foster, L.J., Guarna, M.M., Tarpy, D.R. and Pettis, J.S. (2020). Vulnerability of honey bee queens to heat-induced loss of fertility. *Nature Sustainability*, 3(5), pp.367–376.

McCain, C.M. and Garfinkel, C.F. (2021). Climate change and elevational range shifts in insects. *Current Opinion in Insect Science*, 47.

doi:https://doi.org/10.1016/j.cois.2021.06.003.

McGraw, L.A., Gibson, G., Clark, A.G. and Wolfner, M.F. (2004). Genes Regulated by Mating, Sperm, or Seminal Proteins in Mated Female Drosophila melanogaster. *Current Biology*, 14(16), pp.1509–1514. doi:https://doi.org/10.1016/j.cub.2004.08.028.

Meehl, G.A. (2004). More Intense, More Frequent, and Longer Lasting Heat Waves in the 21st Century. *Science*, 305(5686), pp.994–997.

Meena, A., De Nardo, A., Maggu, K., Sbilordo, S.H., Roy, J., Snook, R.R. and Lüpold, S. (2024a). Fertility loss and recovery dynamics after repeated heat stress across life stages in male *Drosophila melanogaster* : patterns and processes. *Royal Society Open Science*, 11(10). doi:https://doi.org/10.1098/rsos.241082.

Meena, A., Maggu, K., De Nardo, A.N, Kovalov, V., Eggs, B. and Lüpold, S. (2025). Stage-specific and cumulative effects of heat stress on male and female reproductive performance in Drosophila melanogaster. *Journal of Thermal Biology*, 132, pp.104213–104213. doi:https://doi.org/10.1016/j.jtherbio.2025.104213.

Meena, A., Maggu, K., De Nardo, A.N., Sbilordo, S.H., Eggs, B., Al Toma Sho, R. and Lüpold, S. (2024). Life stage-specific effects of heat stress on spermatogenesis and oogenesis in Drosophila melanogaster. *Journal of Thermal Biology*, [online] 125, p.104001. doi:https://doi.org/10.1016/j.jtherbio.2024.104001.parra

Meester, L.D., Stoks, R. and Brans, K.I. (2018). Genetic adaptation as a biological buffer against climate change: Potential and limitations. *Integrative Zoology*, 13(4), pp.372–391.

Menéndez, R., González-Megías, A., Jay-Robert, P. and Marquéz-Ferrando, R. (2013). Climate change and elevational range shifts: evidence from dung beetles in two European mountain ranges. *Global Ecology and Biogeography*, 23(6), pp.646–657.

Merkey, A.B., Wong, C.K., Hoshizaki, D.K. and Gibbs, A.G. (2011). Energetics of metamorphosis in Drosophila melanogaster. *Journal of Insect Physiology*, 57(10), pp.1437–1445.

Mieusset, R. and Bujan, L. (1995). Testicular heating and its possible contributions to male infertility: a review. *International Journal of Andrology*, 18(4), pp.169–184.

Monro, K. and Marshall, D.J. (2016). Unravelling anisogamy: egg size and ejaculate size mediate selection on morphology in free-swimming sperm. *Proceedings of the Royal Society B: Biological Sciences*, 283(1834), p.20160671.

Morton, E.M. and Rafferty, N.E. (2017). Plant–Pollinator Interactions Under Climate Change: The Use of Spatial and Temporal Transplants. *Applications in Plant Sciences*, 5(6), p.1600133.

Nanfack-Minkeu, F. and Sirot, L.K. (2022). Effects of Mating on Gene Expression in Female Insects: Unifying the Field. *Insects*, 13(1), p.69. doi:https://doi.org/10.3390/insects13010069.

Neelam Porwal, Parrett, J.M., Agnieszka Szubert Kruszynska, Pandey, N., Knell, R.J., Cameron, T.C. and Radwan, J. (2025). Fighting Through the Heat: How Sexual Selection Influences Demography Under Recurrent Heatwaves. *bioRxiv (Cold Spring Harbor Laboratory)*. doi:https://doi.org/10.1101/2025.03.31.646346.

Nguyen, A.D., Gotelli, N.J. and Helms Cahan, S. (2016). The evolution of heat shock protein sequences, cis-regulatory elements, and expression profiles in the eusocial Hymenoptera. *BMC Evolutionary Biology*, 16(1). doi:https://doi.org/10.1186/s12862-015-0573-0.

Novais, S.M.A., Nunes, C.A., Santos, N.B., D`Amico, A.R., Fernandes, G.W., Quesada, M., Braga, R.F. and Neves, A.C.O. (2018). Correction: Effects of a Possible Pollinator Crisis on Food Crop Production in Brazil. *PLOS ONE*, 13(5), p.e0197396.

Novotny, V. and Miller, S.E. (2014). Mapping and understanding the diversity of insects in the tropics: past achievements and future directions. *Austral Entomology*, 53(3), pp.259–267.

Oliveira, A., Lopes, A., Correia, E., Niza, S. and Soares, A. (2021). Heatwaves and Summer Urban Heat Islands: A Daily Cycle Approach to Unveil the Urban Thermal Signal Changes in Lisbon, Portugal. *Atmosphere*, 12(3), p.292. doi:https://doi.org/10.3390/atmos12030292.

Oliver, E.C.J., Donat, M.G., Burrows, M.T., Moore, P.J., Smale, D.A., Alexander, L.V., Benthuysen, J.A., Feng, M., Sen Gupta, A., Hobday, A.J., Holbrook, N.J., Perkins-Kirkpatrick, S.E., Scannell, H.A., Straub, S.C. and Wernberg, T. (2018). Longer and more frequent marine heatwaves over the past century. *Nature*

Communications, 9(1).

Ørsted, M., Willot, Q., Olsen, A.K., Viktor Kongsgaard and Overgaard, J. (2024). Thermal limits of survival and reproduction depend on stress duration: A case study of *Drosophila suzukii*. *Ecology Letters*, 27(3). doi:https://doi.org/10.1111/ele.14421.

Pai, A. and Yan, G. (2003). Rapid female multiple mating in red flour beetles (Tribolium castaneum). *Canadian Journal of Zoology*, 81(5), pp.888–896. doi:https://doi.org/10.1139/z03-070.

Paoletti, E., Schaub, M., Matyssek, R., Wieser, G., Augustaitis, A., Bastrup-Birk, A.M., Bytnerowicz, A., Günthardt-Goerg, M.S., Müller-Starck, G. and Serengil, Y. (2010). Advances of air pollution science: From forest decline to multiple-stress effects on forest ecosystem services. *Environmental Pollution*, 158(6), pp.1986–1989.

Park, T., Mertz, D., Grodzinski, W. and Prus, T. (1965). Cannibalistic Predation in Populations of Flour Beetles. *Ecological and Evolutionary Physiology*, 38(3). doi:https://doi-org.uea.idm.oclc.org/10.1086/physzool.38.3.30152840.

Park, Y., Aikins, J., Wang, L.J., Beeman, R.W., Oppert, B., Lord, J.C., Brown, S.J., Lorenzen, M.D., Richards, S., Weinstock, G.M. and Gibbs, R.A. (2008). Analysis of transcriptome data in the red flour beetle, Tribolium castaneum. *Insect Biochemistry and Molecular Biology*, [online] 38(4), pp.380–386. doi:https://doi.org/10.1016/j.ibmb.2007.09.008.

Parmesan, C. (2006). Ecological and Evolutionary Responses to Recent Climate Change. *Annual Review of Ecology, Evolution, and Systematics*, 37(1), pp.637–669.

Parmesan, C., Root, T.L. and Willig, M.R. (2000). Impacts of Extreme Weather and Climate on Terrestrial Biota*. *Bulletin of the American Meteorological Society*, 81(3), pp.443–450.

Parratt, S.R., Walsh, B.S., Metelmann, S., White, N., Manser, A., Bretman, A.J., Hoffmann, A.A., Snook, R.R. and Price, T.A.R. (2021). Temperatures that sterilize males better match global species distributions than lethal temperatures. *Nature Climate Change*, [online] 11(6), pp.481–484. doi:https://doi.org/10.1038/s41558-021-01047-0.

Parrett, J.M. and Knell, R.J. (2018). The effect of sexual selection on adaptation and

extinction under increasing temperatures. *Proceedings of the Royal Society B: Biological Sciences*, 285(1877), p.20180303.

Pascini, T.V. and Martins, G.F. (2017). The insect spermatheca: an overview. *Zoology*, 121, pp.56–71. doi:https://doi.org/10.1016/j.zool.2016.12.001.

Pauls, S.U., Nowak, C., Bálint, M. and Pfenninger, M. (2012). The impact of global climate change on genetic diversity within populations and species. *Molecular Ecology*, 22(4), pp.925–946.

Pembury Smith, M., Latkova, L. and Snook, R.R. (2025). Elevated temperatures have sex-specific effects on nuptial gift behavior. *Behavioral Ecology*, 36(4). doi:https://doi.org/10.1093/beheco/araf049.

Perkins-Kirkpatrick, S.E. and Lewis, S.C. (2020). Increasing trends in regional heatwaves. *Nature Communications*, 11(1). doi:https://doi.org/10.1038/s41467-020-16970-7.

Peterson, A.T., Martínez-Campos, C., Nakazawa, Y. and Martínez-Meyer, E. (2005). Time-specific ecological niche modeling predicts spatial dynamics of vector insects and human dengue cases. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 99(9), pp.647–655.

Phillips, T.W. and Throne, J.E. (2010). Biorational Approaches to Managing Stored-Product Insects. *Annual Review of Entomology*, 55(1), pp.375–397.

Pilakouta, N., Sellers, L.G., Barratt, R. and Ligonniere, A. (2023). The consequences of heatwaves for animal reproduction are timing-dependent. *Functional Ecology*, 37(9). doi:https://doi.org/10.1111/1365-2435.14386.

Pittendrigh, C. and Takamura, R. (1987). Temperature dependence and evolutionary adjustment of critical night length in insect photoperiodism. *Proceedings of the National Academy of Sciences*, 84.

Platts, P.J., Mason, S.C., Palmer, G., Hill, J.K., Oliver, T.H., Powney, G.D., Fox, R. and Thomas, C.D. (2019). Habitat availability explains variation in climate-driven range shifts across multiple taxonomic groups. *Scientific Reports*, [online] 9(1).

