The impacts of heat-wave conditions on reproduction in a model insect, *Tribolium castaneum*

Matthew Edward Dickinson

University of East Anglia

Faculty of Science

School of Biology

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Abstract

Climate change will increase the frequency, intensity and duration of weather extremes including heat-waves, which could have important consequences for biodiversity. This thesis examines the direct influences of thermal stress associated with heat-wave conditions on reproduction in a model insect in order to understand how animal populations might be affected by climate change. Fertility reductions in homeotherms due to thermal stress are well documented, but effects on ectotherms have received little attention. In the first half of this thesis, the flour beetle model *Tribolium castaneum* is used to measure the impacts of heat-wave conditions on reproductive fitness in males and females, and the proximate mechanisms behind any impacts. I find that the reproductive fitness of males, but not females, is impacted by heat-wave conditions. Female fecundity is not affected when mating with heat stressed males, but egg hatch and pupal eclosion rates are reduced. Transgenerational effects were not found beyond the pupal stage.

In experimental examinations of mating behaviour, males exposed to heat-wave conditions were slower to initiate mating and mated less frequently, but still achieving sufficient matings that would normally allow full female fertility. However, ejaculate sperm numbers were reduced more than five-fold following a simulated heat-wave, partly explaining how male reproductive fitness is halved following exposure to a heat-wave.

In addition to an impact on male fertility and sperm production, I also found clear evidence that sperm in female storage were also sensitive to heat-wave conditions, with significant declines in female reproductive fitness if they had already mated and contained sperm, but no effects if the heat-wave was experienced before mating and sperm storage.

This sensitivity of male fertility to heat-wave conditions could generate selection on both males and females to respond. In the second half of the project, the male and female responses to thermal stress and their impacts across generations were investigated. Females were found to be able to rescue their fertility when facing matings with heat stressed males by mating polyandrously, restoring their reproductive output to normal levels. However, I found no evidence that females strategically or facultatively adjusted their remating behaviour to compensate for reductions in fertility condition after thermal stress, and multi-generational male

responses to elevated ambient temperature regimes showed no evidence for an ability to acclimate or adapt to heat-wave conditions.

This thesis advances our knowledge of how one important trait for population viability and biodiversity can be impacted by climate change and increases in extreme weather conditions. It offers directions for future research to investigate the drivers of temperature-induced male fertility loss, and suggestions for how management efforts might be focused to mitigate the impacts of heat-wave conditions on reproduction in ectotherms.

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1 Introduction: heat-waves and thermal impacts on living systems

CHAPTER SUMMARY

Climatological evidence shows the world is warming up. The most probable outcome will not only be an overall increase in temperature, but an increase in extreme temperature events. Future heat-waves are expected to become more intense, more frequent, and longer lasting in the second half of the 21st century. Here, I consider the evidence for increases in heat-waves, and introduce studies which have investigated the impact of extreme heat upon reproduction. Heat-induced sterility has been documented in a variety of species, and studies have repeatedly demonstrated that thermal stress consistently leads to a greater impact on fertility in males than in females. Biologists need to examine how important traits, such as those necessary for reproduction, respond to heat-wave conditions to understand and predict the effects of extreme weather events under climate change upon biodiversity. To understand this phenomenon better, and to examine its impacts within an understudied but important taxonomic group, I introduce the *Tribolium castaneum* flour beetle as a model insect system to study how male reproductive fitness is impacted by thermal stress, and then measure the capacity of individuals and populations to respond.

1.1 Climate change and heat-waves

The world's climate is changing, and the 90% probability interval for global warming from 1990 to 2100 is a predicted average increase of between 1.7°C and 4.9°C (IPCC, 2007). The most probable outcome will not only be an overall rise in temperature, but more frequent heat-waves, droughts and extreme weather events (Karl & Trenberth, 2003).

An extreme (weather or climate) event is generally defined as '*the occurrence of a value of a weather or climate variable above (or below) a threshold value near the upper (or lower) ends ('tails') of the range of observed values of the variable*' (Seneviratne *et al.*, 2012). In this thesis, I use the definition from Frich *et al.* (2002):

five continuous days with the temperature at or above 5°C above previous averages. See Chapter 2.2.2 for a discussion of heat-wave definitions and why I choose this as the most appropriate for my purposes. For the 'previous average' I use the temperature at which population productivity is optimised which, in *T. castaneum*, is 35°C (Sokoloff, 1972-78 and Chapter 3). Climate change means that the frequency, intensity, spatial extent, duration, and timing of extreme weather and climate events can change, and can result in unprecedented extreme weather and climate events. These result in changes in the mean, variance, or shape of probability distributions for environmental temperatures, or a combination of these (IPCC, 2012), although increases in variability will have more direct influences on the frequency of temperature extremes (Katz & Brown, 1992).

The low frequency of extreme events makes it difficult to identify long-term changes, as fewer data are available to model their frequency or intensity. Nevertheless, there is evidence from observations gathered since the 1940s and '50s, of changes in some extremes in many (but not all) regions across the globe (IPCC, 2012, King *et al.*, 2015). In those areas where sufficient data have been recorded, there is medium confidence that the length or number of warm spells or heat-waves has already increased since the middle of the 20th century (Seneviratne *et al.*, 2012). Furthermore, models predict that an increase in heat-wave events over most land areas during the late 20th century was '*likely*' with a future trend of further increase '*highly likely*' (IPCC, 2014). Models predict that future heat-waves are '*virtually certain*' (99–100% probability) to become more intense, more frequent, and longer lasting on a global scale in the second half of the 21st century (Meehl & Tebaldi, 2004; IPCC, 2012). In other words, an extreme temperature record that was a 20-year occurrence (i.e., a value that was exceeded on average only once during the period 1981–2000) is likely to become a 1-in-2-year event by the end of the 21st century in most regions, and this extreme daily maximum temperature will likely increase by about 1°C to 3°C by the mid-21st century, and by about 2°C to 5°C by the late 21st century, depending on the region and emissions scenario (IPCC, 2012). Observed changes on land are consistent with these regional and global analyses of temperature extremes (Smith *et al.*, 2008; Seneviratne *et al.*, 2012).

With the increases in heat-waves that have been observed, and with projections that posit a further future increase in extreme weather events, the question is raised as to how organisms and ecosystems will be affected by extreme

weather events, and how important these are for biodiversity compared with more gradual, average changes. It is important to ascertain the impact of heat-waves on environmental, economic and conservation concerns worldwide. Because the rate of climate change is, by definition, unnaturally high, it is likely that this will hasten population extinctions or range shifts. This has been confirmed in some empirical studies (e.g. McLaughlin *et al.*, 2002), and there is a solid body of evidence that climate change is having ecological consequences (e.g. Visser, 2008). In this thesis, I use laboratory experimentation with an insect model to understand in detail some of the consequences that extreme changes in thermal regime have for reproduction.

1.2 Fertility and temperature

Heat-waves can impact ecosystems, both directly by decreasing production or causing mortality, and indirectly by constraining carbon and nitrogen cycling and reducing water availability (Handmer *et al.*, 2012). Most importantly for the current work, there is now convincing evidence for a number of systems that changes in temperature can cause sterility, usually for males, and through this limit population viability (Rohmer *et al.*, 2004; David *et al.*, 2005).

Sterile males have been used as a way of controlling population size for over fifty years; they 'exert greater influence in regulating animal populations than can be achieved by destroying or removing the same number of individuals from the population' (Knipling, 1959). The effects of exposure to heat on the fertility of male animals has been known for decades in homeotherms (review in Setchell, 1998). Adaptations that allow testicular cooling of 2 to 8°C below core body temperature, such as external testes in many mammals, make it clear that male reproductive physiology and fertility is sensitive to warm temperatures. Even mild increases in the thermal environment can disrupt male fertility: for example, exposing male mice to an air temperature of 32°C for 24h resulted in their fertility declining to 11%, compared with 78-91% for untreated controls (Burfening *et al.*, 1970). Similar fertility decreases were shown by rams exposed to 32°C for just 4 days (Howarth 1969). Direct experimental elevation of testicular temperature also causes fertility declines, even after brief periods of thermal increase; e.g. 20-30 minutes of exposure to warming causes sperm count declines and infertility in mice (Jannes *et al.*, 1998, Paul, Teng & Saunders, 2008). Male reproductive competence is therefore specifically fragile to

changes in temperature that organisms often experience in the natural environment, and since infertility can constrain population viability, this thesis explores the impacts of heat-wave conditions on reproductive fitness.

By contrast with endothermic species, the thermal sensitivity of male fertility in ectothermic taxa has hardly been examined. The work conducted so far shows that similar male sensitivity exists, to the extent that it can be a primary limitation for population viability as the thermal environment changes (Rohmer *et al.*, 2004, David *et al.*, 2005). In *D. melanogaster*, it is thermal sensitivity of male fertility that specifically determines the upper limit of population viability which, in most strains, is 30°C, because this is the threshold at which males become sterile (Rohmer *et al.*, 2004, David *et al.*, 2005).

Ectotherms comprise the majority of global biodiversity; their inability to thermally self-regulate means that they are particularly sensitive to temperature changes. The phenomenon of sex-specific fertility loss is starting to become recognised in ectotherms. Harvey & Viney (2007) demonstrated thermal variation in fecundity in *Caenorhabditis elegans*. Fecundity decreased significantly when isolates developed at, or mature individuals were exposed to, 25°C rather than 19°C. The difference in fecundity at the hotter temperature was not present if mating had already occurred and sperm production was correlated with lifetime fecundity. There was no effect on oocyte development, therefore temperature seems to affect sperm function in this nematode (Harvey & Viney, 2007). Work on another nematode worm, *Caenorhabditis briggsae*, looked at how natural populations adapt to their thermal regimes: strain comparisons between temperate and tropical latitudes show that the temperate strain has reduced reproductive output at higher temperatures compared to the tropical strain (Presad *et al.*, 2011). In addition, heat-treated hermaphrodites mated to control males recovered their fecundity in line with that of control hermaphrodites. These results together strongly suggest that the declines in reproductive fitness were due to an effect on males, which then acts as a limit on natural population viability (Presad *et al.*, 2011). Since the physiology of ectotherms is directly dependent on temperature variation, thermosensitivity of male fertility could have important consequences for their population size when exposed to extreme changes in their thermal environment. Despite this, and in contrast to a lot of work examining ecological consequences of populations to average environmental temperature changes (e.g. Hughes 2000; Thomas *et al.*, 2004; Thomas, Franco & Hill, 2006; Chen *et al.*, 2011), only a small

handful of studies have investigated the '*significant but neglected phenomenon*' (David *et al.*, 2005) of the impact of extreme temperature variation on male fertility in ectotherms.

Although male sterility at extreme temperatures is a problem that is not well documented in insects, evidence that it affects different taxa is growing (David *et al.*, 2005). In insects, there is a gender-specific differential gene expression in the collembolan *Orchesella cincta* when temperature increases by 10°C (Ellers *et al.*, 2008). *Drosophila melanogaster* studies show that high temperature affects individual males, producing a sharp decline in fertility, and thereby limiting population viability (Figure 1.1) (David *et al.*, 2005). Females are not completely immune to this phenomenon, however, Krebs and Loeschcke (1994) found short-term exposure to much higher temperatures (37 to 40°C) reduced female fecundity in *D. melanogaster*, measured as offspring produced over ten days. Male fertility, measured as the progeny produced by a female mated once, differed little among treatments, but these experiments exposed males and females to heat for only 90 to 120 minutes, and may therefore have less relevance to heat-wave conditions in the natural environment.

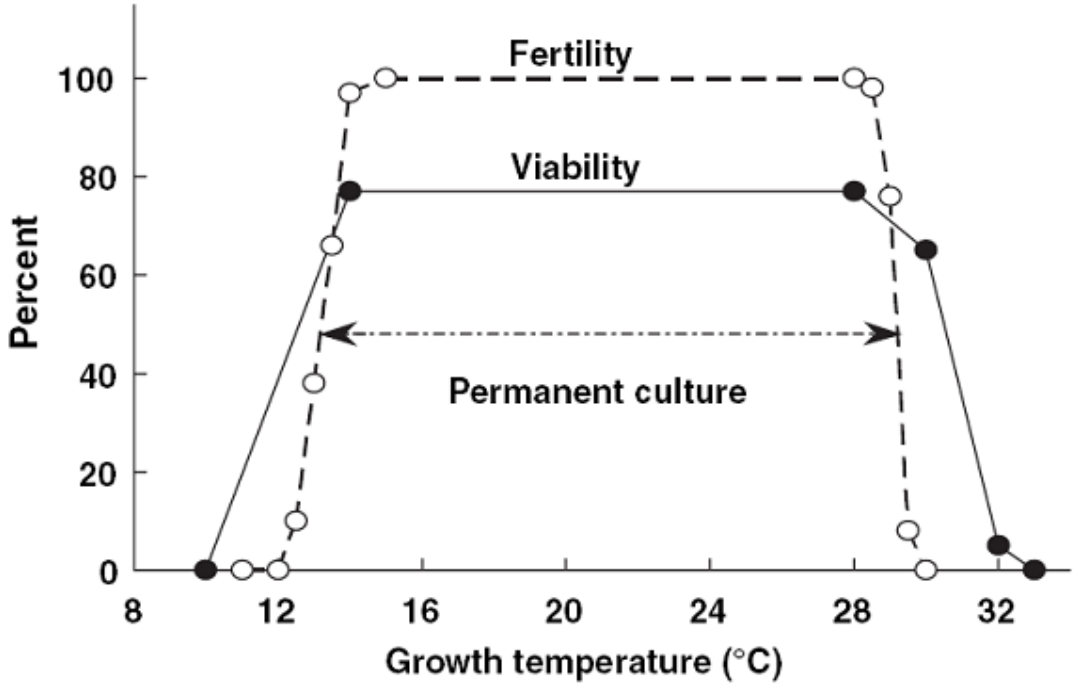

Figure 1.1 Comparison of reaction norms for viability and male fertility according to population growth temperature in a temperate strain of *D. melanogaster* (from David *et al*., 2005).

Among *Drosophila* (fly) species, thermal range is quite variable and correlates with geographic distribution: on average, temperate species are more cold-tolerant, and tropical species more heat tolerant. Upper thermal heat sterility thresholds vary from 23°C in temperate, heat sensitive species, up to 31°C in heat tolerant species. Rohmer *et al.* (2004) showed that different wild populations of *Drosophila* (tropical and temperate) suffered 50% sterility at different temperatures (1°C higher for tropical populations) and provided evidence that this is due to impacts on the male. This finding was shown with back-crosses to introduce the Y chromosome of one population into males of the other, with heat tolerance following the Y chromosome introgression indicating heat-induced sterility is Y chromosome dependent.

In *D. melanogaster,* there is variation between strains in fertility at upper thermal limits (Figure 1.2), with absolute sterility induced at 29.5, 31.0 and 32.5°C for Draveil, Delhi and Panipat derived strains, respectively (Figure 1.2). This strongly suggests a genetic component for fertility tolerance to heat-wave conditions. When this genetic component is further scrutinised, 50% of the differences between geographic populations were confirmed to be due to the Y-chromosome, so the thermal tolerance of a population seems to be linked to male biology (David *et al.*, 2005). Hsp70 expression has been shown to reduce the duration of sterility after thermal stress (Sarup *et al.*, 2004), although this relationship is not conclusive: heat-tolerant strains have been found to have low Hsp70 expression following thermal stress (Zatsepina *et al.*, 2001), still regulation of this protein or other similar plastic responses may be the mechanism that influences differential fertility.

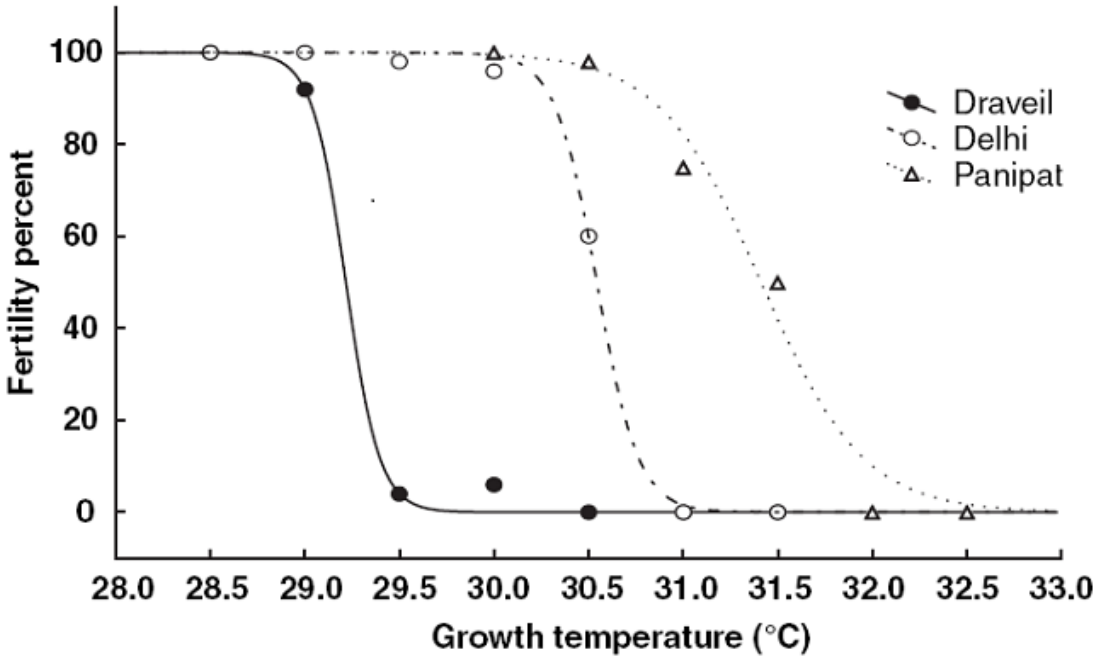

Figure 1.2 Variation in male fertility at high temperatures in three *D. melanogaster* strains (from David *et al*., 2005).

Dolgin, Whitlock & Agrawal (2006) provided evidence that local reproductive adaptation to thermal environment can take place: male *D. melanogaster* have higher mating success at temperatures matching their local thermal environment. Using experimental evolution, males were maintained for ten years at 18 or 25°C, and then reared for a single experimental generation at one of the two temperature regimes, before competing for control females in either an 18 or 25°C mating environment. The mating regime was replicated three times at each temperature treatment using three pairs of populations or 'sets'. Males were significantly more likely to mate successfully in their own experimentally evolved thermal environment, than when trialled in the other, different temperature, for most experimental replicates. In the two cases where no significant advantage was obvious, there was essentially no difference between either type of male (Figure 1.3).

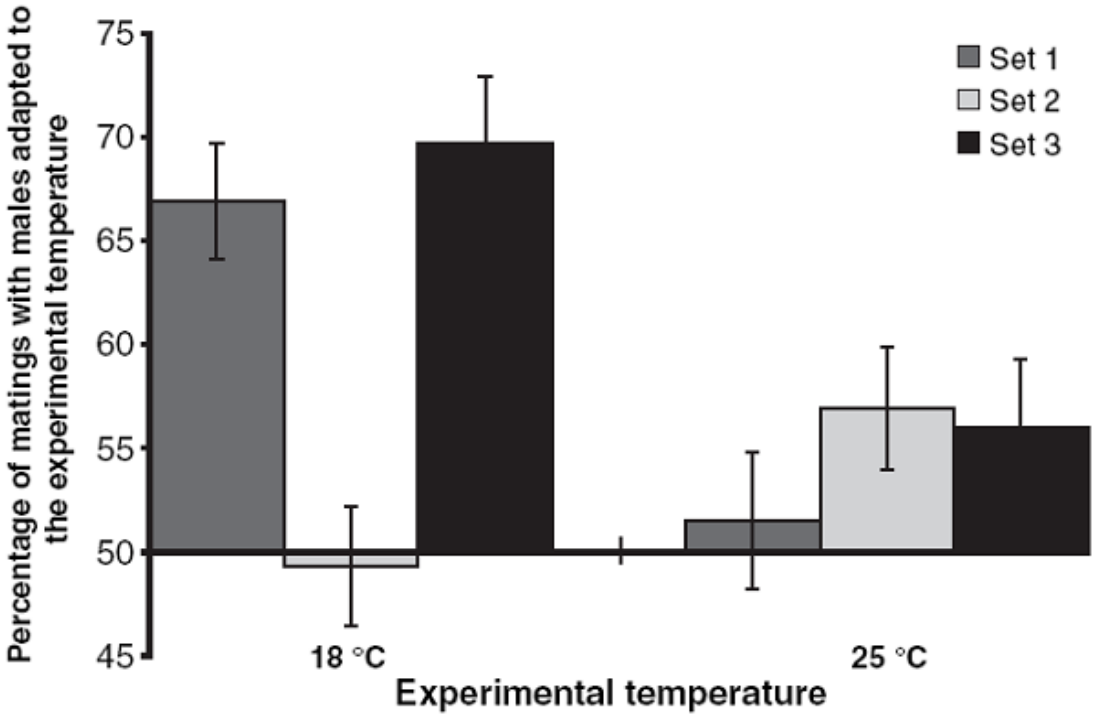

Figure 1.3 Percentage of successful matings by males from the selection line adapted to the local experimental temperature, at 18 and 25°C test temperatures in each set. The results were calculated by weighting by the number of matings per cage. The axis at 50% indicates no difference in mating success between males adapted to the two different temperatures. Error bars denote one standard error (from Dolgin, Whitlock & Agrawal, 2006).

There is therefore abundant evidence that male fertility is sensitive to thermal variation, and this is now beginning to be recognised in ectothermic species. In a world where thermal environments are changing rapidly, it is necessary to understand more precisely how male fertility is impacted by heat stress, and the capacity of male fertility to adapt and evolve to local thermal regimes in a wider range of taxa. Insects account for an estimated 0.8-1.2 million of all animal species on Earth, and it is said, '*with perhaps a little exaggeration, that on first approximation all animals are insects*' (Ruppert, Fox & Barnes, 2004). Insect ecologies are extremely variable, often dominating food chains and food webs in terms of biomass and species richness, and occupying multiple trophic levels (Gullan & Cranston, 1999). As ectotherms, insects are inherently sensitive to temperature changes, but they also have high fecundity and short generation times, giving them relatively high evolutionary potential to cope with selection from climate changes. They therefore represent a model grouping that deserves further attention from the perspective of environmental change. In this thesis, I present a laboratory-based approach to understanding how male fertility is impacted by thermal stress in a model insect

Tribolium castaneum, examine mating pattern behaviours that may increase resilience to heat-wave conditions at a population level, and conclude with a selection experiment that measures the capacity of populations to adapt.