Plesnar-Bielak, A., Skrzynecka, A.M., Prokop, Z.M. and Radwan, J. (2012). Mating system affects population performance and extinction risk under environmental challenge. *Proceedings of the Royal Society B: Biological Sciences*, 279(1747),

pp.4661–4667.

Ploquin, E.F., Herrera, J.M. and Obeso, J.R. (2013). Bumblebee community homogenization after uphill shifts in montane areas of northern Spain. *Oecologia*, 173(4), pp.1649–1660.

Pointer, M.D., Gage, M.J.G. and Spurgin, L.G. (2021). Tribolium beetles as a model system in evolution and ecology. *Heredity*, [online] 126(6), pp.869–883. doi:https://doi.org/10.1038/s41437-021-00420-1.

Porcelli, D., Gaston, K.J., Butlin, R.K. and Snook, R.R. (2016). Local adaptation of reproductive performance during thermal stress. *Journal of Evolutionary Biology*, 30(2), pp.422–429.

Posit team (2024). RStudio: Integrated Development Environment for R. Posit Software, PBC, Boston, MA. URL http://www.posit.co/.

Potter, K., Davidowitz, G. and Woods, H.A. (2009). Insect eggs protected from high temperatures by limited homeothermy of plant leaves. *Journal of Experimental Biology*, 212(21), pp.3448–3454.

Pottier, P., Burke, S., Drobniak, S.M., Lagisz, M. and Nakagawa, S. (2021). Sexual (in)equality? A meta-analysis of sex differences in thermal acclimation capacity across ectotherms. *Functional Ecology*, 35(12), pp.2663–2678. doi:https://doi.org/10.1111/1365-2435.13899.

Potts, S.G., Imperatriz-Fonseca, V.L., Ngo, H.T., Aizen, M.A., Biesmeijer, J.C., Breeze, T.D., Dicks, L.V., Garibaldi, L.A., Hill, R., Settele, J., Vanbergen, A.J., Cunningham, S.A., Eardley, C., Freitas, B.M., Gallai, N., Kevan, P.G., Kovács-Hostyánszki, A., Kremen, C. and Vymazalová, M. (2016). The assessment report of the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services on pollinators, pollination and food production. Bonn, Germany: Secretariat of the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services.

Pureswaran, D.S., Roques, A. and Battisti, A. (2018). Forest Insects and Climate Change. *Current Forestry Reports*, 4(2), pp.35–50.

Putri GH, Anders S, Pyl PT, Pimanda JE, Zanini F. Analysing high-throughput sequencing data in Python with HTSeq 2.0. Bioinformatics. 2022; 38(10): 2943-2945.

R Core Team (2024). _R: A Language and Environment for Statistical Computing_. R Foundation for Statistical Computing, Vienna, Austria. <https://www.R-project.org/>.

Radchuk, V., Reed, T., Teplitsky, C., van de Pol, M., Charmantier, A., Hassall, C., Adamík, P., Adriaensen, F., Ahola, M.P., Arcese, P., Miguel Avilés, J., Balbontin, J., Berg, K.S., Borras, A., Burthe, S., Clobert, J., Dehnhard, N., de Lope, F., Dhondt, A.A. and Dingemanse, N.J. (2019). Adaptive responses of animals to climate change are most likely insufficient. *Nature Communications*, [online] 10(1).

Rahman, M.B., Schellander, K., Luceño, N.L. and Van Soom, A. (2018). Heat stress responses in spermatozoa: Mechanisms and consequences for cattle fertility. *Theriogenology*, 113, pp.102–112.

Ramanathan, V. and Feng, Y. (2009). Air pollution, greenhouse gases and climate change: Global and regional perspectives. *Atmospheric Environment*, [online] 43(1), pp.37–50.

Ratz, T., Tejinder Singh Chechi, Aliki-Ioanna Dimopoulou, Stephanie Daniela Sedlmair and Tuni, C. (2024). Heatwaves inflict reproductive but not survival costs to male insects. *Journal of Experimental Biology*, 227(6). doi:https://doi.org/10.1242/jeb.246698.

Renner, S.S. and Zohner, C.M. (2018). Climate Change and Phenological Mismatch in Trophic Interactions Among Plants, Insects, and Vertebrates. *Annual Review of Ecology, Evolution, and Systematics*, 49(1), pp.165–182.

Resh, V.H. and CardéR.T. (2009). *Encyclopedia of insects*. Amsterdam ; London: Elsevier/Academic Press.

Rezende, E.L., Castañeda, L.E. and Santos, M. (2014). Tolerance landscapes in thermal ecology. *Functional Ecology*, [online] 28(4), pp.799–809. doi:https://doi.org/10.1111/1365-2435.12268.

Robinson, W.H. (2005). *Handbook of urban insects and arachnids*. Cambridge: Cambridge University.

Rodrigues, Y.K. and Beldade, P. (2020). Thermal Plasticity in Insects' Response to Climate Change and to Multifactorial Environments. *Frontiers in Ecology and Evolution*, 8.

Rohmer, C. (2004). Heat induced male sterility in Drosophila melanogaster: adaptive genetic variations among geographic populations and role of the Y chromosome. *Journal of Experimental Biology*, 207(16), pp.2735–2743. doi:https://doi.org/10.1242/jeb.01087.

Roth, Z., Meidan, R., Braw-Tal, R. and Wolfenson, D. (2000). Immediate and delayed effects of heat stress on follicular development and its association with plasma FSH and inhibin concentration in cows. *Reproduction*, pp.83–90.

Roux, O., Le Lann, C., van Alphen, J.J.M. and van Baaren, J. (2010). How does heat shock affect the life history traits of adults and progeny of the aphid parasitoid Aphidius avenae (Hymenoptera: Aphidiidae)? *Bulletin of Entomological Research*, [online] 100(5), pp.543–549.

Ruthrof, K.X., Breshears, D.D., Fontaine, J.B., Froend, R.H., Matusick, G., Kala, J., Miller, B.P., Mitchell, P.J., Wilson, S.K., van Keulen, M., Enright, N.J., Law, D.J., Wernberg, T. and Hardy, G.E.St.J. (2018). Subcontinental heat wave triggers terrestrial and marine, multi-taxa responses. *Scientific Reports*, [online] 8(1). doi:https://doi.org/10.1038/s41598-018-31236-5.

Sailer, B., Sarkar, L., Bjordahl, J., Jost, L. and Evenson, D. (1997). Effects of Heat Stress on Mouse Testicular Cells and Sperm Chromatin Structure. *Journal of Andrology*, 18(3), pp.294–301.

Sales, K., Gage, M.J.G. and Vasudeva, R. (2024). Experimental evolution reveals that males evolving within warmer thermal regimes improve reproductive performance under heatwave conditions in a model insect. *Journal of evolutionary biology*, [online] 37(11), pp.1329–1344. doi:https://doi.org/10.1093/jeb/voae116.

Sales, K., Thomas, P., Gage, M. and Vasudeva, R. (2024). Experimental heatwaves reduce the effectiveness of ejaculates at occupying female reproductive tracts in a model insect. *Royal Society Open Science*, 11(5). doi:https://doi.org/10.1098/rsos.231949.

Sales, K., Vasudeva, R. and Gage, M.J.G. (2021). Fertility and mortality impacts of thermal stress from experimental heatwaves on different life stages and their

recovery in a model insect. *Royal Society Open Science*, 8(3), p.201717.

Sales, K., Vasudeva, R., Dickinson, M.E., Godwin, J.L., Lumley, A.J., Michalczyk, Ł., Hebberecht, L., Thomas, P., Franco, A. and Gage, M.J.G. (2018). Experimental heatwaves compromise sperm function and cause transgenerational damage in a model insect. *Nature Communications*, 9(1).

Sallam, M. (2013). *INSECT DAMAGE: Damage on Post-Harvest*. Rome, Italy: Food and Agriculture Organization of the United Nations.

Sánchez-Bayo, F. and Wyckhuys, K.A.G. (2019). Worldwide decline of the entomofauna: A review of its drivers. *Biological Conservation*, 232(1), pp.8–27.

Sánchez-Guillén, R.A., Córdoba-Aguilar, A., Hansson, B., Ott, J. and Wellenreuther, M. (2015). Evolutionary consequences of climate-induced range shifts in insects. *Biological Reviews*, 91(4), pp.1050–1064.

Schilthuizen, M. and Kellermann, V. (2013). Contemporary climate change and terrestrial invertebrates: evolutionary versus plastic changes. *Evolutionary Applications*, 7(1), pp.56–67.

Schmitz, O.J., Rosenblatt, A.E. and Smylie, M. (2016). Temperature dependence of predation stress and the nutritional ecology of a generalist herbivore. *Ecology*, 97(11), pp.3119–3130.

Scholes, R.J. (2016). Climate change and ecosystem services. *Wiley Interdisciplinary Reviews: Climate Change*, 7(4), pp.537–550.

Schou, M.F., Kristensen, T.N., Kellermann, V., Schlötterer, C. and Loeschcke, V. (2014). A Drosophila
laboratory evolution experiment points to low evolutionary potential under increased temperatures likely to be experienced in the future. *Journal of Evolutionary Biology*, 27(9), pp.1859–1868.

Seebacher, F., White, C.R. and Franklin, C.E. (2014). Physiological plasticity increases resilience of ectothermic animals to climate change. *Nature Climate Change*, 5(1), pp.61–66.

Seneviratne, S.I., Zhang, X., Adnan, M., Badi, W., Dereczynski, C., Di Luca, A., Ghosh, S., Iskandar, I., Kossin, J., Lewis, S., Otto, F., Pinto, I., Satoh, M., Vicente-

Serrano, S.M., Wehner, M. and Zhou, B. (2021). Weather and Climate Extreme Events in a Changing Climate. In Climate Change 2021: The Physical Science Basis. Contribution of Working Group I to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge: Cambridge University Press, pp.1513–1766.

Setchell, B.P. (1998). The Parkes Lecture Heat and the testis. *Reproduction*, 114(2), pp.179–194.