1.3 Temperature, populations and climate change

Temperature is thought to be '*the most important factor*' when looking at the impact of environmental stress on populations (Parsons, 1973). High temperature extremes (i.e. heat-waves), substantially affect ecosystems (Handmer *et al.*, 2012). Increases in the frequency of large-scale disturbances due to extreme weather and climate events can create gaps in, or contractions of, the distribution range for species (Handmer *et al.*, 2012). Changes due to climate extremes can also reduce ecosystem resilience, through the loss of ecosystem services dependent on the previous evolved state (Handmer *et al.*, 2012). It is important to note that climate extremes are not damaging *per se*; many ecosystems are dependent on climate extremes for reproduction, disease control, and in many cases general ecosystem health (e.g., fires or windstorms allowing new growth to replace old (IPCC, 2012)). How such extreme events interact with other trends and circumstances, and what the effects are of more frequent and intense extremes as a result of unnatural climate change, is important to understand (Handmer *et al.*, 2012).

There is abundant evidence that natural populations are responding to climate change: a recent review estimated that 47% of terrestrial threatened mammals (out of 873 species) and 23.4% of threatened birds (out of 1,272 species) '*may have already been negatively impacted by climate change in at least part of their distribution*' (Pacifici *et al.*, 2017), and local extinctions and contractions at the warm edges of species' ranges have been observed in both warm- and cold-blooded taxa (Chen *et al.*, 2011; Cahill *et al.*, 2012), including many insect species, such as mosquitos (Hughes 2000) and butterflies (Hughes 2000; Thomas, Franco & Hill, 2006).

Despite ample evidence for ecological responses to climate change in a variety of systems, the proximate drivers of such extinctions and contractions are poorly understood compared with the widespread evidence for such changes impacting upon biodiversity. In a recent authoritative review, where only 7 of 136 case studies

provided detail on the drivers of significant climate change effects, Cahill *et al.* (2012) stated: '*Our review demonstrates that disturbingly little is known about the proximate causes of extinctions due to recent climate change.*' In the light of these knowledge gaps, Cahill *et al.* (2012) concluded: '*Overall, we argue that understanding the proximate causes of extinction from climate change should be an urgent priority for future research*.' In this thesis, I aim to advance our understanding of the proximate impacts of climate change on reproductive fitness.

Impacts of climate change on biodiversity are determined not only by the magnitude of warming, but also by an organism's physiological sensitivity to that warming, and by their ability to compensate behaviourally, morphologically and physiologically (Handmer *et al.*, 2012). Extreme (and non-extreme) weather or climate events affect a population's vulnerability to future extreme events by modifying resilience, coping capacity, and adaptive capacity (Lavell *et al.*, 2012). Although usage of the term varies (Hodgson, McDonald & Hosken, 2015), in its broadest sense resilience is the ability of a system and its component parts '*to anticipate, absorb, accommodate, or recover from the effects of a hazardous event in a timely and efficient manner, including through ensuring the preservation, restoration, or improvement of its essential basic structures and functions'* (Lavell *et al.*, 2012).

When faced with new or intensified selection pressure from challenges such as changing climate, populations can respond in three fundamental ways, as described by Gienapp *et al.* (2008):

1. **Evade: disperse to suitable habitats elsewhere.**

There is overwhelming evidence that range shift (i.e. extinction at one population boundary and colonisation at another) is correlated with site-specific change in temperature (Gienapp *et al.*, 2008). Two meta-analyses of a wide variety of plant and animal taxa at the start of the last decade underline this: Parmesan & Yohe (2003) demonstrated in global analyses of over 1,500 species '*highly significant, nonrandom patterns of change in accord with observed climate warming in the twentieth century*' and Root *et al.* (2003) reported nearly 1,500 species, or groups of species, showing change, with 80% matching shifts predicted by climate change models. Evasion suggests a physiological constraint on plastic or genetic responses caused by change in local temperature. However, evasion by range shift is not an option open to all organisms, and a number of factors, such as flight capability, visual

acuity and short lifespans, constrain the ability of insects to migrate optimally (Woiwood, Reynolds & Thomas, 2001). The present work does not focus on range-shifts in response changes in climate, but aims to explore what are the consequences for insects when they cannot evade pernicious changes, or before range-shift takes place.

2. Adjust: use phenotypic plasticity, physiological or behavioural changes within a single generation.

The range of temperatures an organism can adjust to using a plastic response is known as the thermal reaction norm (Angilletta, 2009). One of the most well studied physiological responses is the deployment of heat shock proteins (Hsps) that activate in high temperature to protect metabolic processes in tissues and organs (Angilletta, 2009). However, there can be trade-offs to such physiological measures, for example, upregulating Hsp70 reduces the proportion of eggs that successfully hatch (Krebs & Loeschcke, 1996).

The present work, in part, sets out to observe adjustments in behaviour that occur in response to heat stress by both stressed individuals and their mating partners, and it examines whether observed changes can improve a population's coping capacity in response to heat-induced sterility (see Chapter 5).

3. Adapt: alter physiology or behaviour across generations.

Changes in the population's gene pool can take place through selection, favouring progeny that inherit alleles for traits that are better able to cope with extreme environments created by climate change. Analyses have revealed genetic variation for thermo-resistance in laboratory conditions, but evidence for direct selection for thermo-resistance in nature is lacking (Gienapp *et al.*, 2008). The present work will consider whether the size of the gene pool (which can be affected by all three of the coping techniques detailed above) influences resilience (see Chapter 3), but also look at the interaction between plasticity and selection in another way by examining whether there is capacity for populations to evolve a more efficient plastic response to the effects of extreme heat stress (i.e. alter their thermal reaction norm) after exposure to an increased average temperature across generations (Chapter 7).

There is also an additional mechanism that organisms can implement to cope with environmental changes which involves a combination of adjust and adapt: the evolution of added plasticity. This process requires changes that allow widening of the limits of physiological or behavioural reactions across multiple generations, e.g. the evolution of wider thermal reaction norms (Angilletta, 2009). I also explore evidence for this in *Tribolium castaneum* in response to selection from thermal stress across several generations (see Chapter 7).

1.4 Climate change and thermal adaptation

Only relatively recently has progress been made in identifying the genetic and physiological basis of evolutionary shifts in thermoresistance (Hoffman, Sørensen, & Loeschcke, 2003). Plastic, non-genetic responses to thermal stress are usually divided into hardening to short-term exposures, or acclimation to longer-term exposures, both changing reaction norms (Hoffman, Sørensen, & Loeschcke, 2003). However, hardening responses can have costs associated with heat shock protein (Hsp) expression, including age-specific mortality and reproductive constraints, although the latter may be limited to the female: e.g. Hsp70 reduces the proportion of eggs that successfully hatch (Silbermann & Tatar, 2000). Specific costs of acclimation are harder to isolate, as long-term exposures to sub-optimal conditions have deleterious effects on fitness, unrelated to the acclimation response (Hoffman, Sørensen, & Loeschcke, 2003).

Genetic analyses have revealed genetic variation for thermoresistance in laboratory conditions, but although inter- and intra-specific differences in thermoresistance are well known, the genetic variation associated with this capacity has rarely been identified in natural populations (Hoffman, Sørensen & Loeschcke, 2003). A few cases link responses to specific candidate genes (Hoffman, Sørensen & Loeschcke, 2003), and physiological mechanisms and variation in multiple gene expression have been correlated with thermal reaction norms (Ellers *et al.*, 2008). Evidence for direct selection for thermoresistance at a molecular level in nature, however, is lacking (Hoffman, Sørensen & Loeschcke, 2003).

Range shifts are characterised by extinctions at one population boundary and colonisation at another (Parmesan *et al.*, 1999). These two changes suggest a physiological constraint on plastic or genetic responses caused by change in local

temperature (Hoffmann, 2010). In a 2008 review of empirical microevolutionary studies, Gienapp *et al.* (2008) concluded that most studies have been based on phenotypic rather than genetic changes, leaving plastic responses and microevolutionary adaptation poorly delineated as causal factors. Gienapp *et al.* concluded: '*All in all, our understanding of microevolutionary adaptation to climate change is still very much at the same point as it was over 15 years ago when Holt (1990) noted that: "There is almost no species for which we know enough relevant ecology, physiology and genetics to predict its evolutionary response to climate change".'* (Gienapp *et al.*, 2008).

There has been wide debate on whether genetic or plastic changes contribute most to the observed shifts in the distribution of phenotypes associated with climate change (Visser, 2008). The strategy a population will adopt (evade, adjust or adapt), is likely to depend on a variety of factors, such as the timescale considered, the species' life history, the rate and extent of environmental change, the availability of alternative habitats and species' dispersal abilities, as well as underlying genetic variation and its evolutionary past (Gienapp *et al.*, 2008). In this thesis, I use experimental control and evolution to understand the proximate effects of heat-wave conditions on reproductive fitness, advancing our knowledge of how reproduction is impacted, and exploring routes of adaptation by which individuals and populations can avoid impacts of damaging environmental thermal stress.

1.5 Male Tribolium castaneum *fertility and temperature*

Heat sensitive fertility is established from pilot work in *Tribolium castaneum*. Godwin (2010) first established the impacts of heat-wave conditions on male and female fertility in the *Georgia-1* (GA-1) standard laboratory 'wild-type' strain. Offspring production under standardised mating and oviposition conditions was used as a measure of reproductive fitness throughout. Using heat-wave conditions of 5 days of continuous thermal change (Frich *et al.*, 2002) at constant humidity, females or males were thermally treated prior to mating with a control untreated adult of the opposite sex. Females receiving pre-mating thermal treatment showed no significant loss of reproductive fitness at any of the temperatures. However, males exhibited the characteristic fertility sensitivity to increasing temperature. Figure 1.4 (below) identifies the differences between male and female reproductive output (offspring

production) following heat-wave conditions experienced as 10-day old adults. Male reproductive fitness more than halved following a 40°C heat-wave, and complete sterility occurred at 42.5°C. These preliminary data established reproductive thermal sensitivity in this insect model, and provides the basis for which the responses, drivers and adaptations can be understood in greater detail through this thesis.

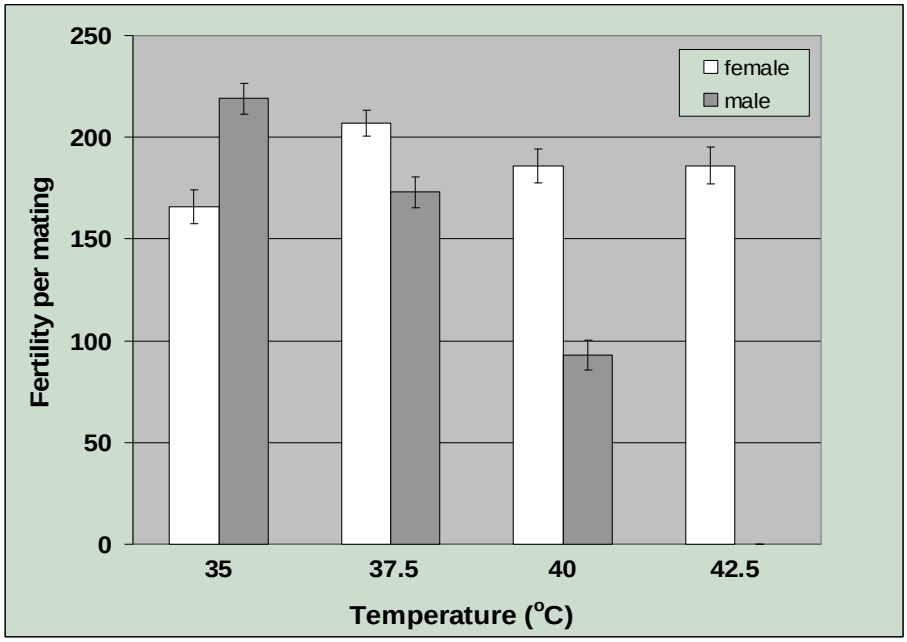

Figure 1.4 The effect of five day 'heat-waves' at four treatment temperatures on the reproductive fitness of *T. castaneum* males and females (fertility per mating = number of offspring produced over 20 days of oviposition following 48h mating access). Means derived from N=20 crosses per sex and treatment (± SE). Godwin 2010.

1.6 Aims and objective of thesis

In this introductory chapter I have reviewed some of the relevant published evidence showing that extreme heat-wave conditions are becoming more likely, that biodiversity is responding to climate change and that male reproduction and fertility are sensitive to thermal stress. This could be one potential driver of population viability in the face of climate change, but that little is known about the proximate drivers of male fertility loss and its impacts on population viability. Using *Tribolium castaneum* as a model, I propose to measure whether intrasexual variation in thermal reaction norms exist, explore the cause of intersexual differences in thermal reaction norms and look at how this variation can allow populations to adapt to heat-waves.

First, I characterise the impacts of heat-wave conditions on reproductive output in both sexes, separately, using an outbred laboratory strain. I then establish whether heat-wave effects impact on female fecundity, male fertility, or offspring development (Chapter 3). Having established male fertility through egg hatch, and pupal eclosion rates, as the likely traits that lead to a reduction in offspring production and male reproductive fitness, I examine whether this was due to a failure of males to mate following a heat-wave, and/or whether impacts on sperm production occurred (Chapter 4). In chapter 5, I explore whether females have evolved pre- or post-copulatory behavioural mechanisms that allow them to avoid male-specific infertility through modulating their own mating pattern. I then explore whether adaptation to heat-waves can occur across a generation, and whether heat stress has any transgenerational effects (Chapter 6), and finally whether acclimation and/or adaptation within laboratory selection lines raised at elevated temperatures can allow increasing ability of males to tolerate heat-waves caused by climate change (Chapter 7). Finally, in chapter 8 I summarise my findings, and use these to suggest further work.

Prior to presenting the results of these experiments, in the next chapter I review the use of *Tribolium castaneum* as a model, and discuss why it is an appropriate tool for the experiments in this thesis. I then detail the methods used throughout this thesis relating to the culture and maintenance of *T. castaneum* populations, different measures of reproductive biology, behaviour and fitness, and the simulation of heat-wave conditions.

2 The *Tribolium castaneum* model and general methods

CHAPTER SUMMARY

In this chapter I introduce and describe *Tribolium castaneum* as a suitable model for addressing questions about thermal biology, behaviour, evolution and reproduction, and outline methods for its culture. I show that *T. castaneum* is an informative model because it is both ecologically and phylogenetically representative, and highly tractable for controlled experimentation in the laboratory using generous sample sizes. I describe the creation of a new outbred strain which was used in an attempt to allow greater relevance to genetically variable natural populations. Finally, I describe how heat-waves are simulated, and the various reproductive fitness assays used throughout this thesis.

Contributors: Alyson Lumley and Joanne Godwin assisted with general culture and maintenance of *T. castaneum*.

2.1 Why use Tribolium castaneum*?*

The red flour beetle *Tribolium castaneum* Herbst (Coleoptera; Tenebrionidae) is a suitable model for studying the impacts of heat-wave conditions on reproduction in an ectotherm. In the following chapter, I summarise its advantageous traits, and detail the general methods used to culture *T. castaneum*. I also describe the general approaches to simulating heat-waves, and bio-assays to measure reproductive fitness, in order to address the questions summarised in chapter 1.

2.1.1 Ecological and physiological suitability

Tribolium castaneum has a worldwide sub-tropical distribution (Sokoloff, 1972-78). This geographic zone is one of the most sensitive to climate change, because it has a narrow climate envelope, where species live closer to their optimal temperature, and

therefore global warming is likely to have the most deleterious consequences in the tropics and sub-tropics (Deutsch *et al.*, 2008). It is one of the earliest global climate zones to have been impacted by climate change, some parts from as early as the 1940's (King *et al.*, 2015). This makes *T. castaneum* a fair representative of how sub-tropical insect species could be impacted by changes in temperature, and of the mechanisms these species might possess to be able to adapt. The majority of biodiversity hotspots are found in tropical and sub-tropical regions, and biodiversity hotspots account for 42% of all terrestrial invertebrate fauna (UNEP WCMC, 2014).

Exact knowledge of the ancestral ecology and geography of *T. castaneum* is lacking, because *Tribolium sp.* have been human commensals, probably since early agriculture (Sokoloff 1972-78), with examples of the genus identified in flour urns of a pharaonic tomb ca. 4,500 years ago (Dawson 1977). *Tribolium* species have evolved to feed on stored grain, to the extent that they are now only found in stored food products (Sokoloff, 1972-78). It is therefore reasonable to assume that stored food, especially flour, is their 'natural' habitat, with an ecology involving major population size variations as infestations change, and assisted dispersal by human food transport. Their populations probably spread by lone female colonisers carrying sperm in storage (Sokoloff, 1972-78). Although adult flight can contribute significantly to active dispersal and genetic mixing over shorter spatial scales (Ridley *et al.*, 2011), their relatively specialised food habitat means they have limited ability to evade climatic changes or extreme events as anthropogenic movement defines their global and local distribution.

Tribolium species are of commercial interest due to their persistence as pest species of stored grain products. Because of their economic importance, there is a large body of literature on how to limit *Tribolium* populations, using an array of environmental factors, including increased temperatures (e.g. Mahroof *et al.*, 2003).

T. castaneum has been an important biological research model for almost 100 years (Brown *et al.*, 2009). It has a generalised insect physiology, making it widely comparable to other insect Orders. As a member of the Order Coleoptera, it belongs to a group which represent almost 25% of all described species on Earth (Hunt *et al.*, 2007): the *'most evolutionarily successful metazoans'* (Richards *et al.*, 2008). The *Tribolium* genome has been sequenced (Richards *et al.*, 2008), and this flour beetle is a popular alternative to *Drosophila sp.* for understanding evolution and genetic

control of development (e.g. Brown, Denell & Beeman, 2003; Denell, 2008). The *Tribolium* genus shows a shorter branch length in phylogenetic trees, compared with other insect genomes, such as the widely used *Drosophila* model. It therefore retains a more primitive set of ancestral genes, especially those relating to embryonic development, which is more representative of other insects (Richards *et al.*, 2008; Schinko, Hillebrand & Bucher, 2012).

2.1.2 Life history and reproduction

As well as the benefits of using *Tribolium castaneum* due to its ecological and phylogenetic representativeness, it is also an ideal model organism for laboratory culture and experimentation. The beetle can be reared easily, in quite dense populations, on a simple medium (organic flour supplemented with brewer's yeast and some oats), and in a wide range of conditions (Sokoloff 1972-78; Richards *et al.*, 2008). It is therefore simple to replicate its 'natural' ecological environment, and it reproduces readily. It is fecund, with *T. castaneum* females producing ten to twenty eggs a day for up to 150 days following a single mating (Sokoloff, 1972-78; Michalczyk *et al.*, 2010). This species therefore has a high intrinsic population growth rate potential, as well as other traits typical of coloniser species which make it useful as a lab model, including: fast maturation, rapid larval development, a short generation time lasting approximately 1 month from egg to sexually mature adult, and a lifespan of about one year (Fedina & Lewis, 2008). Various stages of the short, one month generation cycle can be isolated easily from the flour with sieves of varying mesh size. Eggs take 3-4 days to hatch, and the rest of the life cycle is divided into a larval stage lasting an average of 14 days, a pupal stage lasting 5-7 days, an immature adult which takes 2-3 days for sclerotisation, and the mature adult stage which can live for over a year (see Figure 2.1, below).

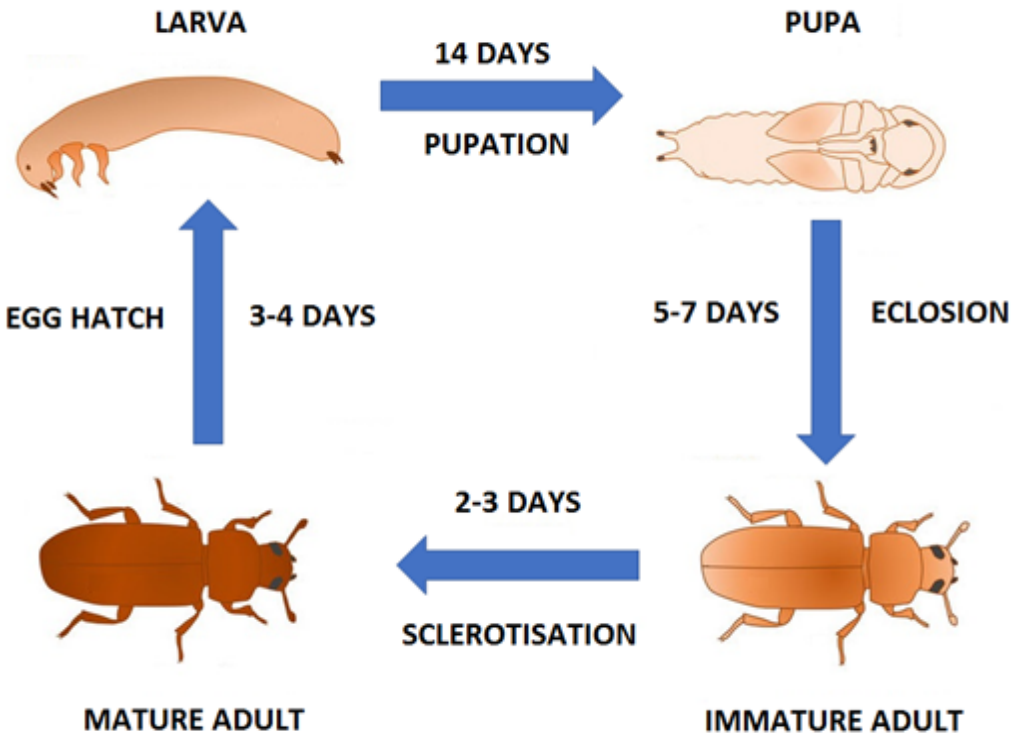

Figure 2.1 Life Cycle of *Tribolium castaneum*. Adapted from Walski *et al.*, 2016.

The mechanisms and dynamics of reproduction have been studied in detail, and are well established in *T. castaneum,* with a successful track record as a model in controlled and replicated experimental evolution studies for understanding phenotypic adaptation (Fedina & Lewis, 2008; Michalczyk *et al.*, 2010; Michalczyk *et al.*, 2011b). It is a model for studying reproduction and sexual selection (Fedina & Lewis, 2008), and meaningful reproductive fitness assays can be consistently conducted with high replication (Arnaud *et al.*, 2001; Michalczyk *et al.*, 2010).

T. castaneum individuals can be easily sexed at the pupal stage (see below, Section 2.2.1), after which adults become more difficult to assign gender. After eclosion, males take up to ten days to mature sexually while they produce mature spermatozoa (Fedina & Lewis, 2008; Sokoloff, 1972-78), so adults that are at least ten days post-eclosion are used throughout my experiments. Both sexes are highly polygamous (Fedina & Lewis, 2008; Lumley *et al.*, 2015) and stable phenotypic markers exist to assign and track parentage (Sokoloff 1972-78, Michalczyk *et al.*, 2010). Female *T. castaneum* store only ~4% of that transferred in each spermatophore in the spermathecae for longer-term oviposition, from which fertile eggs can be produced for up to 150 days (Bloch Qazi, Herbeck, & Lewis, 1996; Bloch Qazi, Aprille,

& Lewis, 1998; Michalczyk *et al.*, 2010). Sperm can be recovered from the male testes and vasa deferentia or female bursa for analysis (Michalczyk, 2008), see Chapter 4.

2.1.3 Krakow Super Strain and a more genetically diverse experimental population

Pilot work with the GA-1 *T. castaneum* strain established that male fertility was sensitive to thermal stress (Godwin 2010, Figure 1.4) with a near complete loss of fertility above 40°C. Godwin's work showed robust preliminary evidence that male fertility is affected by thermal stress in *Tribolium*. However, the GA-1 'wild' type strain has spent seventy years in temperature-controlled laboratories with little selection pressure, and little requirement for the genetic diversity found in a natural population (Michalczyk, 2008). In addition, the abrupt and complete loss of fertility at the higher heat-wave temperature (42.5°C) suggests that minimal standing genetic variation for male fertility at the edge of the thermal range, constraining possibilities for examining genetic adaptation to thermal limits.

Genetic variation and heterozygosity exist within normal healthy wild populations, and these are associated with reduced extinction risk (Frankham, 2005) and the ability to cope with environmental stress and change (Parsons, 1973; Turelli & Ginzburg, 1983; Mopper *et al.*, 1991). Increasing the genetic diversity in my model system will make it more relevant to natural systems facing extreme thermal events, and allow a more relevant understanding of the capacity of natural populations to adapt to heat-wave conditions. I therefore took advantage of a new outbred *T. castaneum* strain created in early 2008 by Dr. Paulina Kramarz in Jagiellonian University, Krakow, which combines the gene pool of *T. castaneum* from across its global range. This 'Krakow Super Strain' (KSS) was produced by placing 35-60 individuals from eleven strains together in a single container, and allowing free mating access and reproduction for two generations. Following this genetic mixing between the 11 different strains, which were derived from diverse geographic backgrounds, they were checked for linkage disequilibrium using microsatellite markers, and demonstrated to be genetically outbred (Kramarz, 2016, pers. comm.). The following strains were mixed and interbred to create KSS:

- GA-1
- GA-2
- *Tiw*-1

- Col-2
- Japan #2
- *cSM*
- San Bernardino (CA)
- Jerez (Spain)
- Banos (Ecuador)
- Schegel Farm (Indiana)
- Bloomington (Indiana)

This is the principal strain used throughout my PhD research, unless otherwise indicated. Figure 2.2 shows that KSS has a similar production curve to GA-1 (see Figure 2 in Michalczyk *et al.*, 2010) albeit with the peak output occurring slightly earlier in KSS, during the first ten days, rather than between 10 and 20 days for GA-1. This tendency for earlier production is also seen in the fact that KSS stops producing offspring after 80 days following a mating, whereas Michalczyk *et al.* (2011a) found that GA-1 females continue until day 90, and another study for 140 days (Michalczyk, *et al.*, 2010). In the former study, GA-1 produced a mean of ~350 adult offspring over 90 days whilst KSS has a greater overall productivity, producing ~450 adult offspring on average in 80 days. Overall, these findings suggest that KSS matures and reproduces slightly faster and more productively than GA-1; this is how a strain adapted to the more variably conditions and greater selection pressures of the wild would be expected to behave in comparison to one adapted to the more constant laboratory conditions (Pianka, 1970).

In order to determine what measurement period of reproductive output can effectively represent total reproductive output following a mating interaction, I measured total reproductive output of 45 pairs of KSS following 48h of mating interaction in 10-day blocks, up to a maximum of 150 days (by which time all females had ceased offspring production). There were significant correlations between lifetime output and both the first 10 and 20 days of offspring production ($R_{sp} = 0.46$ ($p < 0.001$), and 0.55 ($p < 0.001$) respectively; Figure 2.3). Twenty days of reproductive output captures just over half of the total potential output (the shaded area under Figure 2.2 represents 50.71% of the total), so I use twenty days of offspring production as my standard for measuring the reproductive fitness of KSS beetles, unless otherwise indicated.

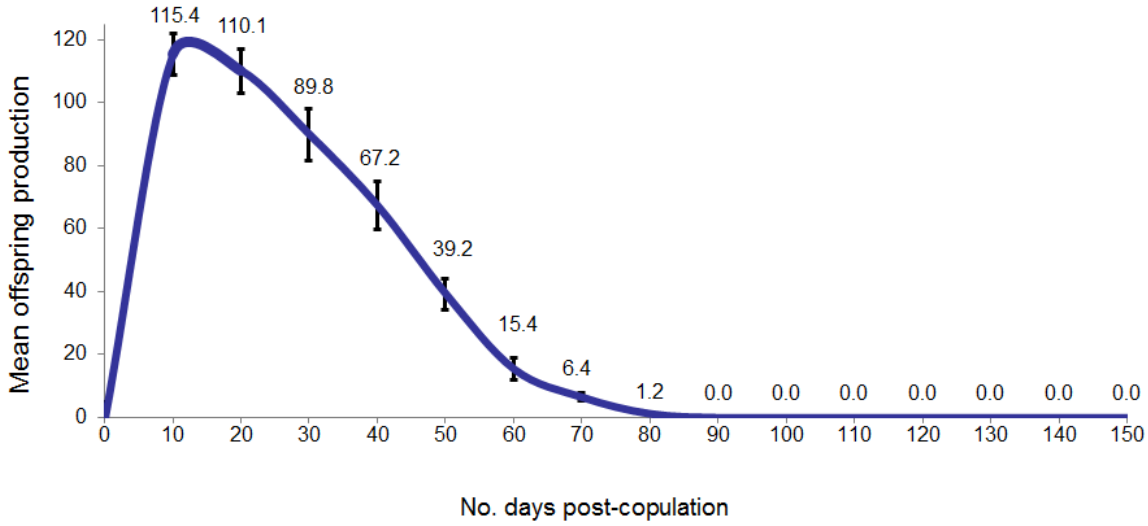

Figure 2.2 Total offspring production of Krakow Super Strain monogamous pairs at control conditions (30°C, 65% humidity) after mating males with females from the same population for 48 hours. Shaded area under graph represents ~50% of an average pair's total production. Sample size: N=45 KSS male-female pairs.

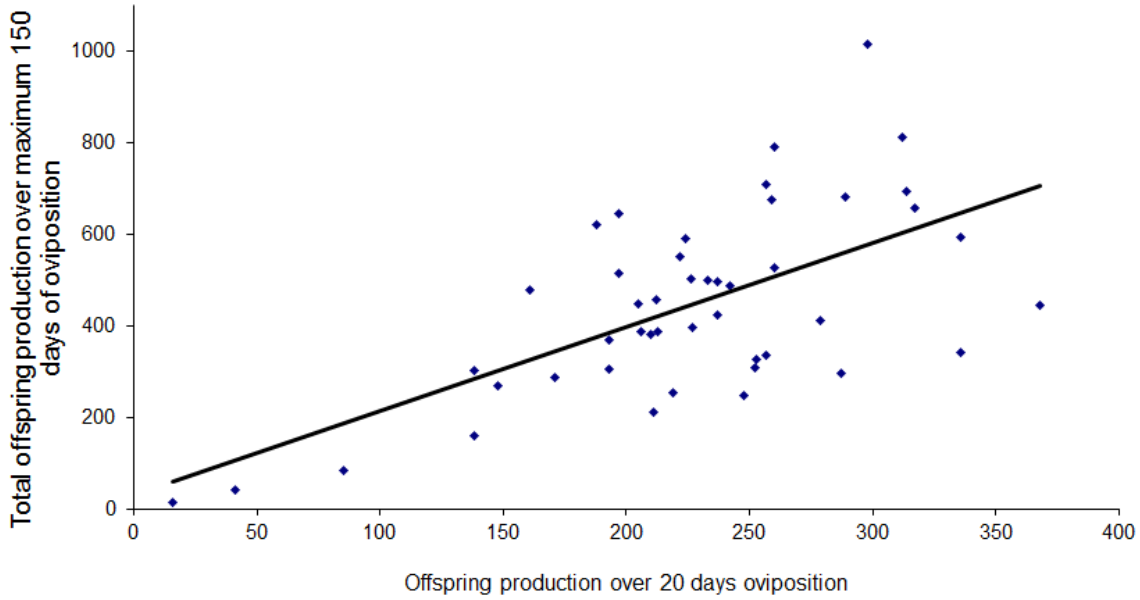

Figure 2.3 Significant correlation across 45 mating pairs between offspring productivity over the first 20 days of oviposition versus total period of offspring production (R_{sp} = 0.55, p < 0.001).

2.2 Methodology

2.2.1 General culture maintenance

Krakow Super Strain populations were maintained in stock populations created by ca. 300 adult individuals, from which experimental populations and individuals were derived as needed. Beetles were kept in 12×12×12 cm plastic boxes (ca. 250ml of fodder) covered with lids containing 7×7 cm windows of fine metal mesh for aeration. All populations were reared in fodder containing nine weight units of white plain organic flour to one weight unit of powdered organic brewer's yeast, provided *ad libitum*. To allow better aeration in the substrate (important for eggs and small larvae, and to allow traction for mating adults) 60ml of large organic rolled oats were mixed in with the fodder. Additionally, a similar amount of oats was spread over the surface of the fodder, sufficient to cover it entirely, to aid adults in locomotion and self-righting at the surface of the substrate (following Sokoloff, 1972).

Under control conditions (used for stock and experimental populations unless otherwise indicated) beetles were maintained in the same constant environment chamber at 30°C, 60% relative humidity and a 16:8 hour light:darkness regime in the Controlled Environment Facility at the University of East Anglia main campus. It is important to note that in a natural environment, temperature drives and interacts with many other environmental factors, humidity, air circulation and wind speed, etc and many of these can influence thermoresistance. For example, prior exposure to different photoperiods can alter thermoresistance in *Drosophila* (Hoffman, Sørensen, & Loeschcke, 2003). Only the effects of increasing temperature were examined in the current work, and all other factors were kept constant within the Controlled Environment Facility.

At least two stock populations of the KSS were maintained throughout. Each main stock population was kept at a size of approximately 300 adults. Every 30 days, pupae were removed from the substrate (separated from substrate by sieving adult *T. castaneum*, 300 of which fill exactly one 30ml microcentrifuge tube) and placed in 250ml of fresh substrate fodder. Sex-ratios were checked every 10 generations by sexing 350 pupae (see below) to ensure 175 of each sex is present for the next generation (the higher number guarantees 300 will eclose to adult imago), and to maintain even sex ratios and a consistent effective population size. After a week, having been left to mate and for females to lay eggs, the adults are removed, allowing

the eggs and early instar larvae within the fodder to develop as a single cohort for the next generation. The removed adults may be discarded, or put into fresh flour (if more individuals are required for experimental populations). This movement of adults into fresh flour-yeast substrate is called a 'rotation'.

If beetles are needed for experiments, the requisite number are removed as pupae, and sexed under the microscope to create unmated adults of known sex and number for the forthcoming experiment (see Figure 2.4). Sometimes attached substrate must be carefully removed from the genital lobes, if the substrate has absorbed moisture it can tear the exoskeleton and the pupa must be discarded. Same-sex small groups are then kept isolated in 5cm diameter, 1 cm deep petri-dishes in groups of twenty, with approx. 15g of flour-substrate mix to help eclosion and provide feeding. After eclosion and cuticular hardening, one sex (usually the female) is marked using a small correcting fluid pen to place a small, white mark on the centre of the dorsal side of the thorax, to allow easy identification of the adult sexes.

Assays where reproductive output is measured usually involved placing individual females, following experimental treatment and mating, into individual small petri-dishes with fodder to oviposit. Unless otherwise indicated, each female is rotated to fresh substrate in a new petri-dish every 10 days, so that density is maintained and differences in larval age structure do not confound experiments. The vacated petri-dish is left in standard rearing conditions for up to 30 days to count offspring production, or a shorter time if counting eggs or pupae, before being frozen at -6°C. Freezing ensures offspring are from a single generation, are motionless for ease of counting, and maintains effective bio-containment and reduces diseases or mite parasites that can take hold in over-conditioned flour.

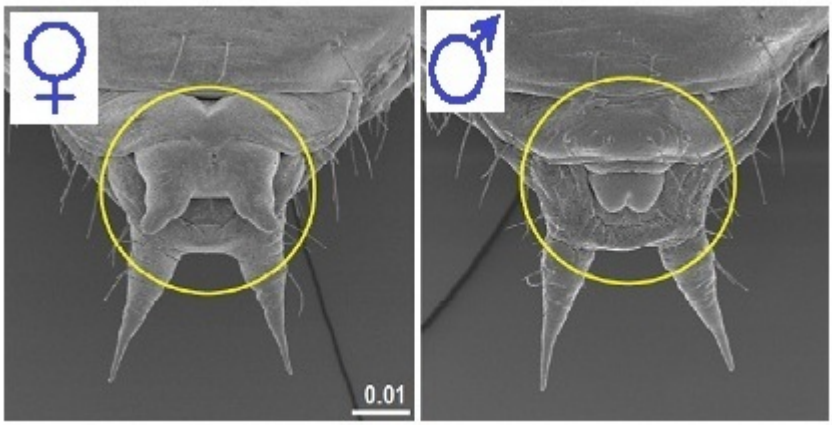

Figure 2.4 Identification of males and females at the pupal stage in *T. castaneum* prior to eclosion; the female (on the left) is distinguished by larger protruding genital lobes. Scanning Electron Microscope images of pupal genital lobes courtesy of Łukasz Michalczyk.

2.2.2 Applying heat-wave conditions

Heat-waves are defined in various ways, using varying values for the minimum required lengths, baseline temperatures and increases in temperature needed to label an extreme weather event. For example, in 1900 Burrows defined a '*hot wave*' for the first time as lasting three or more days, whilst Ward (1925) sets only one day as the minimum length '*to be a heat-wave*'. The widely used definition in Frich *et al.* (2002) defines a heat-wave when the duration is > 5 consecutive days, with T_{max} >5°C above the 1961–1990 daily T_{max} normal. Governmental and meteorological offices use different criteria in different countries regarding the minimum duration and increase in temperature needed to officially be defined as a 'heat-wave'. For example, two days of temperatures more than 32.2°C apply in the United States (National Weather Service, 2011), or three days or more over 28°C apply in Denmark (Cappelen, 2001). In Australia, a heat-wave is five days at 35°C or more, or three days at 40°C or more (Bureau of Meteorology, 2016), and the United Kingdom Met Office uses different thresholds for different regions of the country, all for two days (Met Office, 2015).

'Heat-wave', then, varies globally based on regional temperature and other factors with no standard definition (Baddour, 2010; Dje *et al.*, 2016). This is, of course, sensible; the temperature range that constitutes 'extreme' will change with latitude and other local factors. For the purposes of this project, with this model addressing questions concerning reproduction, I define a heat-wave as constituting 5 days of exposure to thermal regimes that are 5°C above the optimum for population productivity in *T. castaneum*. (This is a modification of the definition from Frich *et al.* (2002), substituting the temperature of optimum *T. castaneum* production in place of the 1961–1990 daily T_{max} normal, which has no relevance in lab conditions.) Although we maintain beetles at our control temperature of 30°C at UEA, other laboratories use 35°C, following Sokoloff (1972-78) who found optimal population productivity at 35°C. Thus, heat-wave conditions in the *Tribolium* model system are defined as those at 40°C and above. In chapter 3 I compare male and female offspring production at both 30°C and 35°C as well as exploring increasing heat-wave temperatures (between 40°C and 42°C) which I then use for the remainder of this thesis. Pilot work established the thermal limit on adult survival over 5 days of exposure is 43 to 44°C.

Adult mortality following 42°C for 5 days is 30 to 40% (K. Sales, unpubl. data; at 30°C there is ~10% mortality), so 42°C represents a more extreme heat-wave condition without high mortality. In terms of environmental relevance, frequencies of absolute heat-wave temperatures are hard to find as most studies present data relative to mean temperatures (as in the Frich *et al.* definition cited above). However, temperatures of 40-42°C are known to have occurred in more than 90 countries (Herrera, 2016), including tropical and sub-tropical locations such as Australia (Bureau of Meteorology, 2016), Taiwan (Kueh *et al.*, 2017) and the United States (National Weather Service, 2011).

For application of heat-wave conditions, petri-dishes were placed into Brinsea Octagon 20DX incubators, positioned in stacks of three dishes, with experimental beetles only held in the middle dish to avoid microclimatic hotspots in the incubator (temperatures peak nearer the heating element at the top of the incubator).

In assays involving offspring production, matings took place over 48 hours (following Arnaud, Gage & Haubruge, 2001). In behavioural observations, pairs were observed over 30-60 minutes together (initially following Nilsson, Fricke & Arnqvist, 2003, but see 5.2). To allow any beetles to recover from the torpor that can occur within a heat-wave (Heath *et al.*, 1971), and to allow any mortality to reveal itself following heat-wave conditions, all treatment beetles were given a 24 hour 'cool down' recovery period before experimental assays were conducted, following Kristensen, Loeschcke and Hoffmann (2007).

2.2.3 General statistical methods

All data were analysed using IBM SPSS Statistics 22. Normal distributions of the residuals were checked before parametric testing based on KOLMOGOROV-SMIRNOV tests (values denoted by D), and parametric tests were performed without transformation of data. Where normality assumptions were not met, data were transformed as appropriate, using the three most common transformations used in biology: Ln(x), square root ($\sqrt{x}$), and arc cosine (arcos(x)) (Fowler, Cohen & Jarvis, 1998). In some rare cases, variations on these transformations were applied: Ln(x + 1), square (x^2). If none of these five transformations resulted in normality, non-parametric tests were employed.

Parametric tests used include WELCH'S *t*-TEST and analysis of variance (ANOVA); non-parametric tests include MANN-WHITNEY U and KRUSKAL-WALLIS. *Post-hoc* testing was performed when comparing more than two variables, using TUKEY or DUNN'S tests for parametric or non-parametric data, respectively, as appropriate. All analyses had unequal sample sizes due to the greater mortality rate of heat-treated beetles, therefore unequal variances were assumed throughout, when comparing two independent samples the WELCH'S *t*-TEST was therefore used as it is more reliable in these circumstances (Ruxton, 2006).

There is some debate in the literature as to whether to use statistical corrections, such as the widely used Bonferroni correction, when carrying out multiple testing on the same dataset (Perneger, 1998; Nakagawa, 2004). Bonferroni corrections lower the power to detect Type II errors to unacceptable levels (Nakagawa, 2004) and there is no formal consensus on when Bonferroni procedures should be employed (Perneger, 1998). To avoid the pitfalls of Bonferroni corrections and circumvent this statistical debate, p-values will be given in relation to a standard α-level of 0.05, and attention drawn to results involving multiple testing and biological and statistical significance discussed. Where Bonferroni corrections would change the significance of any results due to a lower α-level this will be noted and the implications discussed. As stated in Perneger (1998) '*simply describing what was done and why, and discussing the possible interpretations of each result, should enable the reader to reach a reasonable conclusion without the help of Bonferroni adjustments*'.

All statistical output values are presented to 4 significant figures, unless otherwise stated. All 'bar' graphs display error bars, standard errors about the mean. Above the data points sample sizes and homogenous subsets, indicated by letters, are displayed.

3 The impacts of experimental heat-waves on male and female reproductive fitness in *Tribolium castaneum*

CHAPTER SUMMARY

In this chapter I examined how 5-day heat-waves of increasing thermal severity impacted on reproductive fitness of *T. castaneum.* Males and females of the Krakow Super Strain were assayed for impacts of heat-waves by pairing them with a control, unmanipulated adult of the opposite sex, with subsequent offspring counts used to score reproductive fitness. Results showed male-specific sensitivity to heat-wave conditions, with males producing 50 to 60% fewer offspring following a heat-wave at 42°C compared to control conditions at 30° and 35°C respectively. Subsequent assays comparing pre-mating exposures to 30°C versus 40°C 5-day heat-waves examined whether the decline in reproductive fitness could be explained by a reduction in female fecundity (egg number), egg hatch, or offspring survival through the pupal eclosion. Female fecundity was almost identical following matings with 30°C and 40°C treatment males. However, there was a significant 11.3% reduction in egg hatch rate for 40˚C treated males compared with 30°C controls, and a 4.4% reduction in pupal eclosion rate. These ~15% combined reductions can explain the ~25% reduction in reproduction fitness of males following a slightly more severe heat-wave at 41°C, suggesting that heat-wave conditions reduce reproductive fitness by acting mainly on male fertility, and partly on offspring survival through pupal eclosion.

Contributors: Alyson Lumley, James Hayhurst and Laura Browne assisted with implementation and data collection for the assay of male and female reproductive output. Kris Sales assisted with implementation and data collection of life history assays.