Settembre, C., Fraldi, A., Medina, D.L. and Ballabio, A. (2013). Signals from the lysosome: a control centre for cellular clearance and energy metabolism. *Nature Reviews Molecular Cell Biology*, [online] 14(5), pp.283–296. doi:https://doi.org/10.1038/nrm3565.

Sgrò, C.M., Terblanche, J.S. and Hoffmann, A.A. (2016). What Can Plasticity Contribute to Insect Responses to Climate Change? *Annual Review of Entomology*, 61(1), pp.433–451.

Sharma, H.C. (2014). Climate Change Effects on Insects: Implications for Crop Protection and Food Security. *Journal of Crop Improvement*, 28(2), pp.229–259.

Shukla, J.N. and Palli, S.R. (2012). Sex determination in beetles: Production of all male progeny by Parental RNAi knockdown of transformer. *Scientific reports*, [online] 2(1). doi:https://doi.org/10.1038/srep00602.

Smale, D.A., Wernberg, T., Oliver, E.C.J., Thomsen, M., Harvey, B.P., Straub, S.C., Burrows, M.T., Alexander, L.V., Benthuysen, J.A., Donat, M.G., Feng, M., Hobday, A.J., Holbrook, N.J., Perkins-Kirkpatrick, S.E., Scannell, H.A., Sen Gupta, A., Payne, B.L. and Moore, P.J. (2019). Marine heatwaves threaten global biodiversity and the provision of ecosystem services. *Nature Climate Change*, [online] 9(4), pp.306–312.

Song, H., Foquet, B., Mariño-Pérez, R. and Woller, D.A. (2017). Phylogeny of locusts and grasshoppers reveals complex evolution of density-dependent phenotypic plasticity. *Scientific Reports*, 7(1).

Sørensen, J.G., Kristensen, T.N. and Loeschcke, V. (2003). The evolutionary and ecological role of heat shock proteins. *Ecology Letters*, 6(11), pp.1025–1037. doi:https://doi.org/10.1046/j.1461-0248.2003.00528.x.

Soroye, P., Newbold, T. and Kerr, J. (2020). Climate change contributes to

widespread declines among bumble bees across continents. *Science*, 367(6478), pp.685–688.

Southon, R.J., Fernandes, O.A., Nascimento, F.S. and Sumner, S. (2019). Social wasps are effective biocontrol agents of key lepidopteran crop pests. *Proceedings of the Royal Society B: Biological Sciences*, 286(1914), p.20191676.

Stork, N.E. (2018). How Many Species of Insects and Other Terrestrial Arthropods Are There on Earth? *Annual Review of Entomology*, 63(1), pp.31–45.

Symes, L.B., Rodríguez, R.L. and Höbel, G. (2017). Beyond temperature coupling: Effects of temperature on ectotherm signaling and mate choice and the implications for communication in multispecies assemblages. *Ecology and Evolution*, 7(15), pp.5992–6002.

Terlau, J.F., Brose, U., Eisenhauer, N., Angelos Amyntas, Boy, T., Dyer, A., Gebler, A., Hof, C., Liu, T., Scherber, C., Schlägel, U.E., Schmidt, A. and Hirt, M.R. (2023). Microhabitat conditions remedy heat stress effects on insect activity. *Global Change Biology*, 29(13), pp.3747–3758. doi:https://doi.org/10.1111/gcb.16712.

Trenberth, K.E. (2018). Climate change caused by human activities is happening and it already has major consequences. *Journal of Energy & Natural Resources Law*, 36(4), pp.463–481.

Uy, K.L., LeDuc, R., Ganote, C. and Price, D.K. (2015). Physiological effects of heat stress on Hawaiian picture-wing Drosophila: genome-wide expression patterns and stress-related traits. *Conservation Physiology*, 3(1), p.cou062.

van de Pol, M., Jenouvrier, S., Cornelissen, J.H.C. and Visser, M.E. (2017). Behavioural, ecological and evolutionary responses to extreme climatic events: challenges and directions. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 372(1723), p.20160134. doi:https://doi.org/10.1098/rstb.2016.0134.

van Heerwaarden, B. and Sgrò, C.M. (2021). Male fertility thermal limits predict vulnerability to climate warming. *Nature Communications*, 12(1).

Van Klink, R., Bowler, D., Gongalsky, K., Swengel, A., Gentile, A. and Chase, J. (2020). Meta-analysis reveals declines in terrestrial but increases in freshwater insect abundances. *Science*, 368(6489), pp.417–420.

Vasudeva, R., Deeming, D.C. and Eady, P.E. (2014). Developmental temperature affects the expression of ejaculatory traits and the outcome of sperm competition inCallosobruchus maculatus. *Journal of Evolutionary Biology*, 27(9), pp.1811–1818.

Vasudeva, R., Dickinson, M., Sutter, A., Powell, S., Sales, K. and Gage, M.J.G. (2021). Facultative polyandry protects females from compromised male fertility caused by heatwave conditions. *Animal Behaviour*, 178, pp.37–48. doi:https://doi.org/10.1016/j.anbehav.2021.05.016.

Vasudeva, R., Sutter, A., Sales, K., Dickinson, M.E., Lumley, A.J. and Gage, M.J. (2019). Adaptive thermal plasticity enhances sperm and egg performance in a model insect. *eLife*, 8.

Wagner, D.L., Grames, E.M., Forister, M.L., Berenbaum, M.R. and Stopak, D. (2021). Insect decline in the Anthropocene: Death by a thousand cuts. *Proceedings of the National Academy of Sciences*, 118(2). doi:https://doi.org/10.1073/pnas.2023989118.

Walsh, B.S., Parratt, S.R., Hoffmann, A.A., Atkinson, D., Snook, R.R., Bretman, A. and Price, T.A.R. (2019). The Impact of Climate Change on Fertility. *Trends in Ecology & Evolution*, 34(3), pp.249–259.

Wang, L., Yang, S., Han, L., Fan, D. and Zhao, K. (2014). Phenotypic Plasticity of HSP70s Gene Expression during Diapause: Signs of Evolutionary Responses to Cold Stress among Soybean Pod Borer Populations (Leguminivora glycinivorella) in Northeast of China. *PLoS ONE*, 9(10), p.e109465.

Wang, Y.-C., Chang, Y.-W., Yang, F., Gong, W.-R., Hu, J. and Du, Y.-Z. (2024). A potential trade-off between reproduction and enhancement of thermotolerance in Liriomyza trifolii populations driven by thermal acclimation. *Journal of Thermal Biology*, 125, p.103988. doi:https://doi.org/10.1016/j.jtherbio.2024.103988.

War, A., Taggar, G., War, M. and Hussain, B. (2016). Impact of climate change on insect pests, plant chemical ecology, tritrophic interactions and food production. *International Journal of Clinical and Biological Sciences*, 1(2).

Watteyn, C., Fremout, T., Karremans, A.P., Van Meerbeek, K., Janssens, S.B., de Backer, S., Lipińska, M.M. and Muys, B. (2025). Wild Vanilla and pollinators at risk of spatial mismatch in a changing climate. *Frontiers in Plant Science*, 16. doi:https://doi.org/10.3389/fpls.2025.1585540.

Weaving, H., Terblanche, J.S. and English, S. (2024). Heatwaves are detrimental to fertility in the viviparous tsetse fly. *Proceedings of The Royal Society B: Biological Sciences*, 291(2018). doi:https://doi.org/10.1098/rspb.2023.2710.

Weaving, H., Terblanche, J.S., Pottier, P. and English, S. (2022). Meta-analysis reveals weak but pervasive plasticity in insect thermal limits. *Nature Communications*, [online] 13(1), p.5292. doi:https://doi.org/10.1038/s41467-022-32953-2.

Westerman, E. and Monteiro, A. (2016). Rearing Temperature Influences Adult Response to Changes in Mating Status. *PLOS ONE*, 11(2), p.e0146546.

Wickham H, Averick M, Bryan J, Chang W, McGowan LD, François R, Grolemund G, Hayes A, Henry L, Hester J, Kuhn M, Pedersen TL, Miller E, Bache SM, Müller K, Ooms J, Robinson D, Seidel DP, Spinu V, Takahashi K,Vaughan D, Wilke C, Woo K, Yutani H (2019). “Welcome to the tidyverse.” _Journal of Open Source Software_, *4*(43), 1686. doi:10.21105/joss.01686 <https://doi.org/10.21105/joss.01686>.

Wickham H. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York, 2016.

Wiens, J.J. (2016). Climate-Related Local Extinctions Are Already Widespread among Plant and Animal Species. *PLOS Biology*, 14(12), p.e2001104.

Willott, S.J. and Hassall, M. (1998). Life-history responses of British grasshoppers (Orthoptera: Acrididae) to temperature change. *Functional Ecology*, 12(2), pp.232–241.

Wolfner, M.F. (2009). Battle and Ballet: Molecular Interactions between the Sexes in Drosophila. *Journal of Heredity*, 100(4), pp.399–410. doi:https://doi.org/10.1093/jhered/esp013.

World Meteorological Organisation (2020). *Global Climate in 2015–2019*. Geneva, Switzerland: WMO publications, pp.1–22.

World Meteorological Organisation (2024). WMO Global Annual to Decadal Climate Update (Target years: 2024 and 2024-2028). Geneva: World Meteorological Organisation.

Yang, Q. and Lu, Y. (2024). Heat Shock Protein 70 Genes Are Involved in the Thermal Tolerance of Hippodamia variegata. *Insects*, [online] 15(9), pp.678–678. doi:https://doi.org/10.3390/insects15090678.

Yee, D.A., Ezeakacha, N.F. and Abbott, K.C. (2016). The interactive effects of photoperiod and future climate change may have negative consequences for a wide-spread invasive insect. *Oikos*, 126(1), pp.40–51.

Yu, H., Shi, M.-R., Xu, J., Chen, P. and Liu, J.-H. (2021). Mating-Induced Trade-Offs upon Egg Production versus Fertilization and Offspring's Survival in a Sawfly with Facultative Parthenogenesis. *Insects*, 12(8), pp.693–693. doi:https://doi.org/10.3390/insects12080693.

Zajitschek, F., Zajitschek, S. and Manier, M. (2017). Correction to "High-protein paternal diet confers an advantage to sons in sperm competition." *Biology Letters*, 13(7), p.20170297.