3.1 Background

As outlined in the general introduction in chapter 1, there is ample evidence that thermal environment can have negative impacts on male reproductive competence. This phenomenon is well established in endotherms such as mammals, but has received scant attention in ectotherms. This situation is surprising, because (1) male fertility can be essential for population viability, (2) ectotherm physiology is more directly exposed to extremities in the thermal environment, and (3) most biodiversity is composed of ectothermic species. Here, I add to the sparse information on how ectotherm models respond reproductively to heat-wave conditions using an experimental approach with the flour beetle *T. castaneum*. Using a balanced experimental design, I measure impacts of 5-day heat-waves on male and, separately, female reproductive fitness, and compare the relative impacts on either sex.

In addition to the dearth of research into how heat-wave conditions influence reproductive fitness in ectotherms, is a lack of further detail on the actual mechanisms and routes by which heat impacts on reproduction. If, as appears to be the case, males are especially sensitive to elevated thermal conditions, what is the specific target trait that is damaged to result in reduced reproductive output? I explore the details of this phenomenon across two chapters: (1) here in chapter 3 I examine how heat-waves impact on post-mating processes through fecundity, fertility, and offspring development and fitness; (2) in the next chapter, I examine effects of heat-waves upon male mating behaviour, insemination success, and sperm counts.

I first replicate the experiment by Godwin (2010; see Chapter 1.5) using the Krakow Super Strain to clarify the impacts of heat-wave conditions on both male and female adult offspring production, isolated by exposing the sexes separately and mating to control members of the opposite sex. This will also show if and how the new strain reacts differently from the GA-1 used by Godwin (see Chapter 2.1.3). In a further three assays I then look at a number of key points in the production of offspring when males only are stressed, these are intended to look for factors in offspring life history development that may influence the number of offspring produced by stressed males that reach adulthood.

In the first of these life history assays I examine whether both males or females which have experienced heat-wave conditions enter reproductive cycles that have reduced female fecundity, measured as the number of eggs produced through a standardised reproductive output assay (see Chapter 2.2.2). Heat-waves could clearly

act directly on female fecundity but, in many systems, males can also exert an important influence on female fecundity. Males may have to stimulate females to produce eggs and/or offspring via mechanisms such as sexually-selected mating stimulation (Edvardsson & Arnqvist, 2000), or the transfer of seminal fluid proteins (Wolfner, 2000; Chapman *et al.*, 2003; Wigby *et al.*, 2009). By comparing fecundity of females that have either experienced heat-wave conditions themselves, or have mated with males that had been exposed to heat-waves, one mechanism that is known to dictate reproductive fitness can be examined through fecundity. There is a large body of literature regarding the effect of heat stress on egg production in livestock such as hens, which become less keen to lay (see e.g. Mashaly *et al.*, 2004, review in Ayo, Obidi & Rekwot, 2011) and Japanese quail, which have reduced egg production under thermal increases (Yerturk, Avei & Kaplan, 2006). However, these studies tend to focus only on heat stress impacts on females (with or without males also experiencing stress), and tend to expose subjects to raised ambient temperatures over a longer term. This means that other factors caused by heat stress which also affect egg production by mothers, such as reduced feeding, and loss in body mass, are not controlled for (Mashaly *et al.*, 2004). Likewise, a review of the effects of heat stress on life history (Angilletta, 2009) examines impacts on clutch size and egg size, but only in instances when females alone, or both sexes, have been stressed, with little attention to experiments which isolate female and male effects. Work that has assayed egg production when fathers alone have been stressed found that, when mated with heat stressed males, female predatory mites (*Neoseiulus barkeri*) took longer to oviposit, had a reduced oviposition period, and reduced fecundity (Zhang *et al.*, 2016). In the diamondback moth (*Plutella xylostella*), females laid fewer eggs when males were exposed to high temperature (Zhang *et al.*, 2013). In *Bicyclus anynana* butterflies, females showed an increased, rather than decreased early fecundity when mated with heat-stressed males (Janowitz & Fischer, 2011); this may indicate females compensate for male infertility by increasing investment in current reproduction, something I investigate in chapter 5. Evidence is therefore mixed, with insufficient experiments that isolate male and female effects. *T. castaneum* presents an excellent model for such tests as experimental control of specific adult treatments can be applied, and eggs are laid directly into the flour medium to be sieved out and directly assayed.

In addition to fecundity, male and/or female fertility are obvious candidates that could also be sensitive to heat stress. In many species, male reproduction is

known to be thermosensitive, with external testes and gonadal cooling thought to have evolved to protect spermatogenesis and sperm cells from their potential sensitivity to heat damage (Setchell, 1998). By measuring hatch rates of eggs from heat-waved females, and control females mated to males that have experienced heat-wave conditions, impacts of environmental heat on female and male fertility can be assessed. A number of studies have shown that egg development is affected by maternal (Silbermann & Tatar, 2000) or *in vivo* (review in Gharibzadeh *et al.*, 2015) heat stress. Research that has examined sex-specific effects of heat stress on offspring development in predatory mites showed no effect on egg hatch rate when males alone are stressed (Zhang *et al.*, 2016). Again, there is counter-evidence, with a significant reduction in the total number of hatched eggs found when female and/or male diamondback moths were exposed to 40°C for a short period (3-4 hours) (Zhang *et al.*, 2013). In addition, a tendency for declining egg hatch rates with increasing temperature was found in two species of whitefly when both parents were stressed (Cui *et al.*, 2008). One study found a clear effect of paternal temperature on fecundity and egg production, with no significant effect of maternal temperature on either measure, although the differences caused by paternal temperature were small (Huey *et al.*, 1995). Again, isolating male and female effects is required, and *T. castaneum* presents an ideal model for such tests as eggs can be sieved out and counted, and hatch rates then measured through subsequent counts of the presence of early-instar larvae.

Having examined fecundity and fertility, a third possibility by which heat-wave conditions might drive any reduction in reproductive success is via impacts on offspring fitness after fertilization, embryogenesis, and hatch. If heat damages sperm or egg haplotypic DNA (Banks *et al.*, 2005; Pérez-Crespo, Pintado & Gutiérrez-Adán, 2007), or affects direct provisioning to the ovum, or indirect provisioning via male seminal fluids stimulating females to invest in egg provisioning (Chapman & Davies, 2004), heat-wave conditions could impact on reproductive fitness by constraining offspring fitness. Studies at this level have focused mainly on thermotolerance of larvae and pupae (Feder *et al.*, 1996; Feder, Blair & Figueras, 1997), rather than impacts of heat at the adult reproductive stage on the subsequent larvae and pupae. Those studies that have stressed parents have found different results. Maternal heat stress in mice during oocyte maturation caused embryonic mortality, with a large proportion of the ova producing embryos capable of implantation, but not capable of continued development (Baumgartner & Chrisman, 1987), and there is some evidence

that, when heat stressed, aphids produce fewer larval offspring (Montllor, Maxmen & Purcell, 2002). However, the viability of hatched offspring in *D. melanogaster* is not reduced by maternal heat stress (Silberman & Tater, 2000). Again, there is a lack of consistency in results on these questions, calling for further assessment of the impact of heat stress on life history in either sex. Heat stress has been shown to cause *Sarcophaga crassipalpis* flies to fail to eclose when stressed as larvae or pupae (Yocum *et al.*, 1994) and decrease the number of emerging adults when males and females were stressed together in two species of whitefly (Cui *et al.*, 2008). It is interesting to note that, when larval stages are exposed to thermal stress, the older the larvae, the closer they are to adult stage, the greater the curtailing of fertility after maturation (Zhang *et al.*, 2015). However, to my knowledge, no research has isolated the effect on eclosion of offspring when fathers alone have undergone thermal treatment. Depending on whether heat-waves affect fecundity and/or fertility, in males and/or females, an influence on offspring development can be assessed by examining the rates of successful eclosion from pupae into reproductive adults. *T. castaneum* is holometabolous, and undergoes complete metamorphosis through the pupal stage from larva to imago, when major developmental differentiation takes place. DNA damage within gametes might also affect the integrity of complete metamorphosis, so the success of pupal eclosion into adults is also measured to determine whether any reduction in reproductive fitness as a result of heat treatment can impact on the development of offspring.

3.2 Methods

3.2.1 Male reproductive output

Mature (10 days post eclosion) virgin males of the KSS strain were placed into heat-wave conditions at four different temperatures: 38°C, 39°C, 41°C, 42°C; and two control groups were exposed to 30°C (the standard thermal conditions at UEA), and 35°C (the optimum temperature for population productivity in *T. castaneum*, Sokoloff, 1972-78). See Chapter 2.2.2 where I define heat-waves as constituting 5 days of exposure to thermal regimes that are 5°C above the optimum for population productivity in *T. castaneum*. Although under this definition the treatments at 38°C and 39°C do not count as 'heat-waves', I included these temperatures to measure the trend in offspring production across a larger range of temperatures, and because I wanted to determine an appropriate temperature to use for selection lines later in my

PhD research. This latter requirement was for a stress temperature that provided selection when used as the ambient temperature, but one that was not so strong as to cause extinction of populations (see Chapter 7). Temperature exposure was conducted in groups of twenty males kept in single Petri dishes with standard flour mix for 5 days at 60% humidity (+/- 5%).

After a 24-hour recovery period at 30°C following the heat treatment, individual males were given mating access to control virgin females in a perforated microcentrifuge tube, half-filled with standard flour mix, for 48 hours. Females were marked using Pentel Micro Correct Correction Fluid Pen with Needle Point Precision Tip on the upper side of their thoraxes. This leaves a small white mark on the dorsal surface area of the thorax, enabling easy distinction between the sexes. For more detail on methods for distinguishing between the sexes in *T. castaneum* see Section 2.2.1.

After this period, the mated females were then placed in fresh flour in a 5cm petri dish to lay eggs for 10 days, before being rotated to fresh flour, with the previous Petri-dish then left at control conditions for 40 days to allow full offspring development into adults, at which point the dish was frozen to allow scoring of male reproductive success as the number of emerging adult offspring. Mated females were rotated at 10 days between two petri dishes in order to track reproductive output in each treatment over 20 days. The reproductive component of fitness was therefore measured by counting the total number of adult offspring (mature and immature) to emerge in each of the 10-day blocks following the male or female treatment and the mating period.

3.2.2 Female reproductive output

Three generations after the experiment to assess impacts of heat-waves on males, mature virgin females of the KSS received the same heat-wave treatments, twenty per Petri dish, at the same range of temperatures. Following this exposure, females from each treatment were allowed to mate with control (30°C) virgin males in monogamous pairs, as for the male-effect experiment. The mated females were then placed in fresh flour to lay eggs for 20 days across two 10-day blocks as determined in chapter 2.1.3.

3.2.3 Female fecundity and egg hatch rate

Having established that heat-wave effects were male-specific, I focused subsequent measures of impacts on fecundity, fertility and offspring development on adult males, making comparisons between control males exposed to pre-reproductive temperatures of 30°C and heat-wave conditions of 40°C.

Virgin, mature males were exposed to heat-wave conditions in groups of twenty in Petri dishes for five days at 40°C. Following a 24-hour cooling off period, heat-treated and control males were mated to marked control females in microcentrifuge tubes, half filled with flour, for 48 hours. Mated females were then placed to oviposit in standard flour mix, pre-sieved to allow eggs to be more easily separated, in two-thirds full 50ml vials.

Females were allowed to oviposit for ten days with one rotation to fresh flour after five days to further reduce any risk of developing larvae cannibalising unhatched eggs. Fewer days (10) were used than in section 3.1 due to the more time-consuming nature of counting eggs and/or determining if they were hatched. Pilot work has established that ten days of oviposition following a mating period encompasses 26% of a female's total productivity for that mating bout, and productivity over 20-days accounts for 51% of all offspring. Both offspring production scores over 10 days and 20 days are significantly correlated with total offspring production, see Chapter 2.1.3.

After ten days of incubation, by which time all viable eggs would have hatched, the females, eggs and flour mix substrate were then frozen to stop any further development. The fecundity of females mated to heat-treated or control males was measured by sieving the frozen flour mixture and counting the unhatched eggs and larvae under a microscope using a fine paintbrush to separate and manipulate eggs on a black tile which increases the visibility of white eggs. Eggs were determined as unhatched by squeezing and rupturing with fine forceps to determine that they were not hatched egg shells. Thus, the number of unhatched eggs and hatched larvae could be combined to gain measures of both fecundity and fertility (= hatch rate).

3.2.4 Pupal eclosion rate

A second group of males was treated as above, but vials were left for 23 days after removing the female (timed to coincide with early pupation, see Figure 2.1) before making initial counts of pupae and any adults that might have already eclosed. Pupae were removed to fresh flour-substrate mix to eclose, and any larvae were returned to their original flour mix in the vials to pupate. Larvae and pupae were then checked every three days until all larvae had pupated and eclosed into adults, or had failed and died.

3.3 Results

3.3.1 Heat-wave impacts on male and female reproductive fitness

Male reproductive output data contained a high number of zeros, and were therefore non-normally distributed ($D = 0.081$, $p < 0.001$) and heteroscedastic (Levene, $F_{5,256} = 4.695$, $p < 0.001$). Attempts to transform the data (by x^2, Ln(x), Ln(x+1), 1/x and $\sqrt{x}$) were not successful. A KRUSKAL-WALLIS test revealed a significant overall effect of heat-wave temperature on reproductive fitness ($X^2(5) = 67.577$, $p < 0.001$)

Post-hoc DUNN'S tests testing for multiple comparisons showed that males had significantly lower reproductive fitness after being exposed to a heat-wave at 42°C, compared with 30°C control treatments ($Z = 75.549$, $p < 0.001$). The offspring count from males exposed to 42°C was approximately half (52.6%) that of control males at 30°C, and 41% that of control productivity at 35°C. Males exposed to 42°C also produced significantly fewer offspring compared with every other temperature except 41°C (see Figure 3.1).

41°C heat-waves also reduced male offspring production significantly compared with control males at 30°C ($Z = 48.271$, $p = 0.002$). Males treated at 41°C produced less than three-quarters (74.2%) of the offspring produced by males kept at 30°C, and was also significantly different from all other lower temperatures. Following heat-waves of 38°C and 39°C, males produced significantly fewer offspring than males at 35°C, but not from 30°C.

Males exposed to 35°C treatments produced significantly more offspring than males at 30°C (Z = 55.973, p = 0.001) or any higher temperature, confirming that it is an optimal temperature for *Tribolium castaneum* population productivity.

If a Bonferroni correction is applied for multiple testing, including the original KRUSKAL-WALLIS test and each of the 15 pairwise tests, the α-level would become 0.003125 (0.05/16). This would make the differences between 41°C and 38°C and between 41°C and 39°C no longer significant (p = 0.029 and 0.006, respectively). No other results would change showing the effect of thermal stress on fertility at the highest temperatures to be highly significant.

By contrast with effects on males, offspring production by focal females was not significantly affected following exposure to any heat-wave regimes. Data were homoscedastic (Levene, $F_{5,213}$ = 1.968, p = 0.085) and non-normally distributed (D = 0.101, p < 0.001), but transformed by x^2 (D = 0.045, p = 0.200). A one-way ANOVA gave no effect of temperature on female reproductive fitness ($F_{5,213}$ = 1.954, p = 0.087). Females produced fewer offspring following exposure to 42°C heat-waves, but the reduction was not significant (Mean Difference (M) = 31.79, Standard Error (SE) = 18.29, p = 0.509), and females still produced on average 87% that of control females at 30°C, and 82% compared with 35°C females. There was no change in the significance level of the results when Bonferroni correction was applied.

Due to practical limitations, male and female reproductive output could not be assayed at the same time and therefore could not be analysed together. However, Figure 3.1 shows the difference between the sexes' fertility at increasing temperatures is clear, with male fertility significantly declining with increasing temperature past 35°C and females experiencing no significant change in fitness across temperatures.

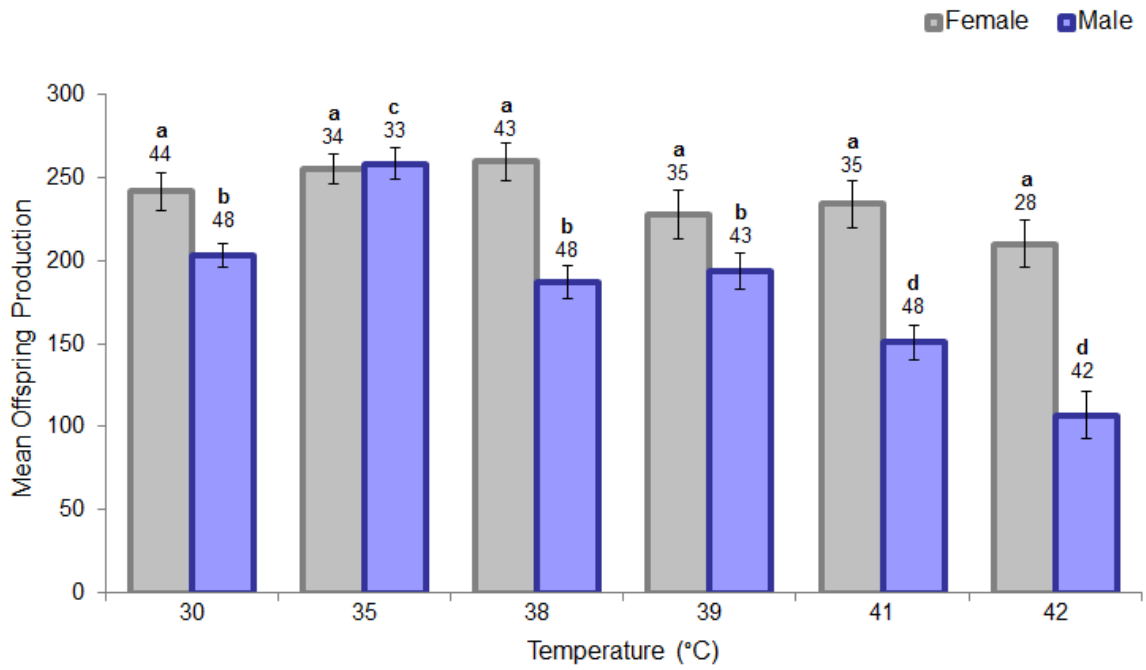

Figure 3.1 Offspring production (total adult output over 20 days) is dependent on level of heat-wave treatment in males, but not females. Both sexes exposed to 5-day thermal treatments, then mated for 48h to control adults of the opposite sex. Error bars show standard error about the mean. Sample sizes and homogenous subsets are displayed above data points. N.B. All female results belong to a single homogenous subset, but since the sexes were analysed separately this does not denote a significant difference from the male subsets.

3.3.2 Variation in male fertility loss

The ~50% fewer offspring produced by males at 42°C could either be due to all of the males halving their reproductive fitness, or half of the males producing normal numbers and the other half producing none. The frequency data for reproductive fitness following 42°C heat-waves compared to 30°C control males was examined in more detail to determine which was the more likely scenario. Frequency counts showed that 23.8% of males produced zero offspring following 42°C treatments, whilst all control males at to 30°C produced some offspring (Figure 3.2). Separating the remaining 76.2% of males that did produce offspring following 42°C treatments, the average offspring produced was 144.6. This count is 71.2% of the average that were sired by the control males. This proportion sits halfway between the scenario where 50% of males suffer total loss of fertility and the one where all males produced 50% of the output of controls. This suggests that the reduction in reproductive fitness following heat-waves is a combination of some males having a proportional reduction in offspring output, and others losing total fertility. Whilst many males stressed at

42°C produced fewer or zero offspring, those with the fertility of the most productive 50% overlapping with that of control males, indicating there is variation in male resistance to heat stress.

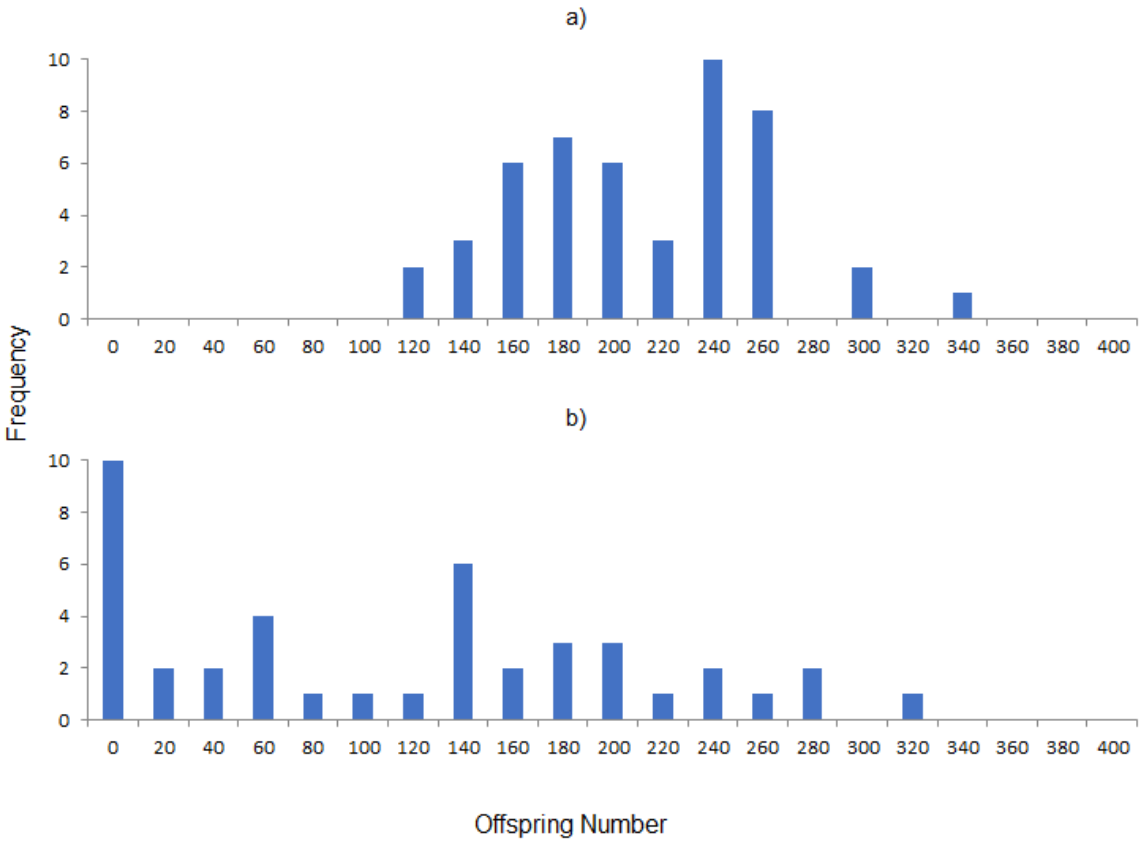

Figure 3.2 Distribution of adult offspring production over 20 days for a) control 30°C males and b) 42°C males. At the highest stress temperature there is a large variation in fertility with ~25% of males (10 out of 42) producing no offspring.

It is important to note that despite high numbers of zeroes often found for the reproductive output of heat-treated males, unpublished data (R. Vasudeva and M. Gage, pers. comm.) showed that every one of N=26 males that had been exposed to a 5-day heat-wave of 42°C had successfully transferred sperm to the female, over a 48-hour mating period, which had become stored in the spermatheca. I look at how insemination success rate after a single mating is affected by heat-stress in chapter 4.

3.3.3 Female fecundity

Data for oviposition rates at both temperatures were normally-distributed according to a Kolmogorov-Smirnov test (30°C D = 0.937, p = 0.200, 40°C D = 0.968, p = 0.200). An independent samples t-TEST assuming unequal variances revealed that there was no significant difference in the mean numbers of eggs laid by females

whether they had mated to control 30°C males versus those mated to males exposed to 40°C heat-wave conditions ($t_{36.582}$ = -0.183, p = 0.856), Figure 3.3, below.

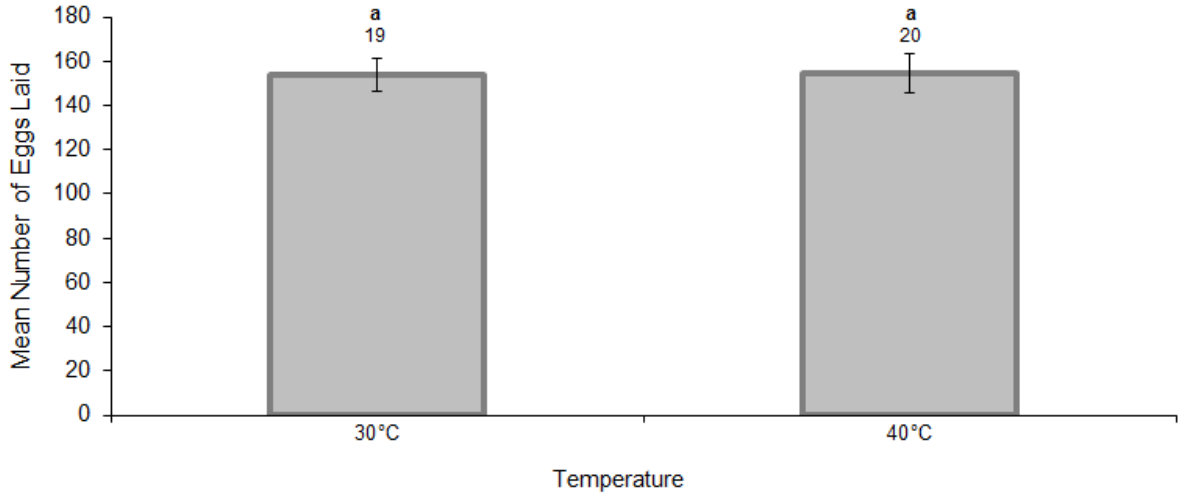

Figure 3.3 Female fecundity across 10 days of oviposition is not affected by the male's previous thermal treatment. Data points represent mean number of un-hatched eggs plus early stage larvae to calculate total fecundity per female. Error bars show standard error about the mean. Sample sizes and homogenous subsets are displayed above data points.

3.3.4 Egg hatch rate

Data for egg hatch rates were non-normally distributed (30°C *D* = 0.218, p = 0.018, 40°C *D* = 0.225, p = 0.013), but could be normalised using an arc-cosine transformation (30°C *D* = 0.158, p = 0.200, 40°C *D* = 0.180, p = 0.106), allowing an independent samples *t*-TEST assuming unequal variances to be run. There was a significant overall 11.3% decrease in the proportion of eggs that hatched when females had been mated to 40°C heat-wave treated males compared with control males ($t_{36.512}$ = -2.229, p = 0.032), Figure 3.4.

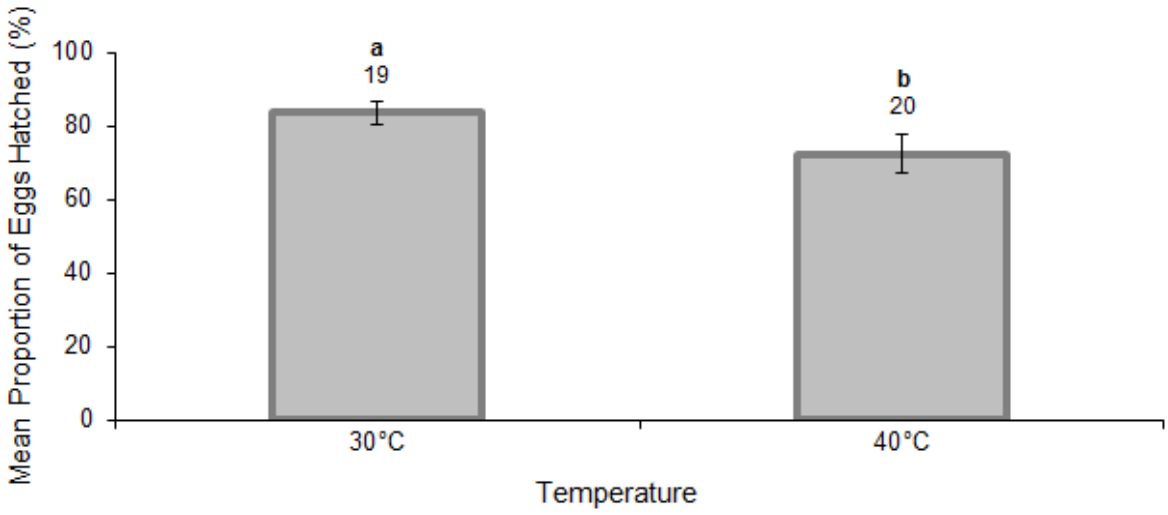

Figure 3.4 Percentage of eggs hatching viable larvae when sired by control versus 40°C heat-wave treated males. Error bars show standard error about the mean. Sample sizes and homogenous subsets are displayed above data points.

3.3.5 Pupal eclosion rate

A MANN-WHITNEY U test was run after both pupal eclosion rate datasets were tested as non-normal (30°C $D = 0.332$, $p < 0.001$, 42°C $D = 0.246$, $p < 0.001$), and all transformation attempts (by x^2, Ln(x), Ln(x+1), arcos(x) and $\sqrt{x}$) also tested as non-normal. There was a significant difference in the distributions of the proportion of pupae that successfully eclosed following analysis using a MANN-WHITNEY U test ($U = 224.50$, $p = 0.004$) with 4.4% fewer offspring successfully eclosing to adults if the father had been exposed to a 40°C heat-wave (Figure 3.5).

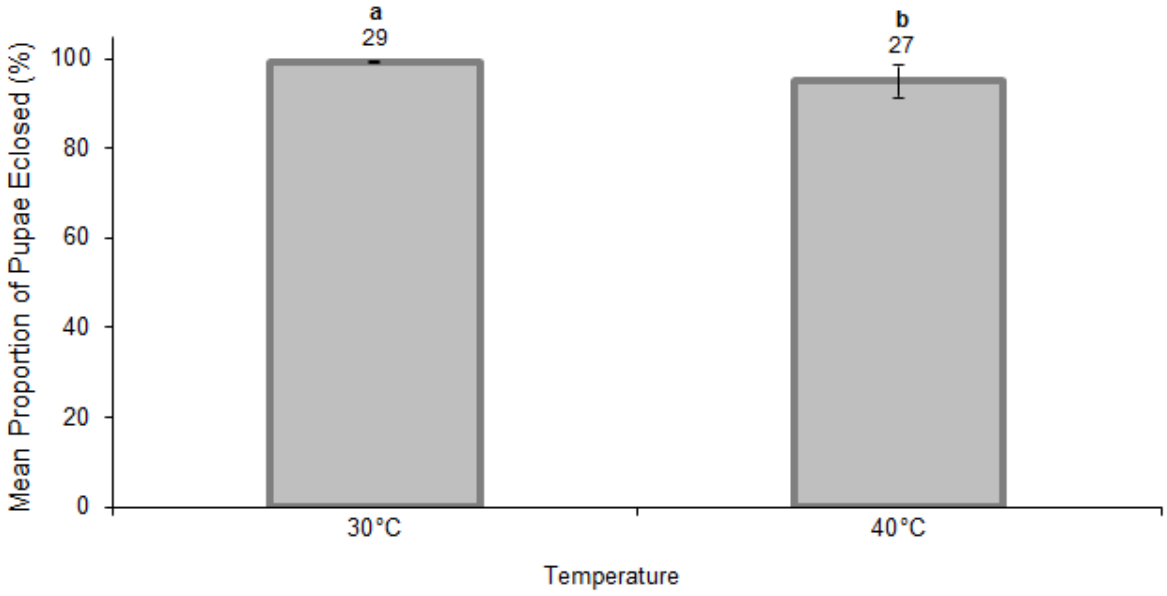

Figure 3.5 Percentage of adults eclosing successfully from pupae is reduced when f0 sires had been exposed to heat-wave conditions. Error bars show standard error about the mean. Sample sizes and homogenous subsets are displayed above data points.

3.4 Discussion

3.4.1 Male reproductive output

In this chapter, I showed that male reproductive fitness is sensitive to thermal stress that arises under heat-wave conditions, while female reproductive output is largely unaffected. These findings are consistent with pilot data for the GA-1 *T. castaneum* strain (Godwin, 2010). The more genetically diverse Krakow Super Strain did show more resilience to thermal stress than the GA-1 standard lab strain. Mean KSS male reproductive output following heat-waves at 42°C was approximately half that of control males, whereas GA-1 males had zero fertility following similar 42.5°C heat-waves (Godwin, 2010). The heat-wave temperature required for an initial effect on male reproductive fitness matches the patterns of gene expression, which shows little change when *T. castaneum* are treated at temperatures below 42°C (Schinko, Hillebrand & Bucher, 2012). Further experiments will use this 42°C temperature to compare effects of heat-wave conditions on reproduction in *T. castaneum*, and the control of 30°C will henceforth be used.

The average 50% reduction in male reproductive output after exposure to 42°C heat-waves occurs as a result of different males showing different degrees of

reproductive failure; a large number of males suffer total fertility loss, whereas others retain higher levels of reproductive fitness (Figure 3.2). This finding implies variation in male ability to resist the damaging effects of thermal stress on reproduction, possibly due to underlying genetic variability. Given this variation, one can hypothesise that females may be able to identify and preferentially mate with males that cope better with thermal stress, in order to protect their own reproductive fitness. In chapter 5 I examine this possibility in more detail, and assess whether it might arise via pre-copulatory or post-copulatory female choice. The evidence for differential male resistance to heat-wave conditions (Figure 3.2) also indicates that insect populations in the wild may have the capacity to adapt and evolve to cope with increasingly common heat-wave conditions and temperature extremes caused by climate change. To explore this possibility in more detail, I use experimental evolution under elevated average temperatures to examine whether male heat-wave resistance can evolve in the laboratory over relatively short time periods (Chapter 7). I used the data from this chapter on offspring production by both sexes at the lower stress temperatures to determine the most suitable temperature for this.

3.4.2 Female reproductive output

In general, the results show that female reproductive output is not significantly affected by thermal stress. This fits with findings from Godwin's testing of GA-1 (2010). There is evidence for some variation in female reproductive fitness under thermal variation, with a small but significant increase in offspring output (5-7%) at lower levels of heat-wave exposure. After this mild increase, heat-wave temperatures greater than 39°C then create a slight downward trend for KSS female reproductive output, but the differences are not significant.

These results suggest that some aspects of female *T. castaneum* reproductive biology favour moderately higher temperatures. Godwin also showed an increase in GA-1 female reproductive output at intermediate heat-wave temperatures of 37.5°C. This may suggest the optimum temperature for female reproductive output is greater than that for males (which peak at 35°C), but limited at 39°C. Although not significant, female reproductive output shows a small decrease when heat-treated at 42°C relative to both control and lower stress temperatures; over 20 days of oviposition, the greatest difference was a 20% drop compared to the optimum at 38°C

(M = 49.67, SE = 18.38, p = 0.079). If male reproductive biology is limiting factor at higher heat-wave temperatures, then there could be sexual conflict over thermal optima, with females reproducing at a sub-optimal temperature at the 30°C used in the laboratory.

As expected, the use of the more genetically diverse Krakow Super Strain effectively increased the 'resolution' of male-specific reproductive fitness loss; there was a significant loss of offspring output at the highest heat-wave temperatures, but it did not cause a complete cessation of reproduction. The major 50% decline took place when males were exposed to 42°C heat-waves, and I will use this treatment as the heat-wave regime for further work in this thesis, unless otherwise indicated, including whether the higher genetic diversity of the Krakow Super Strain allows adaptation under higher average ambient temperatures to allow male thermoresistance under heat-wave conditions.

3.4.3 Sex-specific reproductive sensitivity

The experiment in this section confirms work on *T. castaneum* and other models (see Chapter 1.2 and 1.5) that male reproductive fitness is significantly affected by heat stress, but females are not. This finding is consistent with previous studies showing male sensitivity to heat stress, and ultimately it being a limitation on population viability in both homeotherms (Setchell, 1998) and some ectotherms, such as *Drosophila sp.* (Rohmer *et al.* 2004, David *et al.* 2005). These data add to the knowledge we have on individual male reproduction under heat stress, building on the pilot work exploring differences between male and female offspring production after heat-waves in the *T. castaneum* GA-1 strain (Godwin, 2010). Interestingly, oocytes are the only cells found to contain Hsp70 mRNA without heat shock (Bienz, 1994). Although this may exist because it aids in meiosis (Metchat *et al.*, 2009), it also may equip egg cells with the ability to endure heat stress. Male gametes have no such protection, and I will examine the effects of heat stress on sperm cells in chapter 4.

3.4.4 Female fecundity

Having paired 40°C heat-wave treated and control males to virgin females, I determined that the reduction in a male's subsequent reproductive output is not due to a decrease in female fecundity. This contrasts with studies which have found that females laid fewer eggs when mated to males that had been heat-treated (Zhang *et al.*, 2013; Zhang *et al.*, 2016). These differences may exist because the studies by Zhang *et al.* used a shorter stress period, and mating took place soon thereafter. Males in my present study may have recovered some reproductive function after the heat-wave treatment, during the 24-hour cool-down or 48-hour mating period. Alternatively, female egg production may be unaffected by the reproductive status of the male, and preliminary data (R. Vasudeva and M. Gage unpubl. Data) show that all of 26 heat-waved males given access to virgin control females successfully transferred sperm, even though some of those females then went on to produce zero offspring as a result of male infertility. Since oviposition was not affected when mated to heat-treated males, I find no evidence that females alter their investment in oviposition rate as a result of mating with heat-treated males. To assess the possibility of female compensation for a male's reproductive status further, I examine in chapter 5 whether females can boost their own reproductive output following exposure to heat-treated mates by strategically remating with multiple males. I examine whether polyandry can protect female reproductive fitness from the risk of infertility following matings with heat-wave exposed males. Then, I examine whether a male's heat stress condition affects female mate choice; i.e. are females less 'willing' to mate with heat exposed males, or more willing with control males? Finally, I test whether females can avoid their subsequent reduced reproductive fitness having mated to heat-wave exposed males by strategically mating more polyandrously?

3.4.5 Egg hatch rates and offspring development

By contrast with the fecundity results, I confirmed that egg hatch rate is reduced when females have mated with males exposed to 40°C heat-waves (Figure 3.4). Following heat-wave conditions, egg hatch rate declined from 83% to 72%. These results confirm that heat-wave conditions specifically impact on mechanisms within male fertility.

This finding matches the work in other ectotherms, including moths (Zhang *et al.*, 2013), whitefly (Cui *et al.*, 2008), aphids (Montllor, Maxmen & Purcell, 2002) and fruit flies (Huey *et al.*, 1995; Silberman & Tater, 2000), and strengthens the case for a spermatozoal-specific effect of thermal stress that leads to reductions in male reproductive fitness.

In addition to a reduction in male fertility, I also found evidence that offspring pupal eclosion rate into adult imago is also reduced when male sires have previously experienced a 40°C heat-wave (Figure 3.5). Although the reduction was only 4.4%, the difference was statistically significant, and provides evidence that thermal stress can impact transgenerationally on offspring fitness by somehow disrupting metamorphosis. This agrees with work in whiteflies when both parental sexes were stressed (Cui *et al.*, 2008), but, to my knowledge, is the first to show an effect on eclosion of offspring when fathers have been heat stressed.

The loss of f1 offspring at the threshold of pupal eclosion suggests that thermal stress somehow damages processes that manifest during metamorphosis, either in the transcription of potentially damaged haploid DNA from the father, or at some stage in the process of translation, rather than the mortality of offspring of treated males simply increasing steadily with age. This disruption within metamorphosis could be caused by a pre-zygotic or an epigenetic effect that translates into adult offspring mortality. I will examine further transgenerational effects of heat stress on offspring reproductive fitness in more detail in chapter 6.

I therefore discover, following male exposure to 40°C heat-waves, that both fertility and offspring pupal eclosion rate are impacted. Combining the reductions in reproductive output as a result of these two stages, can explain the reduction in offspring numbers I find following exposure to 39°C and 41°C heat-wave conditions (Figure 3.1). Although the results for adult offspring production in section 3.1 do not have a result at the 40°C treatment, the reduction in adult offspring was 4.6% at 39°C and 25.7% at 41°C, therefore it is reasonable to estimate the decrease at 40°C to be around 15%. It is possible to estimate the cumulative reduction in expected offspring production when males had been exposed to 40°C heat-waves as a result of the declines in fertility (11.3%) and decreases in pupal eclosion (4.4%); combining these effects indicates a 15.7% reduction in adult offspring production. Since there was no difference in female fecundity following mating to males exposed to 40°C heat-waves

compared with controls, the estimated reduction in offspring for 40°C exposed males of ~15% is consistent with my measured reduction in offspring production of 15.7% as a result of the combined damage to male fertility and pupal eclosion.

3.5 Conclusions

In this chapter I explored how reproduction of an outbred lab insect model responded to simulated heat-wave conditions, and found that male reproductive fitness, as measured by offspring production, decreases following increasing heat-wave conditions, being halved after a 42°C heat-wave. Females, by contrast, showed no significant decline in reproductive fitness under similar conditions. Investigating this male-specific impact on offspring production further, I measured the potential mechanisms behind this impact, by specifically measuring heat-wave effects on fecundity, fertility, and offspring development. I found no impact on female fecundity when mating with heat stressed males, but did find significant declines in male fertility through egg hatch rates, and a transgenerational impact on pupal eclosion success rates of offspring fathered by heat stressed males. In subsequent chapters I explore these mechanisms in more detail.

4 What causes the loss of male reproductive performance following heat stress: mating behaviour or sperm numbers?

CHAPTER SUMMARY

The previous chapter 3 established that five-day heat-wave windows of thermal stress caused a significant decline in male reproductive output, apparently due to reductions in male fertility. This chapter investigates two possible causes that drive this male-specific loss of reproductive performance. Male mating behaviour and ejaculate sperm numbers were measured in heat-stressed males, and compared with equivalent controls. Male mating behaviour was assayed in arenas following introduction to virgin females over a one-hour period of recording. After five days exposure to 42°C heat-wave conditions, males were slower to initiate mating and mated less frequently. However, these males still showed ample evidence of motivation to mate, engaging in an average five full matings per hour of observation, and exhibiting a tendency for longer mating durations than the controls. All but one of the heat-wave treatment males, and all the control males, mated for long enough to assume sperm transfer (>35 seconds). I therefore found evidence that heat stressed males had changed their mating behaviour, but that mating activity was still evident, and almost all successfully mated within one hour of access to the female. Following mating, microdissection and counting, ejaculate sperm numbers were found to be significantly lower in males that had experienced a heat-wave (averaging 6,500 sperm per spermatophore), with control males typically ejaculating almost six times as many sperm (37,100 sperm on average). There is therefore a clear impact of heat stress on sperm production, which probably contributes to the reduction in male reproductive performance identified in Chapter 3.

Contributors: Matthew Gage and Łukasz Michalczyk assisted with dissections and sperm counts. Matt Gardiner assisted with implementation and data collection of behavioural assays.

4.1 Background

In the previous chapter I established that the reproductive fitness of adult male *Tribolium castaneum* is especially sensitive to heat, leading to declines in offspring production after exposure for five days to heat-wave conditions. These declines were mainly associated with reductions in egg hatch rate, and some decline in offspring pupal eclosion rates. Here, I explore some potential mechanisms behind this reduction in male reproduction, and investigate whether the cause is associated with pre- or post-copulatory processes. Pre-copulatory causes could be associated with an impact of heat stress on male mating behaviour, and male mating success, potentially including female mate choice. Post-copulatory factors could be associated with the number of sperm transferred to the female at mating, and/or the subsequent function and integrity of those sperm. In this chapter, I therefore examine the impacts of heat-wave conditions on pre-copulatory male mating behaviour, and post-copulatory ejaculate sperm number.

In one of the seminal works on the biology of *T. castaneum,* Sokoloff (1972-78) notes that both males and females of this species are highly promiscuous, and males are especially unselective in their mating habits, having been observed to mount not only females but also other males, dead adults and even clumps of flour that vaguely resemble a beetle shape! Although subsequent research has shown that males display more discerning mate choice (see Arnaud, 1999 and Chapter 5 of the present work), they still mate promiscuously, initiating the vast majority of male-female contacts, and males can mate repeatedly, more than 3-4 times in 30 minutes (Sokoloff, 1972-78; Arnaud, 1999). More recent work has further detailed the high levels of promiscuity exhibited by this species, with males mating successfully with 50 out of 84 virgin females presented in a sequence (Lumley *et al.*, 2015).