Zajitschek, S., Hotzy, C., Zajitschek, F. and Immler, S. (2014). Short-term variation in sperm competition causes sperm-mediated epigenetic effects on early offspring performance in the zebrafish. *Proceedings of the Royal Society B: Biological Sciences*, 281(1785), pp.20140422–20140422.

Zeileis, A., Hothorn, T. (2002). Diagnostic Checking in Regression Relationships. R News 2(3), 7-10. URL https://CRAN.R-project.org/doc/Rnews/

Zeileis, A., Kleiber, C., Jackman, S. (2008). Regression Models for Count Data in R. Journal of Statistical Software 27(8). URL http://www.jstatsoft.org/v27/i08/.

Zeng, B., Zhu, W., Fu, Y. and Zhou, S. (2018). Influence of high-temperature exposure on the mating, oviposition and thermotaxis of Bactrocera cucurbitae (Coquillet) (Diptera:Tephritidae). *PLOS ONE*, 13(9), p.e0204065.

Zhang, M.-H., Shi, Z.-D., Yu, J.-C., Zhang, Y.-P., Wang, L.-G. and Qiu, Y. (2015). Scrotal heat stress causes sperm chromatin damage and cysteinyl aspartate-spicific proteinases 3 changes in fertile men. *Journal of Assisted Reproduction and Genetics*, 32(5), pp.747–755.

Zhang, S., Cao, Z., Wang, Q., Zhang, F. and Liu, T.-X. (2014). Exposing eggs to high temperatures affects the development, survival and reproduction of Harmonia axyridis. *Journal of Thermal Biology*, 39, pp.40–44.

Zhang, Y., Chang, Y.-W., Wang, Y.-C., Yan, Y.-Q. and Du, Y.-Z. (2024). The small heat shock protein Hsp20.8 imparts tolerance to high temperatures in the leafminer fly, *Liriomyza trifolii* (Diptera: Agtomyzidae). *Bulletin of Entomological Research*, 114(2), pp.230–236. doi:https://doi.org/10.1017/s0007485324000026.

Zhang, Y.-B., Yang, A.-P., Zhang, G.-F., Liu, W.-X. and Wan, F.-H. (2019). Effects of Simulated Heat Waves on Life History Traits of a Host Feeding Parasitoid. *Insects*, 10(12), p.419.

Zittis, G., Hadjinicolaou, P., Almazroui, M., Bucchignani, E., Driouech, F., El Rhaz, K., Kurnaz, L., Nikulin, G., Ntoumos, A., Ozturk, T., Proestos, Y., Stenchikov, G., Zaaboul, R. and Lelieveld, J. (2021). Business-as-usual will lead to super and ultra-extreme heatwaves in the Middle East and North Africa. *npj Climate and Atmospheric Science*, [online] 4(1), pp.1–9. doi:https://doi.org/10.1038/s41612-021-00178-7.

Zwoinska, M.K., Rodrigues, L.R., Slate, J. and Snook, R.R. (2020). Phenotypic Responses to and Genetic Architecture of Sterility Following Exposure to Sub-Lethal Temperature During Development. *Frontiers in Genetics*, 11. doi:https://doi.org/10.3389/fgene.2020.00573.

10. Appendices

10.1 Appendix A. Published version of Chapter 4

Cole, B., Vasudeva, R., Dragoi, K., Hibble, J., King, J., Maklakov, A.A., Chapman, T. and Gage, M. (2025). Short experimental heatwaves have sublethal impacts on male reproduction in a model insect. *Journal of Experimental Biology*. 228(15). doi:https://doi.org/10.1242/jeb.250555.

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SHORT COMMUNICATION

Short experimental heatwaves have sublethal impacts on male reproduction in a model insect

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ABSTRACT

Heatwaves are becoming more common and severe. Previous work has highlighted male insects as being particularly vulnerable to multi-day continuous heatwaves, yet our understanding of short duration heatwave impacts on insects is limited. Here, we assessed the impacts of short, simulated heatwave exposures (2, 5 and 10 h) using ecologically relevant temperatures (42, 44, 46, 48 and 50°C) on survival, reproductive output, testes volume and sperm length in *Tribolium castaneum*. We show that reproductive output is compromised at lower temperatures than survival, especially during the shortest heatwaves, supporting the notion that thermal fertility limits are lower than thermal viability limits. Furthermore, testes volume was reduced by 40% after a 10 h exposure at 42°C and sperm length decreased by 2.7% after an exposure of 42°C for just 2 h. This highlights that even short heat exposure can impact male fertility and reproductive trait morphology at temperatures below viability limits.

KEY WORDS: Climate change, Heatwaves, Insect fertility, Male susceptibility, *Tribolium*

INTRODUCTION

Significant changes to Earth's climate are being seen due to human activities, with mean global temperature predicted to reach up to 1.9°C higher than pre-industrial times by 2028 (World Meteorological Organisation, 2024). In addition to increases in mean global temperature, incidences of extreme heat are also rising. Heatwaves are becoming broadly more common and intense, including in regions where they were previously rare (Meehl and Tebaldi, 2004; Perkins-Kirkpatrick and Lewis, 2020). In particular, short duration heatwaves and their impacts on ecosystems and the environment are expected to increase in frequency (Trenberth, 2018). Understanding the ecological and evolutionary implications of these heatwaves is critical as climatic extremes are shifting more rapidly than mean temperature (Seneviratne et al., 2021).

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Received 20 March 2025; Accepted 5 July 2025

With anthropogenic climate change occurring at accelerating rates in all geographical regions (World Meteorological Organisation, 2024), there is emerging evidence that it has caused species extinctions (Ceballos et al., 2015), with insects highlighted as being particularly at risk (Dunn, 2005; Wiens, 2016; Wagner et al., 2021). Understanding the mechanisms underpinning current and future insect declines under climate change is becoming an increasingly important research focus, with extreme heat often linked to decreasing insect survival (Ma et al., 2020). However, sub-lethal impacts of heat on biological functions are less well understood. Recent research has shown that thermal fertility limits (TFLs) are reached at lower temperatures than critical thermal limits (CTLs) in some species (Parratt et al., 2021). Furthermore, male TFLs have been shown to predict species distribution and extinction risk better than CTLs (Parratt et al., 2021; van Heerwaarden and Sgrò, 2021).

Many studies have assessed the effect of extreme heat on multiple aspects of male insect reproduction including spermatogenesis (Canal Domenech and Fricke, 2023), sperm motility, mobility and morphology (Uy et al., 2015; Porcelli et al., 2016; Iossa et al., 2019), sperm number (Vasudeva et al., 2014; Sales et al., 2018), sperm viability (Sales et al., 2018; Martinet et al., 2020; Campion et al., 2023), sperm storage (Sales et al., 2024), testes volume (Vasudeva et al., 2014; Sales et al., 2021), courtship and mating behaviour (Grandela et al., 2023; Ratz et al., 2024) and parental care (Pilakouta et al., 2023). These studies have highlighted the range of fertility traits that can be affected. However, research on the impact of short-term heat remains limited (Dougherty et al., 2024), which is a particular concern given that shorter and more intense heatwaves are increasing in frequency (Trenberth, 2018).

Using *Tribolium castaneum*, a widely used model in evolution and ecology (Pointer et al., 2021; Campbell et al., 2022), Sales et al. (2018) showed that subjecting virgin males, but not virgin females, to a simulated heatwave before mating severely reduced reproductive output. Subjecting males to heatwave conditions of 42°C for 5 continuous days halved male fertility, with a further heatwave sterilising most individuals, which was linked to decreased sperm viability and number. Such long-term heatwaves were also shown to reduce testes volume by half, which recovered to control levels ∼25 days post-heatwave exposure (Sales et al., 2021). Under future climate projections, 'extremely hot days' with temperatures over 50°C are expected to become increasingly common within areas of *T. castaneum*'s global distribution (Campbell et al., 2022; Christidis et al., 2023). It is important to understand the consequences of such intense conditions under ecologically relevant scenarios, as peak temperatures within a heatwave may only be experienced for a few hours in a single day (Holmes et al., 2015; Oliveira et al., 2021). Therefore, there is a pressing need to investigate how exposure to various short duration heatwave conditions may impact male survival and fertility and understand the potential implications this may have on insect populations.

This has been further emphasised by recent studies applying the thermal death time (TDT) framework, which integrates both the duration and intensity of thermal exposures to predict biological damage. While initially focused on describing survival thresholds, such approaches are increasingly being used to understand sublethal impacts on fertility, highlighting that different traits may have distinct thermal sensitivities and damage accumulation rates (Rezende et al., 2014; Jørgensen et al., 2019; Ørsted et al., 2024). Our study contributes to a growing understanding of such interacting factors by testing the impacts of ecologically relevant simulated heatwaves with varying duration (2, 5 or 10 h) and intensity (42, 44, 46, 48 or 50°C) on male survival, reproductive output, testes volume and sperm length using *T. castaneum*. We predict that fertility and associated traits could be more sensitive than survival in general.

MATERIALS AND METHODS

Line maintenance and experimental individuals

Tribolium castaneum (Herbst 1797) beetles used in these experiments were from the Krakow Super Strain (KSS) outbred stock line (details on the set-up are given in Dickinson, 2018). Stock populations were maintained in 12×12×12 cm plastic tubs half-filled with fodder (a 9:1 volume ratio of organic flour and yeast) and topped with an even layer of oats for traction. Populations were kept at constant standard conditions of 30±1°C and 60±10% relative humidity (RH) under a 16 h:8 h light:dark photoperiod. Experimental individuals were obtained as the offspring of ∼300 mature adults selected at random from the stock line. These individuals were allowed to mate randomly and oviposit for 7 days in a fresh tub before the removal of adults by mechanical sieving, leaving only the fodder with oviposited eggs in this new population.

Individuals were sexed at the pupal stage by visual identification of sexually dimorphic genital papillae (on day 18 of development) and then kept in single-sex groups of 20 individuals for a further 10 days to allow for development to sexual maturity. Groups were kept in 6 cm Petri dishes filled with 3 g of fodder and topped with an even layer of oats. Once matured, females were identified with a dot of Uni Posca non-toxic marker (Uni-ball, Tokyo, Japan) on the dorsal thorax.