In common with many insect taxa, *T. castaneum* copulatory behaviour involves several discrete steps described by Arnaud (1999). First the male approaches and makes contact with another beetle using his antennae or maxillary palps to inspect the head or abdomen of the potential mate. The male then attempts to copulate by mounting the female and grasps her prothorax with his first pair of legs. '*The male then lowers his last sternite and extrudes his aedeagus. For successful mating, the female must lower her last sternite to allow intromission*' (Arnaud, 1999). In order to encourage the female to acquiesce and allow intromission, the male may rub his legs

along the female's body (Edvardsson & Arnqvist, 2000; Bloch Qazi, 2003). A review in Fedina & Lewis (2008) concludes that this leg-rubbing behaviour is associated with overcoming female resistance to insemination, but does not alter the likelihood of a female storing or using a male's sperm once inseminated.

Copulation is achieved with the mounted male having his venter on the female dorsum, and it can last from a few seconds to 30 minutes or more (Bloch Qazi, Herbeck & Lewis, 1996), during which time the male transfers his sperm contained in a spermatophore into the female's bursa copulatrix (Bloch Qazi, Herbeck & Lewis, 1996). *T. castaneum* males sometimes mount a female, but do not transfer sperm, with previous work showing that only mating attempts lasting longer than 35 seconds are typically associated with successful sperm transfer (Edvardsson & Arnqvist, 2000; Attia & Tregenza, 2004). I therefore score 'successful mating' in my behaviour assays as those copulations that last at least 36 seconds.

Heat stress is known to impact mating behaviour, reducing mating frequency (Fasolo & Krebs, 2004; Liao, Qian & Liu, 2014) and mating success (Janowitz & Fischer, 2011; Jerbi-Elayed *et al.*, 2015) in a number of insect models. However, many studies observe mating behaviour actually during heat treatment (e.g. Katsuki & Miyatake, 2009; Nguyen & Amano, 2010), which masks differences between male and female effects. In the current chapter, I isolate the effects of heat stress on male mating behaviour, and then look at female mating behaviour in chapter 5.

A relationship between sperm production and temperature has long been inferred since homeothermic mammals have various adaptations to keep sperm and their production site cooler than core body temperatures, with a common adaptation being the descent of the testes into a scrotal sack during maturation (Setchell, 1998). If an abnormality within development prevents the testes from descending to a cooler location, males are commonly sterile, but if this condition is alleviated early enough, spermatogenesis will begin (Setchell, 1998). Not all mammals have a scrotum, with some retaining their testes inside the abdomen, and all birds and other animals do the same, despite birds having higher average body temperatures (Setchell, 1998). In these taxa, it is clear that the cost of carrying the gonads outside the abdomen is greater than the risk of heat damage or the cost of developing and maintaining spermatozoa at higher core body temperatures. It is not known what, if any, adaptations birds have to keep spermatozoa at lower temperatures, but it is known

that, in poultry, heat stress readily affects sperm viability and sperm quality (Karaca *et al.*, 2002), all phases of semen production and sperm function (Ayo, Obidi & Rekwot, 2011), and heat delays the onset of spermatogenesis during development, and decreases the sperm stock of male birds at maturity (Chirault *et al.*, 2015).

Thermal stress is known to damage spermatozoa and spermatogenesis in a number of invertebrate species, although evidence as to the nature of the damage is somewhat contradictory. In the nematode *Caenorhabditis briggsae*, the number of sperm recovered from the gonads of mature individuals that had been heat-treated to 30°C did not differ significantly from control (20°C) or between temperate, tropical and equatorial strains, suggesting that mature sperm are resistant to thermal stress. Furthermore, worms heat-treated at a stage of the lifecycle where spermatogenesis is occurring showed permanent defects in sperm fertility, whereas fertility recovers for mature individuals, indicating that thermal stress impacts spermatogenesis, rather than mature sperm function (Prasad *et al.*, 2011). In *C. elegans*, thermal stress prevents spermatozoa from fertilising eggs (Ward & Miwa, 1977). Although normal sperm numbers are transferred *in copula*, compared to control males, heat-stressed male gametes do not reach the spermathecae and males are therefore infertile (Ward & Miwa, 1977).

In *D. melanogaster*, males reared throughout their entire development at the thermal threshold of fertility seemed to become sterile because spermatogenesis is impaired, with complete maturation of sperm being constrained, and more sperm cells having abnormal nuclei and greater levels of cell death (Rohmer *et al.*, 2004). However, a similar study on *D. simulans*, in which species-specific morphology allows small changes in spermatozoal length to be more easily measured, found no large discontinuity caused by thermal stress. Therefore, the variation with temperature '*might be more the consequence of a normal progressive plasticity than of the sudden disruption of a specific physiological process*' (David *et al.*, 2005).

If sperm cells themselves are at risk from thermal stress, adaptations should be expected to increase thermal resistance. Indeed, there is evidence in *D. melanogaster* that heat stress protein Hsp90 regulation alters sperm production, and form and function is different between normal and mutant flies which have reduced expression (Yue *et al.*, 1999). The reduction in Hsp90 function in the mutant flies had a large effect on all stages of spermatogenesis involving microtubule function, from

early mitotic divisions to later stages of sperm maturation, individualization, and motility. Furthermore, linking these *in vitro* microscopy confirmations of sperm damage to *in vivo* effects, these mutant males were shown to be sterile (Yue *et al.*, 1999).

Here, I investigated male mating behaviour by observing the effect that thermal treatments have on subsequent reproductive interactions between males and control virgin females. Through one-hour behaviour assays I specifically measured: 1) how long males take to attempt their first mating, 2) the frequency of attempts, 3) how often males are successful (as measured by an established mating duration threshold), and 4) how long on average they copulate for. I then examined whether the heat-wave conditions that cause a halving in male reproductive fitness also impact on sperm production. To determine how thermal stress impacts on *T. castaneum* spermatozoa and spermatophore transfer, I conducted *in vitro* studies of ejaculate sperm numbers recovered from females mated to heat-wave treated males, compared to control males. I used the same five-day heat-wave conditions established in chapters 2 and 3 to determine whether this environmentally relevant condition affects the number of sperm transferred to the female, and therefore whether this post-copulatory damage explains the significant reduction in male reproductive performance at raised temperatures.

4.2 Methods

4.2.1 Male mating behaviour

Virgin males of the Krakow Super Strain (KSS) were either placed under heat-wave conditions of 42°C (as per Chapter 3.4.2) or kept at a 30°C control, both for 5 days at 60% humidity. (For full heat stress methodology, see chapter 2.2.2.) All females were also KSS and kept under control conditions. Accounting for some mortality and unexpected contingencies, excess males were exposed to their treatment aiming to generate a sample of N=30 males in either treatment (see Chapter 2.2.2 for a discussion of mortality rates as temperature increases). Following eclosion, and until introduction to the female, males were kept in isolation to maintain unmated virgin status, and avoid any pre-trial homosexual interactions or competitiveness which could influence subsequent male mating behaviour (Nilsson, Fricke & Arnqvist, 2003). Females were marked with a spot of white paint on the dorsum of their thorax.

Males received five days of the heat-wave or control treatment, and then given a 24-hour cooling-off period. Any dead males were then removed and the surviving males placed with control females in 1cm^2 plastic mating cells, with smoothed sides to prevent escape, and scratched bases to allow traction and self-righting (see Figure 4.1). Since males can mate for up to 32 minutes (Bloch Qazi, Herbeck & Lewis, 1996) their mating behaviour was recorded for an hour to ensure that the start and end of any copulations with sperm transfer were likely to be recorded, following Nilsson, Fricke & Arnqvist (2003). Footage of the mating behaviour was recorded using three tripod-mounted Sony digital camcorders. To balance the behaviour experiment, both treatments were filmed simultaneously using 6 mating arena cells per camcorder (Figure 4.1), the three camcorders recording 18 male-female pairs per hour. Analysis of the footage was performed blind to the treatments (a third party gave treatments random numbers as labels and did not reveal which code denoted which treatment until after the data had been collected from the footage and analysed). Although the mating arenas were prepared to make the sides difficult to climb, some beetles managed to escape during the assays, and occasionally climbed into neighbouring cells; pairs with escapees or invaders were then discounted from analysis. These eliminations gave sample sizes of N=25 and N=14 for the control and heat stress treatment respectively.

Figure 4.1 Example of arenas for recording mating behaviour. Note scratched floor of plastic, painted surfaces and marked females. Photo credit: M. Dickinson.

Mating behaviour was scored and collated by replaying each 1-hour recording. Females were identified with a small white spot on the dorsal thorax. A mating attempt was defined as when the male's ventral side was positioned to completely cover the female's dorsal side, with anterior and posterior ends aligned in the same direction (Arnaud, 1999); if this position was achieved the times of the mount and dismount were recorded. Pausing and replaying the video while transcribing behaviour ensured no activity was missed while recording the data point. Mating attempts that could not be assumed to last long enough to allow spermatophore transfer, i.e. lasting 0-35 seconds, were defined as 'mating attempts'. 'Mating success' was defined when copulations lasted long enough to assume successful sperm transfer, i.e. $\geq 36s$ (as per Edvardsson & Arnqvist, 2000).

Analysis of the videos allowed quantification of: 1) latency to attempt mating, 2) number and 3) duration of attempts, as well as 4) latency to successful mating, 5) successful mating number, and 6) duration(s), although not all of these measures were used in final analyses (see results section below). Mating attempts provide a proxy for male motivation to copulate, which females do not always cooperate with; whereas mating success indicates copulatory cooperation between male and female, and which should relate more directly to subsequent male reproductive fitness.

4.2.2 Ejaculate sperm counts and insemination success rate

Following the methods established in chapters 2 and 3 (see 2.2.2 and 3.4.2), males were exposed to heat-wave conditions for five days at 42°C, or left at control temperatures of 30°C, followed by 24 hours cooling off before being given access to virgin unmated females. Each male was provided with five females to mate with in sequence, to counteract the risk of an observed mating but no sperm transfer, and to provide information on failed insemination rates between treatments. Males were moved to the next female after the first mount and dismount, or after 15 minutes if no mounting took place. The females were frozen within 5 minutes of mating at -20°C, before movement of sperm from the ejaculated spermatophore into less accessible female spermathecal storage (Bloch Qazi, Aprille & Lewis, 1998).

Spermatozoa were recovered and counted from spermatophores deposited in the bursae of defrosted females, allowing a count of mature sperm transferred to females. To extract spermatophores, females were taken straight from the freezer, pinned ventral side up in a wax dish using fine titanium entomology needles, and submersed in saline buffer (1% NaCl solution). Intact spermatophores were recovered from the bursa using a dissecting stereomicroscope (Zeiss Discovery V.12) at 10-20x magnification, removing the entire female reproductive tract by pulling the ovipositor terminally using fine forceps, separating the intact bursa and lower tract from the abdomen. The spermatophore-containing bursa was then isolated from the tract through excision using fine spring scissors, and moved to a cavity slide containing a 100 µl drop of saline buffer. Here, the spermatophore, visible as a white ovoid, was removed intact from the bursa, and moved to another cavity slide containing 100 µl of saline. The spermatophore was then ruptured using fine needles and the sperm mass released and dispersed using gentle stirring. Once the sperm mass was dispersed, which could be assessed under x20 magnification, the entire solution contents of the cavity slide were washed off the slide using 20 to 40 µl of saline buffer from a P200 autopipette into a pre-weighed microcentrifugal tube. Depending on whether there was a low, medium or high density of sperm present on dispersal from the spermatophore, the sperm solution was then diluted further with 1 to 3 ml of saline buffer and gently mixed.

Sperm counts were achieved by taking three 20 µl subsamples of the diluent and allowing these to air dry on plain glass slides, which makes the sperm stick to the glass in a two-dimensional plane. Once dried, and before counting, the slides were gently dipped in distilled water to wash off desiccated salt crystals. The total number of sperm in each smear was then counted under dark-field phase contrast microscopy at x200 (see Arnaud, Gage & Haubruge, 2001; Michalczyk 2008). Using this method, sperm tail membranes were often ruptured and the two mitochondrial derivatives separated and clearly visible. To count sperm, I therefore scored the total number of intact sperm, plus the total number of mitochondrial derivatives divided by two (there are two per cell). Total sperm count was then calculated by taking the mean of the three smear counts multiplied by their 20 µl dilution factor in the total 1 to 3 ml diluent (calculated by subtracting the weight of the empty tube from that of the tube with solution in it).

4.3 Results

4.3.1 Male mating behaviour: initial attempts and latency

All males attempted to copulate with the female, and all but one successfully mounted for more than 35 seconds. Only three out of 25 control males made a first attempt that was not successful (i.e. 22 out of 25 males mounted for >35 seconds). By contrast, half of the 42°C heat-wave treated males (7/14) took more than one attempt for the female to allow >35 second matings, indicating a significant difference in first-mating success rates between controls and heat-treated males (U = 101.00, p = 0.015). One of the heat treatment males failed to mate altogether with the female for more than 35 seconds across the whole 60-minute duration and was excluded from relevant analyses.

Because of differences in first mating success rates, I focused latency analyses on the first mating attempt. These data were non-normally distributed for the 42°C treatment (30°C D = 0.146, p = 0.182, 42°C D = 0.332, p < 0.001) and could not be normalised using a number of common transformations (x^2, Ln(x), Ln(x+1), 1/x or $\sqrt{x}$), so an independent samples MANN-WHITNEY U test was used, revealing highly significant differences in the mean latency of a male's first attempt between control and heat treatment males (U = 28.50, p < 0.001). Regardless of mating success, the latency to first mating attempt was over twelve times longer for heat stressed males compared to that of control males (Figure 4.2).

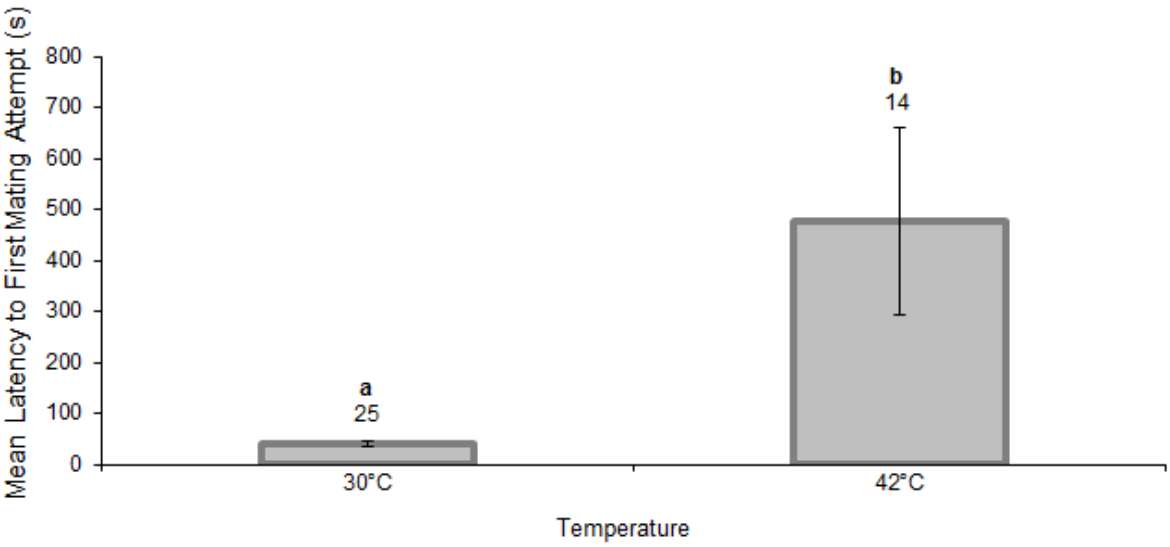

Figure 4.2 Latency (seconds) for males to attempt their first mating within 1 hour observation periods comparing control with heat-wave treated (42°C) males. Error bars show standard error about the mean. Sample sizes and homogenous subsets are displayed above data points.

4.3.2 Male mating behaviour: number of mating attempts

The number of mating attempts and their success levels in achieving >35 second copulations provide important information about male mating persistence and/or female choice. The distributions of the number of mating attempts (excluding successes) were normal for both temperature treatments (30°C D = 0.124, p = 0.200, 42°C D = 0.173, p = 0.200). An independent samples t-TEST assuming unequal variances showed significantly fewer attempts were made by heat-treated males, with a decrease of over 60%, $t_{36.989}$ = 4.413, p < 0.001, Figure 4.3.

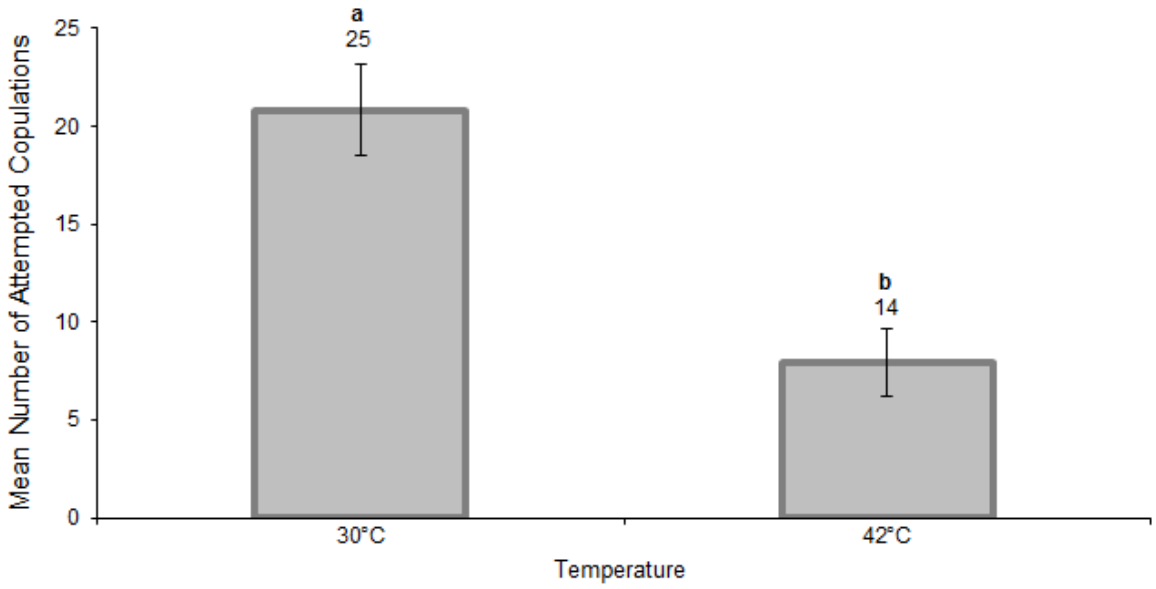

Figure 4.3 The number of attempted copulations by males (across 1 hour observation periods) decreases by more than half following heat-wave conditions. Error bars show standard error about the mean. Sample sizes and homogenous subsets are displayed above data points.

4.3.3 Male mating behaviour: mating success

For both prior temperature treatments, the number of successful mating events were not normally distributed (30°C D = 0.164, p = 0.082, 42°C D = 0.156, p = 0.200). Using a square-root transformation in an independent samples t-TEST assuming unequal variances, there was a significant difference in the number of successful mating events ($t_{19.555}$ = 2.724, p = 0.004). Heat stressed males mated ~40% less frequently compared to control males, Figure 4.4, but it is important to note that these males still mated on average ~5 times with the female over each 1-hour observation period, compared with ~8 times for control males.

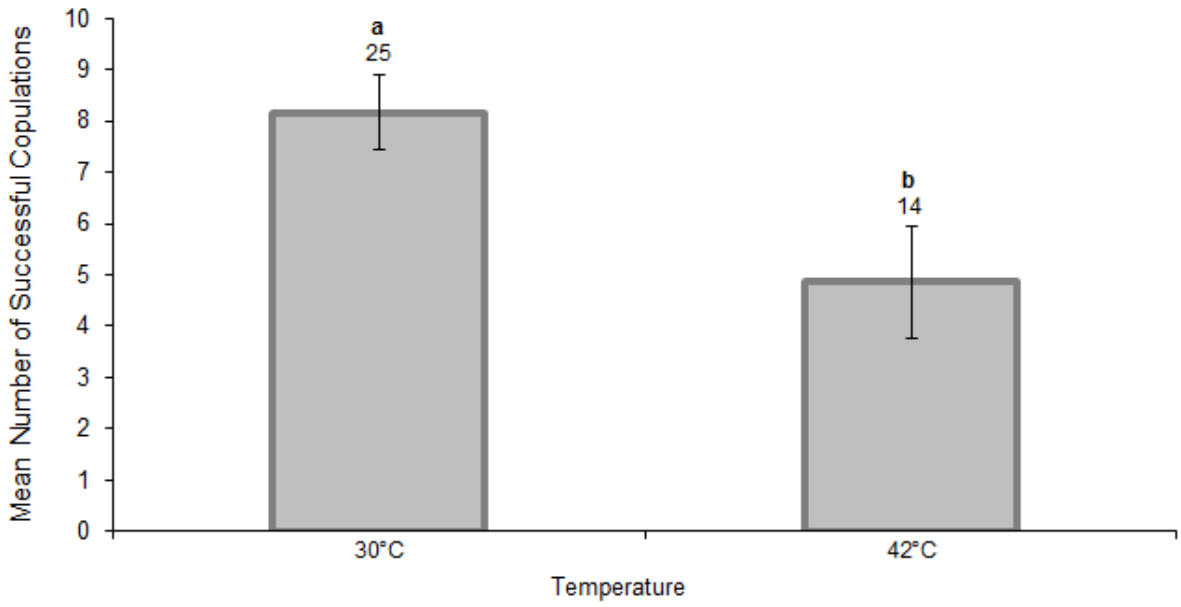

Figure 4.4 Number of successful mating events (as defined by copulations that lasted at least 35 seconds) for control and heat-wave treated males across 1-hour observation periods. Error bars show standard error about the mean. Sample sizes and homogenous subsets are displayed above data points.