Experimental heatwave treatments

Heatwave conditions were applied using either an A.B. Newlife 75 Mk4 forced air egg incubator or an A.B. Newlife 75 Mk 4 moving air incubator (A.B. Incubators, Suffolk, UK). These conditions were selected based on previous research, which established the reproductive optimum of males (see Sales et al., 2018). Virgin male beetles were exposed to either heatwave conditions (42, 44, 46, 48 or 50°C ±1°C, 60±10% RH) or control conditions (30±1°C, 60±10% RH) for 2, 5 or 10 h in single-sex groups of 20 individuals in 6 cm Petri dishes filled with 3 g of fodder and a layer of oats. Temperature was checked every 30 min using a digital thermometer integrated into the incubator and an additional mercury thermometer placed in the incubator. No recorded temperature was above or below 1°C from the set point.

Experimental protocol

Survival and fertility

Virgin, sexually mature males (48–72 h post-eclosion) were subject to heatwave conditions with controls run in parallel (conditions described above). All individuals then experienced a further rest period at 30±1°C for 24 h. Females were sourced from the same population but were maintained constantly under standard conditions in identical density. Individual males were visually assessed at this time, and survivors were paired with an age-matched virgin female for 48 h at 30±1°C in a 7 ml mating vial. Sample sizes for survival and reproductive output assays are summarised in Table 1. These mating vials were filled with ∼2.4 g of fodder and topped with a few pieces of oats for traction. These mating vials were retained to ensure successful ejaculate transfer, assessed by the visual presence of larval tracks/offspring.

Table 1. Number of *Tribolium castaneum* individuals subject to each heatwave temperature/duration condition for the survival assay and number of survivors that subsequently went through the reproductive output assay

		Sample size		
	Temperature (°C)	2 h	5 h	10 h
Survival	30	58	60	60
	42	40	40	60
	44	35	38	60
	46	34	40	60
	48	70	69	60
	50	70	61	60
Reproductive output	30	53	54	51
	42	38	37	56
	44	28	32	60
	46	32	32	59
	48	65	65	32
	50	68	52	0*

*No individuals were assayed because of 100% mortality during the heatwave exposure.

Females from the above mating assays were then moved into individual 6 cm Petri dishes (filled with 3 g of fodder and topped with an even layer of oats) and allowed to oviposit. Oviposition occurred across two separate 10 day blocks (20 days in total) to reduce cannibalism associated with overlapping offspring developmental life stages (Park et al., 1965). After 20 days, the females were removed, and the oviposited eggs were allowed to develop until maturity for an additional 35 days under standard developmental conditions (30±1°C, 60±10% RH). The reproductive output of each pair was then assessed as the number of mature adults produced from 20 days of oviposition (equating to ∼51% of a female's lifetime reproductive output; see p. 31, Dickinson, 2018).

Testes and sperm measurements

An additional cohort of males that experienced identical experimental conditions were allocated to quantifying testes volume and total sperm length. These individuals were frozen at −20°C immediately after being subject to either heatwave or control conditions (as described above). All samples were blinded to the user by a random code at this point to avoid any unconscious bias during morphological measurements. Measurements were taken from individuals that survived the heatwaves for all experimental groups apart from those subject to 50°C for 10 h, where all males had died during the exposure period. Testes dissections and measurements were carried out as described in Sales et al. (2021). The testes of 10 males were measured per duration/heatwave condition. Total sperm length measurements were taken as described in Godwin et al. (2017) and Vasudeva et al. (2019). Twenty individual mature intact sperm were measured per male (N=10 males per duration/heatwave condition).

Statistical analysis

All data were analysed using R version 4.4.1 (http://www.R-project.org/) in RStudio Version 2024.04.2+764 (https://posit.co). Plots were created using 'ggplot2' (Wickham, 2016). Data manipulation was done using 'tidyverse' (Wickham et al., 2019). The impact of

heatwave temperature on survival for each of the three heatwave durations was initially assessed using a binomial GLM. Firth's penalised logistic regression from the brglm2 package was used where near-separation or complete separation issues caused model convergence issues (https://CRAN.R-project.org/package=brglm2). Fit of the models was then assessed using 'performance' (Lüdecke et al., 2021) and residuals were visually evaluated.

Reproductive output data were first censored where individuals escaped from or died during the reproductive output assay (N=31). Zero-inflated negative binomial generalised linear models (GLMs) (Zeileis et al., 2008) were used to analyse data after assessing models for over- or under-dispersion and comparing goodness of fit using 'performance' and 'lmtest' (Zeileis and Hothorn, 2002).

Testes volume data were analysed using Gaussian GLMs and models, and residuals were tested. Where residuals showed heteroscedasticity, gamma GLMs were employed, and models were compared for goodness of fit. Average sperm length data, grouped by heatwave condition, were analysed using gamma GLMs and models and residuals were tested using 'performance' and 'lmtest'.

RESULTS AND DISCUSSION

Our findings show that reproductive traits were impacted at sublethal temperatures when male *T. castaneum* beetles were exposed to heatwave conditions from 42°C to 50°C for 2, 5 and 10 h. Some reproductive traits were also more thermally sensitive than others. For example, sperm length showed reductions at lower temperatures than reproductive output for all heatwave durations. Our results are in line with existing findings across taxa, which report sensitivity to heat in the reproductive traits of males (e.g. Canal Domenech and Fricke, 2023; Grandela et al., 2023; Meena et al., 2024; Ratz et al., 2024). Statistics are summarised in Table 2.

The survival of males exposed to heatwave conditions for 2 or 5 h was high across all groups, with no significant reduction compared with controls, except for a 5 h exposure at 46°C. However, this difference was not observed at higher temperatures. Survival started to drop dramatically compared with controls in males exposed to higher temperatures for 10 h (Fig. 1A–C). Males exposed to heatwave conditions of 48°C and 50°C for 10 h showed a marked reduction in survival. At 48°C, only 53% of males survived, and at 50°C, none survived (compared with 93.3% in the control group). Here, we demonstrate that male *T. castaneum* survival was resilient to heatwave conditions until ∼11–13°C above their optimum at 35°C (Sales et al., 2018) before hitting an upper limit when exposed to very intense heat (48°C and 50°C for 10 h).

By contrast, male reproductive output and reproductive morphology were less resilient across a range of short duration heat exposures. As exposure duration and intensity increased, we observed increasingly negative impacts on reproductive output. We found that temperatures as low as 46°C caused a significant reduction in reproductive output after exposure to heatwave conditions for 10 h. At 48°C and 50°C, there were significant reductions following heat exposure of only 5 h and 2 h, respectively. Heatwave exposure at 50°C for 2 h reduced male reproductive output by 33% compared with controls (Fig. 1D). Heatwave exposure at 48°C and 50°C for 5 h reduced male reproductive output by 23% and 55%, respectively (Fig. 1E). Heatwave exposure at 46°C and 48°C for 10 h reduced male reproductive output by 17% and 66%, respectively (Fig. 1F). All individuals exposed to 50°C for 10 h died before the reproductive assay; therefore, no reproductive output data were collected.

The relationship between heatwave duration and intensity shown in this study further highlights the importance of considering the two factors simultaneously when assessing the impact of heat on insects. This is particularly relevant when considering future climate change scenarios where both short duration and more intense heatwaves are predicted to become more prevalent (Trenberth, 2018). Moreover, there is a need to consider trait-specific responses to combinations of these factors.

Consistent with previous studies (Sales et al., 2021; Hu et al., 2022), we found that testes volume was significantly impacted by heatwave exposure. This effect was observed following exposure to very brief heatwave conditions. Males exposed to heatwave conditions of 50°C for 2 h showed reduced testes volume by 29% compared with controls (Fig. 1G). For 5 h heatwave exposure, 48°C and 50°C reduced testes volume by 26% and 36%, respectively

Table 2. Summary statistics for survival, reproductive output, testes volume and sperm length data under various heatwave conditions

	Survival			20 day reproductive output			Testes volume (mm^3)			Sperm length (μm)		
Treatment	Survivors (n/N)	z	P	Mean±s.e.m.	z	P	Mean±s.e.m.	t	P	Mean±s.e.m.	t	P
2 h exposure												
30°C	54/58	–	–	207±15.3	–	–	0.260±0.014	–	–	86.4±0.758	–	–
42°C	38/40	0.291	0.771	218±19.8	1.058	0.290	0.299±0.018	1.579	0.120	84.2±0.640	−2.099	0.041
44°C	35/35	1.158	0.247	183±15.9	−1.843	0.065	0.210±0.021	−1.969	0.054	81.5±0.968	−4.560	<0.001
46°C	32/34	0.085	0.932	186±16.7	−0.908	0.364	0.224±0.023	−1.427	0.159	81.8±0.821	−4.346	<0.001
48°C	65/70	−0.025	0.980	217±12.4	−0.230	0.818	0.225±0.012	−1.375	0.175	81.1±0.623	−4.977	<0.001
50°C	69/70	1.387	0.165	139±8.7	−4.302	<0.001	0.184±0.017	−3.018	0.004	80.7±0.623	−0.5352	<0.001
5 h exposure												
30°C	59/60	–	–	262±10.7	–	–	0.273±0.011	–	–	88.1±0.577	–	–
42°C	39/40	−0.290	0.772	222±19.6	−0.536	0.592	0.254±0.019	−0.764	0.448	83.6±0.686	−4.367	<0.001
44°C	35/38	−1.381	0.167	217±11.6	−1.541	0.123	0.226±0.017	−1.881	0.065	81.4±0.727	−6.581	<0.001
46°C	34/40	−2.128	0.033	190±20.9	−1.450	0.147	0.245±0.020	−1.097	0.278	82.8±0.763	−5.162	<0.001
48°C	67/69	−0.457	0.647	201±12.6	−1.985	0.047	0.201±0.018	−2.847	0.006	79.8±0.668	−8.344	<0.001
50°C	54/61	−1.875	0.061	120±12.5	−6.111	<0.001	0.176±0.021	−3.865	<0.001	81.8±0.740	−6.267	<0.001
10 h exposure												
30°C	56/60	–	–	264±10.7	–	–	0.292±0.010	–	–	84.4±0.587	–	–
42°C	57/60	0.362	0.717	255±12.8	0.292	0.770	0.174±0.009	4.471	<0.001	82.5±0.711	−1.836	0.072
44°C	60/60	1.496	0.135	235±12.3	−0.840	0.401	0.183±0.007	4.077	<0.001	80.1±0.839	−4.228	<0.001
46°C	59/60	1.187	0.235	219±10.2	−2.026	0.043	0.205±0.020	3.147	0.003	81.5±0.915	−2.836	0.006
48°C	32/60	−4.302	<0.001	90±19.4	−2.837	0.005	0.155±0.010	5.356	<0.001	80.5±0.668	−3.824	<0.001
50°C	0/60	−4.837	<0.001	–	–	–	0.086±0.011	8.754	<0.001	81.7±0.524	−2.684	0.010

Statistics represent a comparison between each group and their respective control.