4.3.4 Male mating behaviour: mating duration

There was a trend for mating durations to be longer for heat-treated than control males, although wide variance in the mating durations of the heated males limited the ability of statistics to determine clear differences. Analysing the duration of a male's first successful mating (>35 seconds in duration), regardless of how many attempts took place before this, mean duration increases by more than 50% for males that were heat-treated. Untransformed data were non-normal (30°C D = 0.313, p < 0.001, 42°C D = 0.309, p = 0.001). Using 1/x transformed data (30°C D = 0.103, p = 0.200, 42°C D = 0.167, p = 0.200) an independent samples t-TEST assuming unequal variances showed that there was no significant difference between the two treatments ($t_{18.362}$ = -0.120, p = 0.906; Figure 4.5).

The mean duration of a male's first mating attempt, regardless of whether it was successful or not (i.e. duration >1 second), showed no difference between heat-treated and control males, using an independent samples t-TEST assuming unequal variances on Ln(x) transformed data ($t_{36.886}$ = -0.960, p = 0.343).

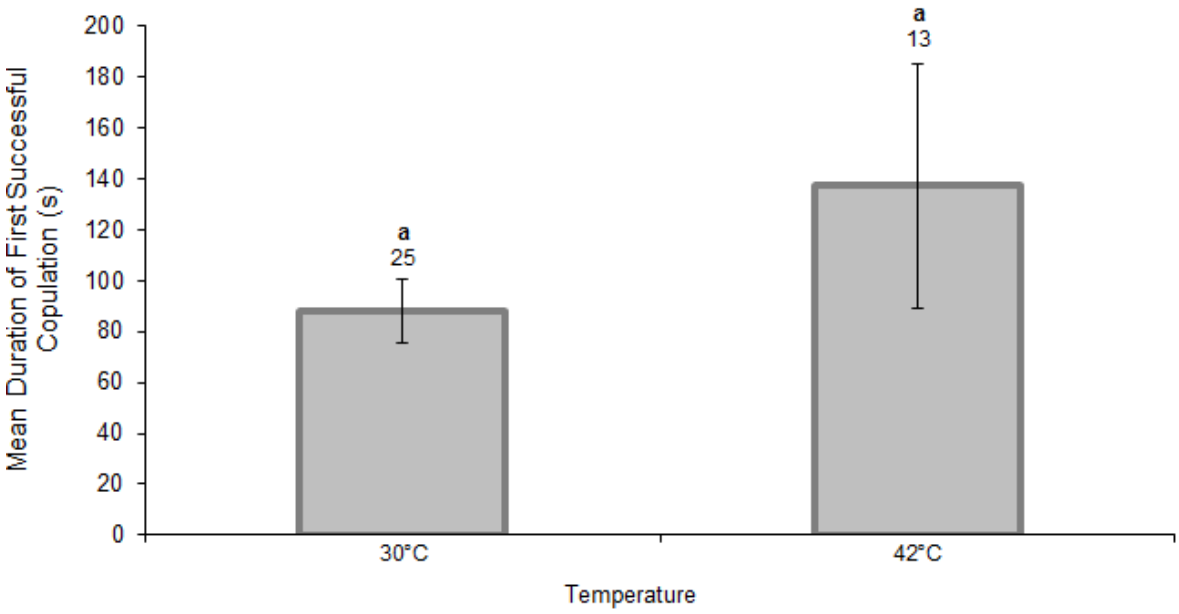

Figure 4.5 Time taken from mounting to sperm transfer (duration of first copulation >35 seconds) increases when males are heat-wave treated. Error bars show standard error about the mean. Sample sizes and homogenous subsets are displayed above data points.

4.3.5 Ejaculate sperm counts and insemination success rate

Sperm count data were non-normally distributed in control males (30°C D = 0.280, $p < 0.001$, 42°C D =.162, $p = 0.140$) and could not be transformed. A non-parametric MANN-WHITNEY U test revealed that there was a highly significant difference between control and heat-wave treated males in their ejaculate sperm numbers (U = 145.0, $p = 0.009$), with control males producing on average five times more sperm than heat-treated males (Figure 4.6).

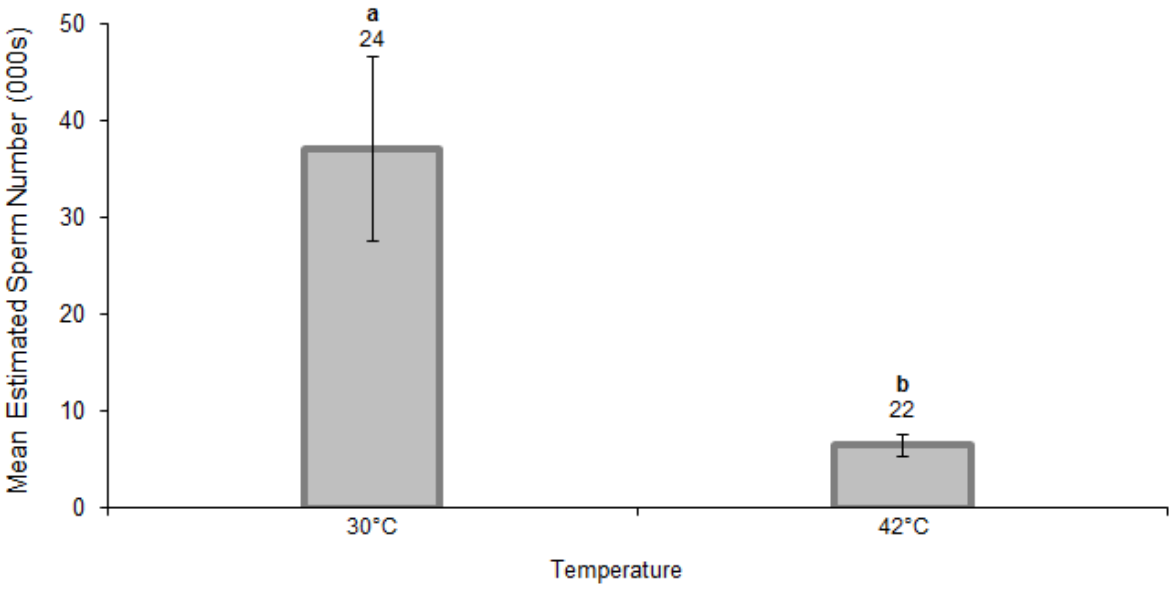

Figure 4.6 Ejaculate sperm number is significantly reduced following male exposure to heat-wave conditions. Error bars show standard error about the mean. Sample sizes and homogenous subsets are displayed above data points.

There was a significant difference in insemination success between control and heat-wave treated males: 19 out of 24 control males successfully inseminated their first female, whereas only 7 out of 22 heat-treated males inseminated the first female (U = 116.50, $p < 0.001$). Of the 15 heat-treated males that failed to transfer sperm to the first females, 6 inseminated the second female, 5 required a third female, 3 males did not inseminate successfully until their fourth female and 1 male only inseminated the fifth female.

4.4 Discussion

4.4.1 Male mating behaviour

In this chapter, I find changes in male mating behaviour, with males previously experiencing heat-wave conditions being slower to start mating (Figure 4.2), and mating less frequently (Figure 4.3), and showing reduced mating success (Figure 4.4). These results suggest an overall impact on general male breeding condition, potentially as a result of reduced male libido, an effect on their ability to pursue and mount the female, or on their effectiveness in inducing the female to cooperate prior to and during copulation. It is therefore possible that some pre-copulatory factors within male mating behaviour contribute somehow to the halving

of reproductive performance I observed in chapter 3 for males following heat-wave conditions.

The results for reduced mating number matches those of other studies in different systems. Short-term exposure to higher temperature in *D. melanogaster* greatly reduced the mating frequency of males and females (Krebs & Loeschcke, 1994). On the other hand, my results for male mating latency contradict earlier studies, such as Janowitz & Fischer (2011), who found no significant effect of heat stress on time to copulation in *Bicyclus anynana* butterflies, but concurs with others finding that time to mating was slower following heat stress of male predatory mites (Zhang *et al.*, 2013) and the springtail *Orchesella cincta* (Zizzari & Ellers, 2011). When stressed with a low nutrition diet *T. castaneum* males have reduced pheromone production and olfactory attractiveness (Ming & Lewis, 2010), indicating these factors act as a condition-dependent mating signal. If heat-stress has the same effect then this may also explain female resistance to thermally stressed males.

The *copula* duration results showed an increase in mating duration following the 42°C treatment (Figure 4.5) which, under normal circumstances, would suggest a higher probability of successful sperm transfer (Bloch Qazi, 2003; Fedina & Lewis, 2006). If this behaviour is driven by males, it could be interpreted as heat-treated males needing longer to transfer spermatozoa and may be supported by the reduction in insemination success rate. Some past work has also found a sex-specific effect of short-term heat exposure on mating behaviour, with copulation duration increased when males were stressed (Zhang *et al.*, 2013; Liao, Qian & Liu, 2014). Other studies have found no effect of heat stress on copulation duration (Janowitz & Fischer, 2011).

Despite the general indications that heat stressed males have lower mating performance across the behaviour assays, these males did still achieve, on average, 5 matings per one-hour observation period (compared with 8 successful matings by control males). In *T. castaneum*, only one mating is sufficient for females to fertilize ~700 eggs across up to four months of oviposition (Bloch Qazi *et al.*, 1996). The male mating behaviour analyses therefore, at best, only provide a part-explanation for why male reproductive performance is halved following exposure to heat-wave conditions. One factor which was not possible to isolate in these experiments was the possibility for female action in response to longer latency and duration after thermal stress; females may seek to avoid mating with poor quality males or they may reject sperm

from a male which somehow performs poorly, either in courtship stimulation or copulation ability. Alternatively, they may even submit to mate longer with treated males in an attempt to increase sperm transfer and increase their own fertility. Thus, the thermally-induced decline in male reproductive performance could be explained by female mating behaviour, or some form of post-copulatory female control (Edvardsson & Arnqvist, 2000; Bloch Qazi, 2003), rather than being controlled directly by the male. I investigate the possibility for female effects in this system in the next chapter, exploring whether female *T. castaneum* alter their pre- or post-copulatory behaviour in response to the condition of thermally stressed males.

4.4.2 Sperm counts

This experiment presents clear evidence of a substantial effect of heat-wave conditions on sperm production and insemination success rates. Following a heat-wave at 42°C, only 32% of males successfully inseminated the first virgin female they met, whereas 80% of control males were successful. Moreover, when heat-treated males did deposit a spermatophore, it contained only one fifth the sperm population of control males (Figure 4.6). These findings provide an explanation for why male fertility (egg hatch rate) declines after exposure to 40°C (Chapter 3.3.5), and why male reproductive output also declines following heat-wave conditions, including an increase in the number of males that failed to produce any offspring following 42°C heat-waves (Chapter 3.3.2).

Anecdotally, it was also obvious during microdissection that the sperm mass within spermatophores of males exposed to heat-wave conditions typically contained large amounts of globular detritus, while control ejaculates only contained sperm cells. It was not possible to quantify this detritus as it was held within the sperm mass, and after dispersal was not clearly visible, but its position and appearance was consistent with the possibility that this was degraded and disrupted sperm cell material. Presumably this damage to sperm cells is what led to the reduction in the number of intact cells counted, it may also mean that heat stress reduces the quality as well as the quantity of sperm in males.

What might explain this reduction in sperm count and insemination success? There are several possibilities: damage to spermatogenesis, increased sperm

mortality, damage to sperm function including damage to the flagellum affecting motility or membrane disruption, damage to haploid DNA or damage to seminal fluid proteins. None of these possibilities are mutually exclusive; in the mouse model *Mus musculus* heat shock treatment of testes *in vivo* at 42°C resulted in a lower concentration of spermatozoa with reduced viability and low motility compared with a control of 33°C (Pérez-Crespo, Pintado & Gutiérrez-Adán, 2007). The same experiment also showed DNA integrity of the spermatozoa resulting from spermatocytes was degraded, matching other work by Banks *et al.* (2005). The highest degree of DNA damage was found among spermatozoa resulting from spermatids present within the testis at the time of heat stress (Pérez-Crespo, Pintado & Gutiérrez-Adán, 2007), suggesting earlier stage spermatogenesis to be especially sensitive. My results suggest the cause is most likely spermatozoal, but DNA damage is also implied due to the reduction in hatch rate and the transgenerational effect on pupal eclosion rates shown in chapter 3.3.6, which points to haploid DNA damage that might manifest itself during zygote development and metamorphosis. In chapter 6, I look to see whether there is a transgenerational loss of fertility in sons whose fathers were heat stressed, which would further indicate damage to DNA or to epigenetic modifiers.

Separating out these intimate gamete-level mechanisms is unfortunately beyond the scope of the present work, but future research would benefit from examining the effects of heat stress on: sperm longevity; sperm viability; sperm length; spermatogenesis as separate from the effects on mature sperm, by using immature males before they have their full contingent of mature sperm; DNA quantity; DNA structural damage and seminal fluid proteins composition. I discuss these further with outlines of methodology in chapter 8.

The results of this chapter provide strong evidence that effects on sperm are the main cause of the male-specific heat-induced fertility-loss phenomenon, and that male mating behaviour could be a contributing factor. The past two chapters have therefore demonstrated impacts of heat stress on male reproduction and offspring development in an insect model, delineating effects on mating behaviour, sperm cells and insemination success, zygote development, metamorphosis and offspring production. In the next two chapters I investigate possible responses by females, both in protecting their own reproductive fitness when facing risks of mating with low

fertility males after heat-waves, and whether storing sperm from multiple males also protects sperm in spermathecal storage from heat-wave effects on mated females.

5 Female reproductive responses to male infertility following heat-wave conditions

CHAPTER SUMMARY

Although previous chapters demonstrate that females are resistant to reproductive constraints following heat-waves, they may suffer reductions in output as a consequence of mating with less fertile, heat stressed males. In this chapter, I assess whether females can rescue their own infertility when mating with heat-stressed males by mating polyandrously. From a post-copulatory perspective, I examine if sperm in female storage are susceptible to heat-wave damage, and whether storage of sperm from multiple males can reduce this damage. From a pre-copulatory perspective, I examine whether females adjust their remating behaviour following fertility-reducing heat-waves acting on males or sperm in storage, to assess whether polyandry acts as a fertility-improvement mechanism in the context of thermal stress.

I find evidence that polyandry can rescue female fertility that has been limited by reduced male fertility from heat-stress. Mating with five males that were exposed to heat-waves allows females to rescue their reproductive output back up to levels achieved with one or five control males. Separately, I find that heat-waves applied to pre-mated females damage sperm in female storage, but this was regardless of the number of males a female has previously mated with (and hence of the diversity of stored sperm). Finally, although it is clear that forced polyandrous mating can avoid male infertility following heat-waves, when given the option in behavioural assays I find that females do not facultatively adjust their remating behaviour after exposure to males or sperm that have suffered heat stress.

Contributors: Alyson Lumley, Paul Holman and Nick Coleman assisted with implementation and data collection for all polyandry and remating assays.

5.1 Background

Polyandry was proposed as a possible insurance mechanism against male infertility more than 20 years ago (Sheldon, 1994). Experimental work with *T. castaneum*, for example, has shown that it is advantageous for females facing direct risks of reproductive damage from inbreeding to mate promiscuously, somehow

increasing their own reproductive fitness by promoting male-male competition or opportunities for female choice (Michalczyk *et al.*, 2011b).

Polyandry has both potential benefits and potential costs, which can be direct or indirect (Martin & Hosken, 2003). Mating with multiple males could provide females with an opportunity to obtain direct benefits from several males, and/or to obtain indirect genetic benefits by allowing sperm competition and the opportunity for cryptic female choice (Edvardsson & Arnqvist, 2000; Bloch Qazi, 2003; Fedina & Lewis, 2008; Pitnick & Hosken, 2010). Polyandry may cost the female primarily because courtship and copulation takes time and energy, potentially exposing females to a range of risks including predation (Pitnick & Hosken, 2010), toxic seminal fluid products that have evolved as a result of male:male competition (Bernasconi & Keller, 2001), or from catching diseases carried by the male (Bernasconi & Keller, 2001; Pitnick & Hosken, 2010). In *T. castaneum,* polyandry may be beneficial for females because their sons inherit the genes for being superior offensive sperm competitors when second males in sperm precedence experiments, but the same genes may be linked with reduced success when in the defensive first male sperm precedence position (Bernasconi & Keller, 2001; Martin & Hosken, 2003), so there may be intricate balances throughout the reproductive process. Whether the costs of polyandry outweigh the potential benefits will likely vary between taxa, and even between populations within species (Taylor *et al.*, 2008).

Mating behaviour has been studied in some detail in *T. castaneum*. Because of their high promiscuity, there appears to be little pre-mating discrimination among potential mates by either sex. However, during mating, *Tribolium* females *'reject spermatophore transfer and limit sperm numbers transferred by males with low phenotypic quality'* (Fedina & Lewis, 2008). This female assessment is probably dependent on a mix of male olfactory attractiveness (Ming & Lewis, 2010) and certain behaviours that males perform that encourage the female to acquiesce to copulation (Edvardsson & Arnqvist, 2000). Mounted male *Tribolium* have been observed to produce a rubbing movement with up to four of their legs moving distally along the female elytra (Bloch Qazi, 2003), which is theorised to stimulate setae on the female's abdomen (Sokoloff, 1972-78). This leg rubbing is likely a signalling strategy evolved by males to induce quiescence in females allowing males to stay mounted for longer (Bloch Qazi, 2003). The increase in latency to mate, and the reduction in the number of copulations observed for stressed males in the previous chapter (Figures 4.2 and

4.5), suggests they are less successful at inducing female quiescence, in this chapter I look at whether females assess heat-stressed males to be of lower quality, possibly via their reduced mating activity. In polyandrous species, mating success does not equal fertilization success (Parker, 1970), but since this cessation in female movement is believed to correspond to internal events such as spermatophore transfer, male leg-rubbing performance may be used by males to increase their chances of fertilisation, and by females to exercise cryptic female choice (Edvardsson & Arnqvist, 2000; Bloch Qazi, 2003). Indeed, Bloch Qazi (2003) demonstrated that female quiescence determines sperm fate. The pathway to spermathecal storage is also preceded by a complex of long, twisting tube which sperm cells must navigate through to reach storage, where they remain until released to fertilise oocytes at oviposition (see Figure 5.3).

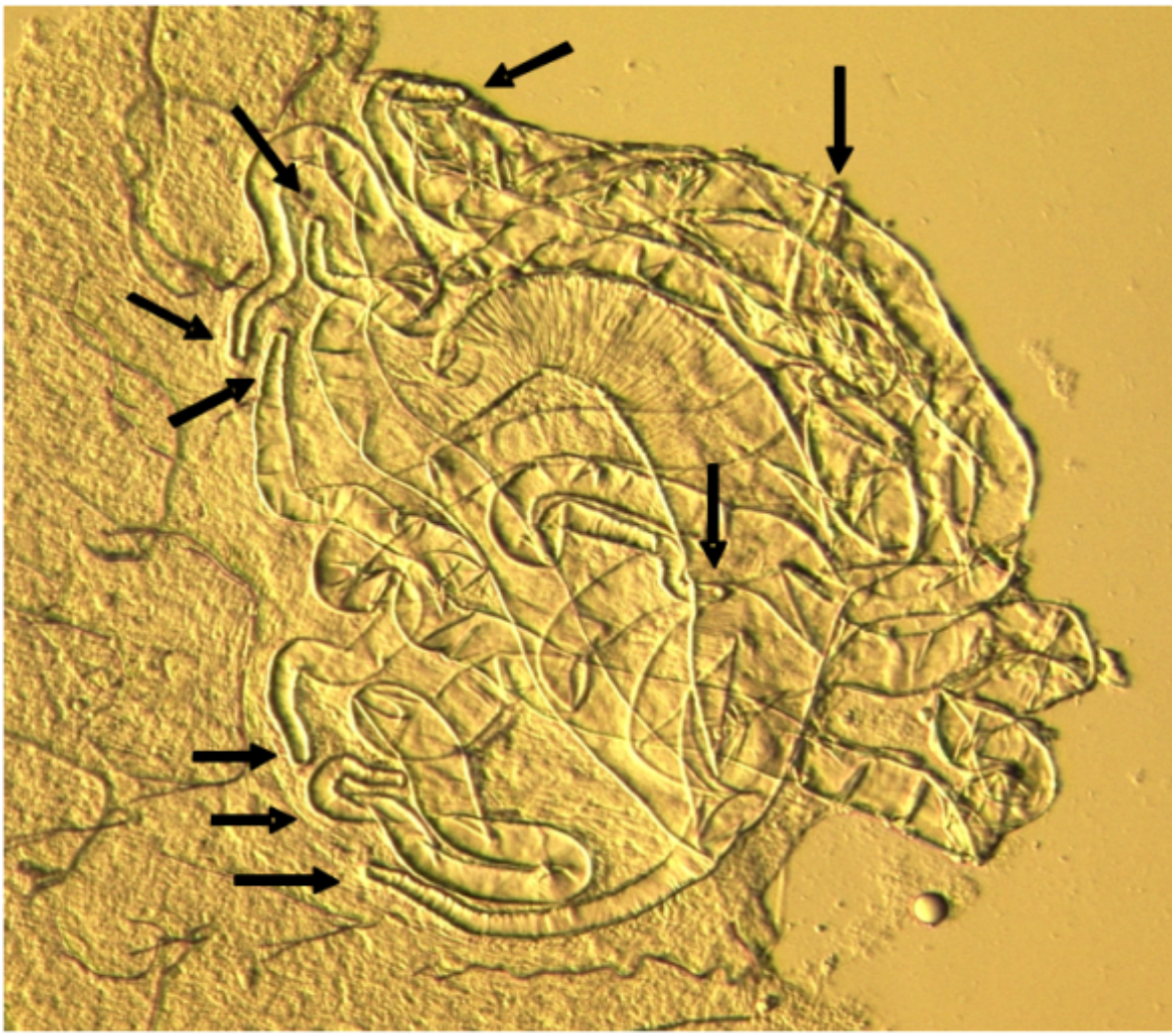

Figure 5.1 Light microscope image showing spermathecal complexity in *T. castaneum*. Arrows identify nine blind-ending tubes or sheaths, within which sperm cells reach longer-term storage from the bursa where spermatophores are deposited. Females vary in the number of

sheaths and therefore spermathecal complexity and storage space (40x magnification. Information and photo credit: Michalczyk, 2008).