Journal of Experimental Biology (2025) 228, jeb250555. doi:10.1242/jeb.250555

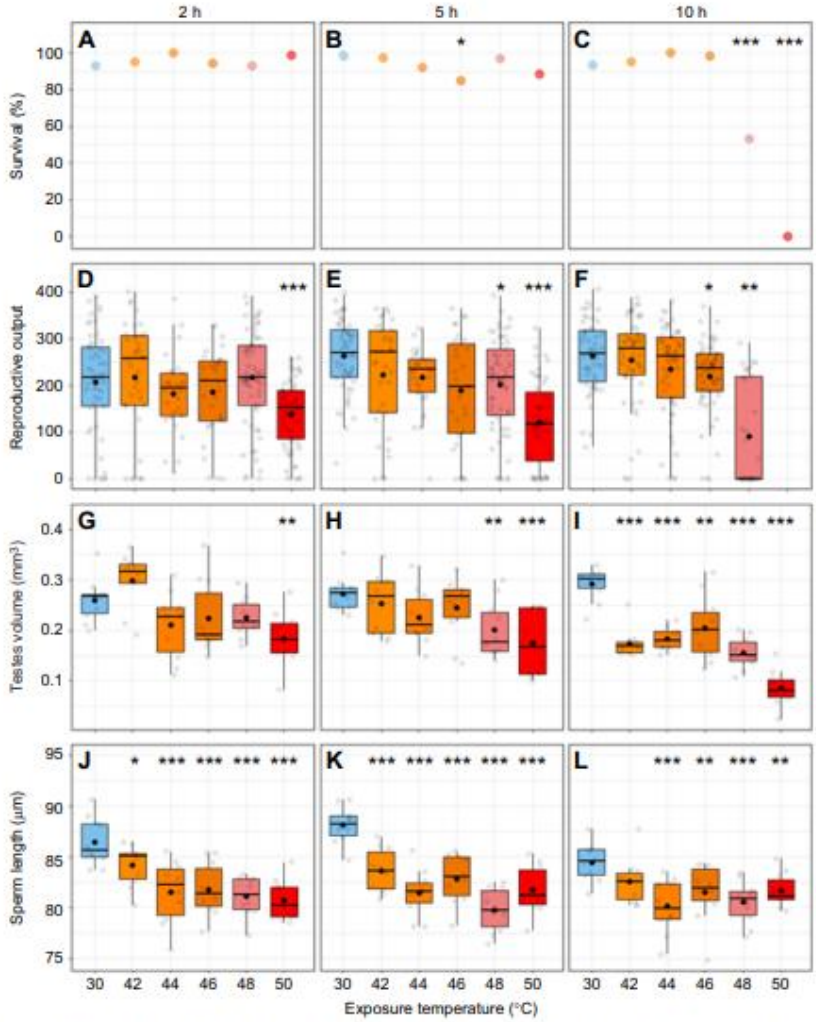

Fig. 1. Effects of varying heatwave temperature/duration conditions on male *Tribolium castaneum*. (A–C) Survival, (D–F) 20 day reproductive output, (G–I) testes volume and (J–L) sperm length following exposure to 30, 42, 44, 46, 48 and 50°C for 2, 5 and 10 h. For D–I, raw data points are plotted as open jittered circles. For J–L, the mean sperm length per male is plotted as open jittered circles. Boxplots contain a median line, mean dot and interquartile range box. Significance values representing a comparison between 30°C and the experimental heatwave condition are denoted by asterisks: **P*<0.05, ***P*<0.01, ****P*<0.001. Sample size for survival from left to right for each exposure duration: 2 h (A): 58, 40, 35, 34, 70, 70; 5 h (B): 60, 40, 38, 40, 69, 61; 10 h (C): 60, 60, 60, 60, 60, 60. Sample size for reproductive output for each exposure duration: 2 h (D): 53, 38, 28, 32, 65, 68; 5 h (E): 54, 37, 32, 32, 65, 52; 10 h (F): 51, 56, 60, 59, 32 (all individuals exposed to 50°C for 10 h died before the reproductive assay). Sample size for testes volume was 10 individuals per group. The sample size for sperm length was 20 individual sperm from 10 males per group.

(Fig. 1H). For 10 h exposure, all heatwave temperatures reduced testes volume compared with controls, with even the lowest exposure temperature of 42°C resulting in a 40% drop in testes volume (Fig. 1I). This reduction in testes volume remained relatively consistent over 44°C (37%) and 46°C (30%), and further reductions were seen at 48°C (47%) and at 50°C, where testes volume was reduced by 70%. For both 2 h and 5 h heatwaves, the temperatures that resulted in significant reductions in testes volume were the same as those at which significant reductions in reproductive output occurred (50°C and 48°C, respectively). For 10 h exposures, testes volume was significantly reduced at a lower temperature (42°C) than that at which reproductive output was first compromised (46°C). Broadly, these findings suggest that reductions in testes volume are associated with reduced male

reproductive output and that testes volume is highly thermally sensitive.

Heat-related damage to insect sperm has been documented previously (Martinet et al., 2020; Sales et al., 2021; Canal Domenech and Fricke, 2023; Lv et al., 2024). In *T. castaneum*, a 5 day, 42°C heatwave reduced sperm survival by ∼60% and sperm count by 75% (Sales et al., 2018). Here, we found that even the shortest and least intense heatwave exposures tested in this study caused a decrease in sperm length. All heatwave exposures for 2 h reduced sperm length compared with controls (Fig. 1J), with exposure at 42°C reducing sperm length by 2.7%. This reduction in total sperm length generally worsened with increasing temperature, seeing decreases at 44°C (5.7%), 46°C (5.4%), 48°C (6.2%) and 50°C (6.7%). Similarly, for both 5 h and 10 h exposures, we observed reduced sperm length compared with controls (Fig. 1K,L). For 5 h exposure, 42°C resulted in a 5.1% reduction, with further decreases at 44°C (7.6%), 46°C (6%), 48°C (9.5%) and 50°C (7.3%). For 10 h exposure, all heatwave temperatures, except 42°C, resulted in significantly reduced sperm length compared with controls. After 44°C exposure, there was a 5.1% reduction, with similar decreases at 46°C (3.5%), 48°C (4.7%) and 50°C (3.3%). It is unclear exactly what mechanism gives rise to these sperm length reductions, and future work on this will be insightful.

Spermatogenesis occurs throughout the adult life stage in *T. castaneum*, but the exact duration of spermatogenesis and spermiogenesis is unknown. However, in other insects, these processes occur over a number of days (e.g. 10 days for spermatogenesis and 5 days for spermiogenesis in *Drosophila melanogaster*: Rohmer, 2004; Fabian and Brill, 2012; and 12 days for spermatogenesis in *Haematobia irritans*: Basso et al., 2011). Considering the short duration between the initiation of heat exposure and freezing of samples in this study (2–10 h), we suggest that the mechanisms underpinning sperm length change in response to heat exposure may be linked to disruption of spermiogenesis in nearly mature sperm or through direct impacts on mature sperm. This is supported by an observed increase in sperm length variation within individuals, associated with increasing temperatures of heatwave conditions (see Fig. S1). Further work assessing the specific morphological changes associated with such sperm length variation may elucidate the mechanisms behind the impacts on sperm and male reproductive output observed in this study.

Broadly, we have highlighted that the thermal sensitivity of male reproductive traits is even greater than previously demonstrated (e.g. Sales et al., 2018, 2021). Our results showing trait-specific sensitivity also align with recent work on other taxa. For example, in *Drosophila suzukii*, traits (survival, coma induction and productivity) were shown to have different sensitivities to heat stress exposure, and the stress durations required to produce a 50% reduction in each trait often varied greatly (Ørsted et al., 2024). That study, utilising thermal dose–time models, builds on earlier work showing that the time–temperature relationship underlying thermal damage differs across traits (Jørgensen et al., 2019), and future work integrating such approaches would be insightful.

It would be interesting to expand future heatwave studies to test whether trait-specific variability changes after multiple days of exposure, with and without a rest phase. This may reveal the relative vulnerability of specific traits to natural heatwaves, whose intensity would vary over several days (Frich et al., 2002; Christidis et al., 2023). It is also unclear whether some traits can recover or harden after exposure to extreme conditions (e.g. 48°C and 50°C) under natural heatwave scenarios, as potentially compounding effects from repeated exposures may be modulated by periods of non-stressful conditions (e.g. benign cooler night-time and daytime temperatures), allowing time for physiological repair and fitness recovery (Bai et al., 2019). Previous work found that males can recover reproductive function after longer exposure to less intense experimental heatwave conditions (Sales et al., 2021). Therefore, it will also be important to understand whether there is a general recovery of reproductive potential in males exposed to shorter but more intense heatwave conditions as used in this study.

Our study focused on the effects of heatwave conditions that may be experienced by this species in its natural environment, and which are likely to be increasingly common in the future (Zittis et al., 2021; Campbell et al., 2022). We show that reproductive output is sensitive to sublethal short heatwaves. However, we recognise that the thermal homogeneity and potential lack of interacting factors in our study may have constrained strategies such as moving to more benign microhabitats (e.g. dropping behaviour in aphids; Ma and Ma, 2012), which may alleviate the impact of extreme heat (Terlau et al., 2023). In future studies, it will be necessary to explore these factors and assess whether different species can adapt to increasingly severe short-term heat exposures (Kellermann and van Heerwaarden, 2019) or whether they are likely to be overwhelmed by the transient and unpredictable nature of extreme thermal events (van de Pol et al., 2017).

Acknowledgements
This work is dedicated to Prof. Matt Gage (1967–2022). We also thank members of the Gage lab who have helped to develop this study and members of the European Thermal Fertility Network (an ESEB funded Special Topics Network to study thermal fertility impacts; 2020–present) for general research discussions about fertility impacts.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: B.C., R.V., M.J.G.G.; Data curation: B.C., R.V., K.D., J.H., J.K.; Formal analysis: B.C., R.V.; Funding acquisition: M.J.G.G.; Investigation: B.C., R.V., K.D., J.H., J.K.; Methodology: B.C., R.V., K.D., J.H., J.K., M.J.G.G.; Project administration: B.C., R.V.; Resources: B.C., R.V.; Software: B.C., R.V.; Supervision: R.V., A.A.M., T.C., M.J.G.G.; Validation: B.C., R.V.; Visualization: B.C., R.V.; Writing – original draft: B.C., R.V.; Writing – review & editing: B.C., R.V., K.D., J.H., J.K., A.A.M., T.C.

Funding
B.C. was supported by the UKRI Biotechnology and Biological Sciences Research Council Norwich Research Park Biosciences Doctoral Training Partnership [BB/T008717/1]. R.V. was funded by a Commonwealth Rutherford Fellowship (INRF-2017-254). Open Access funding provided by University of East Anglia. Deposited in PMC for immediate release.

Data and resource availability
The raw data files along with the associated R scripts with the codes for analyses are available from the GitHub repository: https://github.com/rvasudeva83/sHW-fertility.git

References
Bai, C.-M., Ma, G., Cai, W.-Z. and Ma, C.-S. (2019). Independent and combined effects of daytime heat stress and nighttime recovery determine thermal performance. *Biol. Open* **8**, bio038141. doi:10.1242/bio.038141

Basso, A. L., Forneris, N. S., Filiberti, A., Argaraña, C. E., Rabossi, A. and Quesada-Allué, L. A. (2011). Metamorphosis and gonad maturation in the horn fly *Haematobia irritans*. *J. Insect Sci.* **11**, 174. doi:10.1673/031.011.17401

Campbell, J. F., Athanassiou, C. G., Hagstrum, D. W. and Zhu, K. Y. (2022). *Tribolium castaneum*: a model insect for fundamental and applied research. *Annu. Rev. Entomol.* **67**, 347-365. doi:10.1146/annurev-ento-080921-075157

Campion, C., Rajamohan, A. and Dillon, M. E. (2023). Sperm can't take the heat: short-term temperature exposures compromise fertility of male bumble bees (*Bombus impatiens*). *J. Insect Physiol.* **146**, 104491. doi:10.1016/j.jinsphys.2023.104491

Canal Domenech, B. and Fricke, C. (2023). Developmental heat stress interrupts spermatogenesis inducing early male sterility in *Drosophila melanogaster*. *J. Therm. Biol.* **114**, 103589. doi:10.1016/j.jtherbio.2023.103589

Ceballos, G., Ehrlich, P. R., Barnosky, A. D., García, A., Pringle, R. M. and Palmer, T. M. (2015). Accelerated modern human–induced species losses: entering the sixth mass extinction. *Sci. Adv.* **1**, e1400253.
Christidis, N., Mitchell, D. and Stott, P. A. (2023). Rapidly increasing likelihood of exceeding 50°C in parts of the Mediterranean and the Middle East due to human influence. *NPJ Clim. Atmos. Sci.* **6**, 1-12. doi:10.1038/s41612-023-00377-4
Dickinson, M. (2018). The impacts of heat-wave conditions on reproduction in a model insect, *Tribolium castaneum*. *PhD thesis*, University of East Anglia. https://ueaeprints.uea.ac.uk/id/eprint/67673
Dougherty, L. R., Frost, F., Maenpaa, M. I., Rowe, M., Cole, B. J., Vasudeva, R., Pottier, P., Schultner, E., Macartney, E. L., Lindenbaum, I. et al. (2024). A systematic map of studies testing the relationship between temperature and animal reproduction. *Ecol Solut. Evid.* **5**, e12303. doi:10.1002/2688-8319.12303
Dunn, R. (2005). Modern insect extinctions, the neglected majority. *Conserv. Biol.* **19**, 1030-1036. doi:10.1111/j.1523-1739.2005.00078.x
Fabian, L. and Brill, J. A. (2012). *Drosophila* spermiogenesis. *Spermatogenesis* **2**, 197-212. doi:10.4161/spmg.21798
Frich, P., Alexander, L., Della-Marta, P., Gleason, B., Haylock, M., Klein Tank, A. and Peterson, T. (2002). Observed coherent changes in climatic extremes during the second half of the twentieth century. *Clim. Res.* **19**, 193-212. doi:10.3354/cr019193
Godwin, J. L., Vasudeva, R., Michalczyk, Ł., Martin, O. Y., Lumley, A. J., Chapman, T. and Gage, M. J. G. (2017). Experimental evolution reveals that sperm competition intensity selects for longer, more costly sperm. *Evol. Lett.* **1**, 102-113. doi:10.1002/evl3.13
Grandela, A., Antunes, M. A., Santos, M. A., Matos, M., Rodrigues, L. R. and Simões, P. (2023). Detrimental impact of a heatwave on male reproductive behaviour and fertility. *Acta Ethol.* **27**, 1-11. doi:10.1007/s10211-023-00431-7
Holmes, T. R. H., Crow, W. T., Hain, C., Anderson, M. C. and Kustas, W. P. (2015). Diurnal temperature cycle as observed by thermal infrared and microwave radiometers. *Remote Sens. Environ.* **158**, 110-125. doi:10.1016/j.rse.2014.10.031
Hu, J., Medison, R. G., Zhang, S., Ma, P. and Shi, C. (2022). Impacts of non-lethal high-temperature stress on the development and reproductive organs of *Bradysia odoriphaga*. *Insects* **13**, 74. doi:10.3390/insects13010074
Iossa, G., Maury, C., Fletcher, R. M. and Eady, P. E. (2019). Temperature–induced developmental plasticity in *Plodia interpunctella*: Reproductive behaviour and sperm length. *J. Evol. Biol.* **32**, 675-682. doi:10.1111/jeb.13447
Jørgensen, L. B., Malte, H. and Overgaard, J. (2019). How to assess Drosophila heat tolerance: Unifying static and dynamic tolerance assays to predict heat distribution limits. *Funct. Ecol.* **33**, 629-642. doi:10.1111/1365-2435.13279
Kellermann, V. and van Heerwaarden, B. (2019). Terrestrial insects and climate change: adaptive responses in key traits. *Physiol. Entomol.* **44**, 99-115. doi:10.1111/phen.12282
Lüdecke, D., Ben-Shachar, M. S., Patil, I., Waggoner, P. and Makowski, D. (2021). performance: an R package for assessment, comparison and testing of statistical models. *J. Open Source Softw.* **6**, 3139. doi:10.21105/joss.03139
Lv, W., Jiang, C., Wang, F., Liu, H., Stanley, D. and Zhang, L. (2024). Experimental heatwaves impair male fertility in oriental armyworm, *Mythimna separata*. *Entomol. Gen.* **44**, 915-925. doi:10.1127/entomologia/2024/2476
Ma, G. and Ma, C.-S. (2012). Climate warming may increase aphids' dropping probabilities in response to high temperatures. *J. Insect Physiol.* **58**, 1456-1462. doi:10.1016/j.jinsphys.2012.08.012
Ma, C.-S., Ma, G. and Pincebourde, S. (2020). Survive a warming climate: insect responses to extreme high temperatures. *Annu. Rev. Entomol.* **66**, 163-184. doi:10.1146/annurev-ento-041520-074454
Martinet, B., Zambra, E., Przybyla, K., Lecocq, T., Anselmo, A., Nonclercq, D., Rasmont, P., Michez, D. and Hennebert, E. (2020). Mating under climate change: impact of simulated heatwaves on the reproduction of model pollinators. *Funct. Ecol.* **35**, 739-752. doi:10.1111/1365-2435.13738
Meehl, G. A. and Tebaldi, C. (2004). More intense, more frequent, and longer lasting heat waves in the 21st century. *Science* **305**, 994-997. doi:10.1126/science.1098704
Meena, A., Maggu, K., De Nardo, A. N., Sbilordo, S. H., Eggs, B., Al Toma Sho, R. and Lüpold, S. (2024). Life stage-specific effects of heat stress on spermatogenesis and oogenesis in *Drosophila melanogaster*. *J. Therm. Biol.* **125**, 104001. doi:10.1016/j.jtherbio.2024.104001
Oliveira, A., Lopes, A., Correia, E., Niza, S. and Soares, A. (2021). Heatwaves and summer urban heat islands: a daily cycle approach to unveil the urban thermal signal changes in Lisbon, Portugal. *Atmosphere* **12**, 292. doi:10.3390/atmos12030292
Ørsted, M., Willot, Q., Olsen, A. K., Kongsgaard, V. and Overgaard, J. (2024). Thermal limits of survival and reproduction depend on stress duration: a case study of *Drosophila suzukii*. *Ecol. Lett.* **27**, e14421. doi:10.1111/ele.14421
Park, T., Mertz, D., Grodzinski, W. and Prus, T. (1965). Cannibalistic predation in populations of flour beetles. *Ecol. Evol. Physiol.* **38**. doi:10.1086/physzool.38.3.30152840
Parratt, S. R., Walsh, B. S., Metelmann, S., White, N., Manser, A., Bretman, A. J., Hoffmann, A. A., Snook, R. R. and Price, T. A. R. (2021). Temperatures that sterilize males better match global species distributions than lethal temperatures. *Nat. Clim. Change* **11**, 481-484. doi:10.1038/s41558-021-01047-0
Perkins-Kirkpatrick, S. E. and Lewis, S. C. (2020). Increasing trends in regional heatwaves. *Nat. Commun.* **11**, 3357. doi:10.1038/s41467-020-16970-7
Pilakouta, N., Sellers, L. G., Barratt, R. and Ligonniere, A. (2023). The consequences of heatwaves for animal reproduction are timing–dependent. *Funct. Ecol.* **37**, 2425-2433. doi:10.1111/1365-2435.14386
Pointer, M. D., Gage, M. J. G. and Spurgin, L. G. (2021). Tribolium beetles as a model system in evolution and ecology. *Heredity* **126**, 869-883. doi:10.1038/s41437-021-00420-1
Porcelli, D., Gaston, K. J., Butlin, R. K. and Snook, R. R. (2016). Local adaptation of reproductive performance during thermal stress. *J. Evol. Biol.* **30**, 422-429. doi:10.1111/jeb.13018
Ratz, T., Chechi, T. S., Dimopoulou, A.-I., Sedlmair, S. D. and Tuni, C. (2024). Heatwaves inflict reproductive but not survival costs to male insects. *J. Exp. Biol.* **227**, jeb246698. doi:10.1242/jeb.246698
Rezende, E. L., Castañeda, L. E. and Santos, M. (2014). Tolerance landscapes in thermal ecology. *Funct. Ecol.* **28**, 799-809. doi:10.1111/1365-2435.12268
Rohmer, C. (2004). Heat induced male sterility in Drosophila melanogaster: adaptive genetic variations among geographic populations and role of the Y chromosome. *J. Exp. Biol.* **207**, 2735-2743. doi:10.1242/jeb.01087
Sales, K., Vasudeva, R., Dickinson, M. E., Godwin, J. L., Lumley, A. J., Michalczyk, Ł., Hebberecht, L., Thomas, P., Franco, A. and Gage, M. J. G. (2018). Experimental heatwaves compromise sperm function and cause transgenerational damage in a model insect. *Nat. Commun.* **9**, 4771. doi:10.1038/s41467-018-07273-z
Sales, K., Vasudeva, R. and Gage, M. J. G. (2021). Fertility and mortality impacts of thermal stress from experimental heatwaves on different life stages and their recovery in a model insect. *R. Soc. Open Sci.* **8**, 201717. doi:10.1098/rsos.201717
Sales, K., Thomas, P., Gage, M. and Vasudeva, R. (2024). Experimental heatwaves reduce the effectiveness of ejaculates at occupying female reproductive tracts in a model insect. *R. Soc. Open Sci.* **11**, 231949. doi:10.1098/rsos.231949
Seneviratne, S. I., Zhang, X., Adnan, M., Badi, W., Dereczynski, C., Di Luca, A., Ghosh, S., Iskandar, I., Kossin, J., Lewis, S. et al. (2021). *Weather and Climate Extreme Events in a Changing Climate*. In Climate Change 2021: The Physical Science Basis. Contribution of Working Group I to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge: Cambridge University Press, 1513-1766.
Terlau, J. F., Brose, U., Eisenhauer, N., Angelos Amyntas, B., Dyer, T., Gebler, A., Hof, A., Liu, C., Scherber, T., Schlägel, C. et al. (2023). Microhabitat conditions remedy heat stress effects on insect activity. *Glob. Change Biol.* **29**, 3747-3758. doi:10.1111/gcb.16712
Trenberth, K. E. (2018). Climate change caused by human activities is happening and it already has major consequences. *J. Energy Nat. Res. Law* **36**, 463-481. doi:10.1080/02646811.2018.1450895
Uy, K. L., LeDuc, R., Ganote, C. and Price, D. K. (2015). Physiological effects of heat stress on Hawaiian picture-wing *Drosophila*: genome-wide expression patterns and stress-related traits. *Conserv. Physiol.* **3**, cou062. doi:10.1093/conphys/cou062
van de Pol, M., Jenouvrier, S., Cornelissen, J. H. C. and Visser, M. E. (2017). Behavioural, ecological and evolutionary responses to extreme climatic events: challenges and directions. *Philos. Trans. R. Soc. B Biol. Sci.* **372**, 20160134. doi:10.1098/rstb.2016.0134
van Heerwaarden, B. and Sgrò, C. M. (2021). Male fertility thermal limits predict vulnerability to climate warming. *Nat. Commun.* **12**, 2214. doi:10.1038/s41467-021-22546-w
Vasudeva, R., Deeming, D. C. and Eady, P. E. (2014). Developmental temperature affects the expression of ejaculatory traits and the outcome of sperm competition in *Callosobruchus maculatus*. *J. Evol. Biol.* **27**, 1811-1818. doi:10.1111/jeb.12431
Vasudeva, R., Sutter, A., Sales, K., Dickinson, M. E., Lumley, A. J. and Gage, M. J. (2019). Adaptive thermal plasticity enhances sperm and egg performance in a model insect. *eLife* **8**, e49452. doi:10.7554/elife.49452
Wagner, D. L., Grames, E. M., Forister, M. L., Berenbaum, M. R. and Stopak, D. (2021). Insect decline in the Anthropocene: Death by a thousand cuts. *Proc. Natl Acad. Sci. USA* **118**, e2023989118. doi:10.1073/pnas.2023989118
Wickham, H. (2016). *ggplot2: Elegant Graphics for Data Analysis*. Springer-Verlag New York.
Wickham, H., Averick, M., Bryan, J., Chang, W., McGowan, L. D., François, R., Grolemund, G., Hayes, A., Henry, L., Hester, J. et al. (2019). Welcome to the tidyverse. *J. Open Source Softw.* **4**, 1686. doi:10.21105/joss.01686
Wiens, J. J. (2016). Climate-related local extinctions are already widespread among plant and animal species. *PLoS Biol.* **14**, e2001104. doi:10.1371/journal.pbio.2001104
World Meteorological Organisation (2024). *WMO Global Annual to Decadal Climate Update (Target years: 2024 and 2024-2028)*. Geneva: World Meteorological Organisation.
Zeileis, A. and Hothorn, T. (2002). Diagnostic checking in regression relationships. *R News* **2**, 7-10. https://CRAN.R-project.org/doc/Rnews/
Zeileis, A., Kleiber, C. and Jackman, S. (2008). Regression models for count data in R. *J. Stat. Softw.* **27**. http://www.jstatsoft.org/v27/i08/
Zittis, G., Hadjinicolaou, P., Almazroui, M., Bucchignani, E., Driouech, F., El Rhaz, K., Kurnaz, L., Nikulin, G., Ntoumos, A., Ozturk, T. et al. (2021). Business-as-usual will lead to super and ultra-extreme heatwaves in the Middle East and North Africa. *NPJ Clim. Atmos. Sci.* **4**, 1-9. doi:10.1038/s41612-021-00178-7

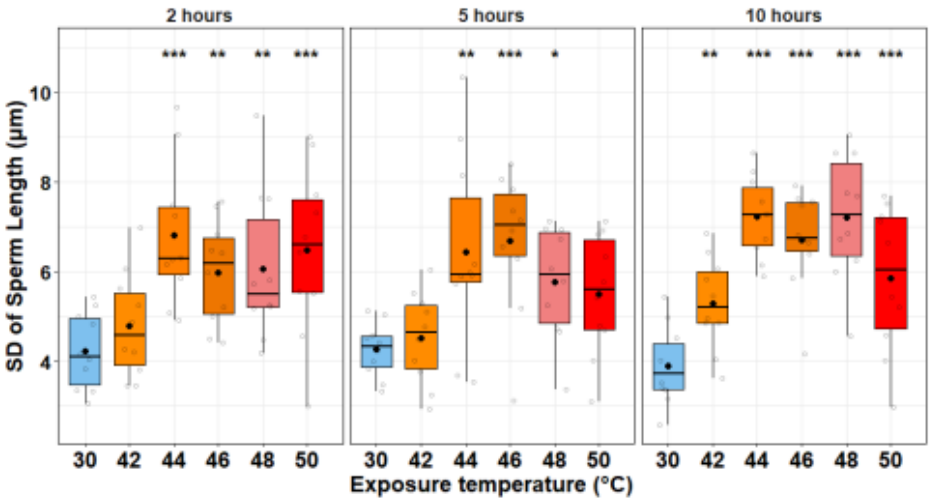

Fig. S1. Effects of varying heatwave conditions on the standard deviation of sperm length within males. The mean sperm length standard deviation per male is plotted as open jittered circles. Boxplots contain a median line, mean dot and interquartile range box. Significance values representing a comparison between 30°C and the experimental heatwave condition are denoted by stars: * = P <0.05, ** = P < 0.01, *** = P < 0.001. The sample size for sperm length variation consisted of 20 individual sperm from 10 males across all groups.

The standard deviation of sperm length for each male was calculated. Linear mixed models were fitted separately for each exposure duration with temperature as a fixed effect and Male ID as a random effect. Our results show a significant increase in sperm length variability at higher temperatures across all durations (Fig. S1). All heatwave exposures for 2 h increased the standard deviation of sperm length when compared to controls, except for 42°C ($t = 0.933$, $p = 0.356$). Variability increased by 61.1% at 44°C ($t = 4.234$, $p < 0.001$), 41.7% at 46°C ($t = 2.881$, $p = 0.006$), 43.3% at 48°C ($t = 3.008$, $p = 0.004$), and 53.1% at 50°C ($t = 3.683$, $p < 0.001$), relative to controls. In line with the 2 h exposure results, a 5 h exposure at 42°C did not significantly affect sperm length variation when compared to controls ($t = 0.415$, $p = 0.679$). In contrast, exposure to 44°C resulted in a 51.3% increase ($t = 3.459$, $p = 0.001$). Variability remained high at more extreme temperatures, with increases of 57.2% at 46°C ($t = 3.862$, $p < 0.001$), 35.3% at 48°C ($t = 2.390$, $p = 0.020$), and 29.3% at 50°C ($t = 1.974$, $p = 0.053$), although the latter only approached significance. Exposure to

heatwave conditions for 10 h significantly increased sperm length variability at all temperatures tested. Variability increased by 36.0% at 42°C ($t = 2.792$, $p = 0.008$), with larger increases at higher temperatures: 85.5% at 44°C ($t = 6.637$, $p < 0.001$), 72.5% at 46°C ($t = 5.618$, $p < 0.001$), 85.3% at 48°C ($t = 6.614$, $p < 0.001$), and 49.9% at 50°C ($t = 3.883$, $p < 0.001$), relative to controls.

These findings demonstrate that prolonged heat exposure consistently elevates sperm length variability, highlighting a potential sensitivity of sperm morphology to heatwave conditions. This increase in variability may have a role in the observed reductions in male reproductive output.