These factors create a conflict between the sexes, with males trying to entice females to mate, and females resisting and exercising choice. Female multiple mating leads to sperm competition between males as the haploid cells vie, post-copulation, for fertilisation success. In *Tribolium castaneum,* the last male to mate in a sequence typically achieves highest sperm precedence, around 80% (Arnaud, Gage & Haubruge, 2001), generating an incentive for males to mate with previously inseminated females, and for females to remate if subsequent males are more desirable than previous mates. Past research shows an effect of sperm-deficiency and heat stress on remating behaviour in other species. Females remate more frequently when mated with sperm-deficient males in red garter snakes (*Thamnophis sirtalis parietalis*), although fewer matings take place overall (Friesen, Uhriq & Mason, 2014). In the predatory mite (*Neoseiulus barkeri*), the distribution of copulations across time was affected by heat stress, with sex-specific variation: 29% of matings occurring later when heat stress was imposed only on males, and 84% of matings were delayed when females or both sexes were stressed (Zhang *et al.*, 2016). There is therefore some evidence that females can drive mating behaviour following stress, and here I explore in this promiscuous species whether females mate strategically to bias fertilisation success away from heat-stressed, low fertility males.

In my first set of experiments, I showed that female *T. castaneum* suffer no significant loss of reproductive output when they endure thermal stress (Chapter 3.3.1). However, in populations exposed to heat-waves, female fertility will be curtailed indirectly if their mating options are limited to heat-stressed males who suffer a 50% decline in their own offspring production, which I show in chapter 3 is mainly the consequence of male infertility. It seems likely that females cannot recover their own fertility by mating multiply with the same male: in the reproductive output assays in chapter 3, males are given 48 hours to mate with females, and my results in chapter 4 show that heat stressed males copulate long enough to transfer sperm on average 5 times in a single hour (Chapter 4.3.3). In this chapter, therefore, I investigate whether female mating behaviour can protect against male infertility following heat-wave conditions.

There will be strong selection for females to counter any fertility losses with either plastic and/or evolutionary responses; one option is for females to rescue their fertility by mating with multiple males. In this chapter I test whether polyandry can rescue fertility and whether females modulate their polyandry as a plastic response to elevated risk of infertility under heat-waves.

In this chapter, I examine whether such mechanisms exist when the loss of reproductive fitness is not genetic, but environmental. In this regard, the requirement for fertility rescue may vary throughout a female's lifespan, depending on when heat-waves might occur (by contrast within an inbred population where reproductive depression may be constant within a generation).

I explore whether mating with multiple males allows females to regain their fertility through two experiments. The first looks at whether females can rescue fertility through pre-mating mechanisms when reproducing within a population of thermally-treated males, by allowing mating with a single male (as per the reproductive fitness assay in Chapter 3) versus mating with five, heat stressed males. The second experiment examines post-mating possibilities, by comparing female reproductive fitness, whether they are heat stressed with sperm in storage from single or multiple males. This second experiment also provides information on whether sperm are damaged in storage, an important consideration for insect species where it is commonplace to store sperm after mating(s) in specialised spermathecae. In another set of experiments, I then examine whether females have evolved plastic responses to males who have suffered heat-wave exposure, and measure variation in female remating behaviour.

To determine whether female infertility can be rescued by polyandry, I provide females with mating access to either one (monandry) or five (polyandry) males in two experiments. In the first experiment, half the treatments use males from thermally-stressed backgrounds (as in Chapter 3), comparing offspring production with control, non-stressed treatment males; within either treatment, females are mated to one or five males. This experiment will show whether rescue can occur, and whether females benefit by mating with a wider pool of males, some of which may have differential resilience to heat stress. I refer to this experiment as fertility rescue following pre-copulatory heat stress, as males are stressed before exposure to females and before copulation takes place.

The second fertility rescue experiment measures offspring production when females are mated to one or five control (non-stressed) males, and half the females are then exposed to heat-waves with sperm in storage, or kept under control conditions. This experiment will provide information on whether sperm is damaged in storage and determine, albeit indirectly, if female polyandry can improve or protect the amount of fertile sperm in storage after thermal stress. I refer to this as post-copulatory fertility rescue.

To compliment the investigation of the effects of polyandry under thermal stress on reproductive output, I next measured whether females compensate for low anticipated male fertility by facultatively changing their mating behaviour depending on the thermal stress history of the males they have been previously paired with. I therefore ask whether female *T. castaneum* can detect the low potential fertility of their previous mates, and are increasingly motivated to re-mate as a consequence. The first set of experiments investigate whether polyandry can allow fertility rescue to take place, and this second set looks at whether it females vary their polyandry facultatively to protect their own reproductive fitness.

I examine whether female remating behaviour becomes more polyandrous across two experiments: first, when given a sequence of two males that have been heat stressed or not, and second when given the opportunity to re-mate after females themselves have been heat stressed, or not. The first experiment tests whether females can assess male reproductive 'quality' following the male's heat treatment, and the second, whether females can assess the quality of sperm in storage following heat stress, and re-mate preferentially to promote fertility as a response.

In the first remating behaviour experiment, I will test the prediction that female mating behaviour favours polyandry to compensate against infertility of the first male, if he has suffered exposure to heat-wave conditions that will reduce his fertility. I measure female motivation to mate with a second male, who is either also heat-stressed or has normal fertility. If mated by a low fertility, heat stressed male, females should be keener to remate than if the first male was an unmanipulated control with normal high fertility, and who will successfully transfer a full complement of functional and fertile sperm. In addition, I hypothesise that the condition of the second male could also change female mating preferences: if the

second male is also heat stressed, females could be less keen to remate, or she may compensate for both males possessing low fertility by mating more frequently. This is the pre-copulatory stress remating assay, as males are stressed before being placed with the females.

In the second remating assay I will test the prediction that female mating behaviour favours polyandry and remating if sperm are damaged by heat-wave conditions in female storage. If sperm are damaged in female storage by heat-waves, females should be more eager to remate with additional males. Females could also change their mating behaviour to compensate for suffering thermal stress themselves. I supplement the behavioural measures in the second assay with a virgin condition, in which mating behaviour is compared between females who have had no prior mating experience before they are exposed to thermal stress. Using virgin females will separate whether remating behaviour changes due to female self-assessment following heat-waves, or whether females assess damage to sperm in storage from a heat-wave condition.

Both assays use the study by Nilsson, Fricke & Arnqvist (2003), who measured the time until copulation after introduction of the second male as a measure of female willingness to remate in *T. castaneum*, combined with methodology from my previous chapter (4.2.1). To ensure all data were collected on the same day, and avoid confounding factors associated with different times and conditions, the recording of mating assays per female was run over 30-minute observation periods, rather than the 60-minute period used in chapter 4. Since all but two males mated within 30 minutes in the previous chapter, this change in recording time should allow relevant measures of remating behaviour. Video recordings were analysed for latency to mate (=copulations lasting longer than 35 seconds), the number of mating attempts, and number of matings where sperm transfer could be assumed, and the duration of the first successful mating.

For ease of description, I will use standard sperm competition nomenclature, referring to the first male as the defensive or P1 male, the second as offensive or P2 male. I will continue referring to mating events that last more than 35 seconds as 'successes', indicative of sperm transfer, and those equal to or less than 35s as 'attempts' as explained in chapter 4.2.1.

5.2 Methods

5.2.1 Fertility rescue: pre-copulatory stress

To account for losses due to natural mortality rates and deaths caused by heat-waves, 900 males were sexed as pupae, and after reaching 10 days post-eclosion maturity, a third were kept at control and two-thirds at 42°C for five days (see Chapter 2.2 for details of sexing at the pupal stage and for methods relating to heat stress, including sample size adjustments for different stress temperatures). Following 24 hours post-heat-wave cool-down, males were placed with control, virgin females, either singly in a microcentrifuge tube, or in a Petri dish in groups of 5, for 48 hours. The males given access to females were either all heat-treated at 42°C, or 30°C control, giving four mating regime treatments: 1 x 30°C control male; 5 x 30°C control males; 1 x 42°C treated male; 5 x 42°C treated males. Offspring production to adult emergence was then measured following reproduction and mating over 10 days, which encompassed ~26% of total offspring production (see Chapter 2.1.3).

5.2.2 Impacts of post-copulatory thermal stress on sperm in storage

Female *T. castaneum* were mated to one or five control males for 48 hours, in microcentrifuge tubes or Petri dishes, as in the rescue assay above. Half of the females from each breeding regime were then treated post-copulation at 42°C for five days, the other half were kept at control temperature, generating four regimes: 1) monandrous mating and females exposed to 30°C control; 2) polyandrous mating and females exposed to 30°C control; 3) monandrous mating and females exposed 42°C heat stress treatment; 4) polyandrous mating and females exposed to 42°C heat stress treatment. Thermal stress of the females was timed to ensure they occurred at the same age (36 days from hatch) as the male stress in 5.2.1 when they were exposed to heat-wave conditions. Offspring production to adult emergence was measured over 10 days following heat-wave exposure.

5.2.3 Remating: pre-copulatory thermal stress

Groups of males were exposed to simulated heat-waves at 42°C for five days, together with a control kept at 30°C. After 24 hours recovery time, half of each group

were mated to virgin control females in monogamous pairs in a microcentrifuge tube for 48 hours. Immediately following this, the females were taken and placed in a mating arena (see Figure 4.1) with a second virgin male from the remaining half of the treatment groups. Each female was filmed for 30 minutes with the new male, which was either 30°C control or 42°C thermally treated, producing a full factorial design. For more information on methods regarding the preparation of beetles for, and employment in, behavioural assays and on data collection from video recordings see chapter 4.2.1.

Sample size was ~20 for each of the four regimes: 1) both males 30°C control; 2) first male 30°C control, second male 42°C treated; 3) first male 42°C treated, second male 30°C control; and 4) both males 42°C treated. Data for unsuccessful mating attempts, for males that did not mate at all during the 30 observed minutes, and for those that started but did not finish within the 30 minutes (so total duration could not be calculated) were discarded.

The latency of females to engage in the first copulation with successful sperm transfer (>35 seconds) was used to measure female motivation versus resistance to mate, rather than the latency to a mating attempt. Attempts may be a fair measure of male mating motivation (as in the previous chapter), but successful matings >35 seconds are better measures of female motivation to mate. Although the number of attempts and copulations provide information about female behaviour, it is not clear whether a male mating attempt signals female resistance or acceptance of males. Duration of the first successful mating therefore remains a relevant measure of mating motivation, as well as latency to the start of that event.

5.2.4 Remating: post-copulatory stress

A series of virgin control females were given mating access to virgin, control males for 48 hours, and a second group of females remained virgins. Half of the females from either group were then exposed to heat-wave conditions at 42°C for five days. Mating behaviour of these females from all four groups were then recorded over 30 minutes with a second male, all of which were control. As above, the recording of mating behaviour was collected on the same day and analysed by the same person, and all other methodology regarding filming and collecting data remained the same as

in the first remating assay, with the exception that more time passed between the first and second matings in the current assay, which was the time when half the females were exposed to heat-wave conditions.

The experimental designs used in this chapter feature 2 variables (factors) and 2 levels within each variable. Two-way, univariate analyses of variance (ANOVAs) were therefore applied to explore differences between treatments and regimes, with breeding regime and stress temperature as independent variables, and offspring production as the dependent variable where possible (i.e. where parametric assumptions held). However, this does not allow pairwise comparisons, since *post-hoc* analyses such as TUKEY testing can only be used on designs with 3 levels of more (Fowler, Cohen & Jarvis, 1998), preventing pairwise comparisons. To ensure as much information as possible was gathered from the results, a second one-way ANOVA (or non-parametric equivalent) was run to allow *post-hoc* pairwise comparisons. These rearranged the test treatments into 1 factor with 4 levels to identify specific differences between the combinations of breeding regime and thermal stress if the ANOVA yielded an overall significance value less than $p = 0.05$. Where non-parametric tests were required only a KRUSKAL-WALLIS test with pairwise DUNN'S tests were carried out, since there is no non-parametric equivalent of a two-way ANOVA. When Bonferroni correction is applied to parametric tests the standard α-level of 0.05 was divided by 8 (= 0.00625), to account for the two ANOVAs plus 6 pairwise comparisons, for non-parametric tests the α-level was divided by 7 (0.00714)

5.3 Results

5.3.1 Fertility rescue: pre-copulatory stress

The data distributions in offspring number following different mating treatments were non-normal ($D = 0.893$, $p < 0.001$) and transformed by x^2 ($D = 0.056$, $p = 0.069$). Data were homoscedastic ($F_{3,234} = 1.351$, $p = 0.258$). A two-way ANOVA with male temperature and female mating regime as independent factors, gave a significant effect of temperature ($F_{1,234} = 2.963$, $p = 0.008$) and a significant interaction ($F_{1,234}=11.265$, $p < 0.001$), but no effect due to the number of males ($F_{1,234} = 2.327$, $p = 0.128$), Figure 5.2. Pairwise TUKEY comparisons showed that there was no statistical difference in reproductive fitness between monandrous and polyandrous treatments,

when reproducing with males that had not experienced a heat-wave (Mean Difference (M) = 2362, Standard Error (SE) = 1824, p = 0.567), but that females mated polyandrously to males exposured to a heat-wave had significantly increased reproductive fitness compared with monandrous matings following male heat stress (M = 6297, SE = 1824, p = 0.004). Moreover, there was no difference between offspring production following polyandrous matings following a heat-wave compared with females mated with either monandrous control males (M = 252.5, SE = 1817, p = 0.999) or with polyandrous control males (M = 2109, SE = 1824, p = 0.655). These findings show that the polyandry treatment when mating with heat-stressed males allowed females to recover their reproductive fitness up to normal control levels, indicating that fertility rescue through polyandry is possible.

When Bonferroni correction was applied the effect of temperature in the two-way model no longer significant, but the interaction between temperature and mating regime and the pairwise comparisons remained significant.

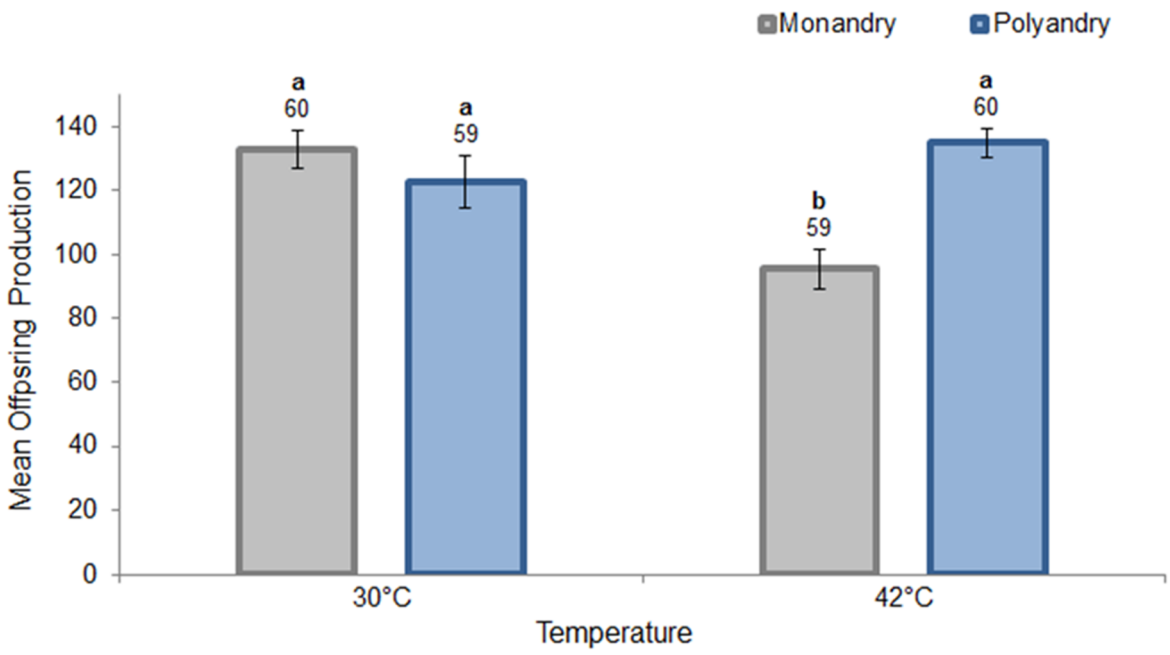

Figure 5.2 Polyandry can rescue heat-induced fertility reduction in females to control levels over 10-days offspring production. Females mated to one or five, control or heat-wave treated males for 48h. Error bars show standard error about the mean. Sample sizes and homogenous subsets are displayed above data points.

5.3.2 Impacts of post-copulatory thermal stress on sperm in storage

Data were normally distributed (D = 0.049, p = 0.200) and homoscedastic ($F_{3,161}$ = 0.798, p = 0.497). A two-way ANOVA between female temperature and mating regime gave only an effect of female treatment temperature ($F_{1,161}$ = 72.164, p < 0.001), with no effect of the mating pattern treatment ($F_{1,161}$ = 0.544, p = 0.462) and no interaction ($F_{1,161}$ = 0.383, p = 0.537), Figure 5.3. *Post-hoc* TUKEY testing showed that there was a significant loss of fertility for heat stressed females mated monandrously, compared to control 30°C monandrous matings (M = 44.46, SE = 8.102, p < 0.001) and control 30°C polyandrous females (M = 52.11, SE = 8.356, p < 0.001). However, unlike the experiment above where males were heat-treated before mating either polyandrously or monandrously (5.3.1, above), heat-wave treatment of females after polyandrous mating also caused a significant reduction in offspring production compared to both monandrous 30°C control females (M = 43.79, SE = 7.592, p < 0.001) and polyandrous 30°C control females (M = 51.45, SE = 7.862, p < 0.001). Indeed, there was no difference in offspring production between heat-stressed females after monandrous or polyandrous mating (M = 0.669, SE = 8.488, p = 1.000), indicating that fertility rescue did not take place under polyandrous sperm storage. As in the first rescue assay, there was also no difference between breeding regimes when females were not exposed to heat-waves but kept in 30°C control conditions (M = 7.655, SE = 7.443, p = 0.733). There was no change in the significance level of the results when Bonferroni correction was applied.

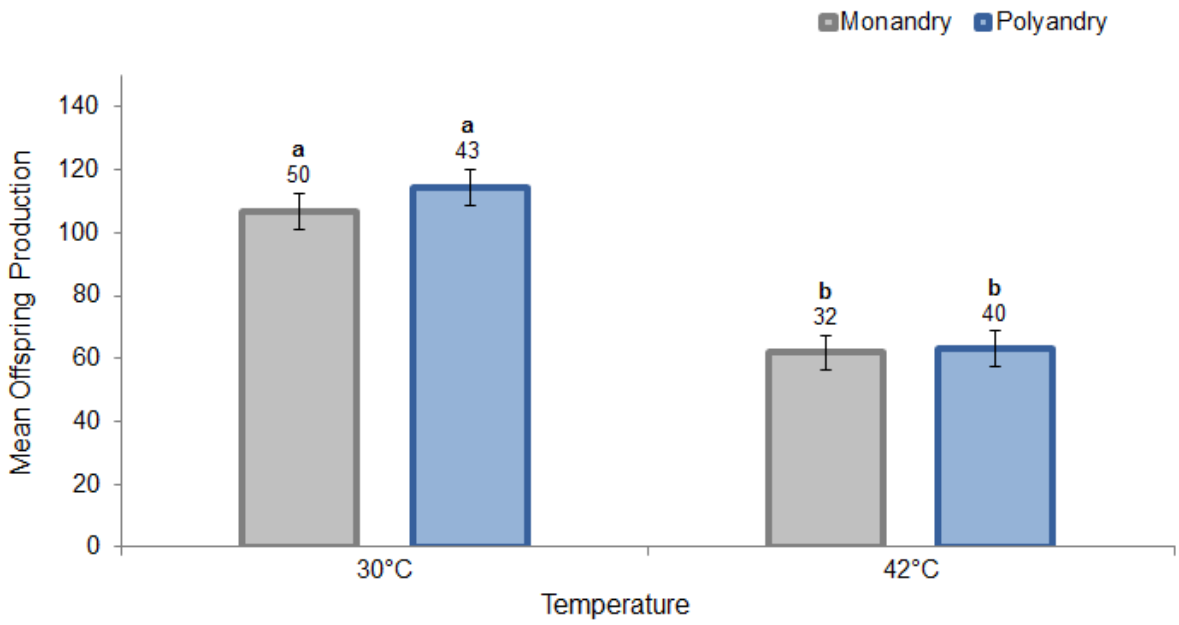

Figure 5.3 Post-copulatory impacts of heat-waves indicates that sperm in spermathecal storage are damaged, regardless of the female's previous mating pattern. Females mated to 1 or 5 males for 48h before treatment. Offspring production measured over 10-days of oviposition post-mating. Error bars show standard error about the mean. Sample sizes and homogenous subsets are displayed above data points.

5.3.3 Remating: pre-copulatory thermal stress

Latency to first successful mating

Data were non-normal (D = 0.269, p < 0.001) until Ln(x) transformed (D = 0.095, p = 0.092), data were homoscedastic ($F_{3,71}$ = 2.129, p = 0.104). A two-way ANOVA with P1 and P2 temperatures as independent factors showed that latency to mate was significantly increased if the P2 male had been exposed to heat-wave conditions, ($F_{1,71}$ = 36.247, p < 0.001), but the temperature the P1 male was exposed to did not affect the female's subsequent latency ($F_{1,71}$ = 0.050, p = 0.825), Figure 5.4. There was also no interaction between first and second male temperature treatments on the latency to mate ($F_{1,71}$ = 0.072, p = 0.783). Pairwise comparisons gave differences if the P2 male experienced a different heat stress level, but not if the P1 males differed. There was no change in the significance level of the results when Bonferroni correction was applied.

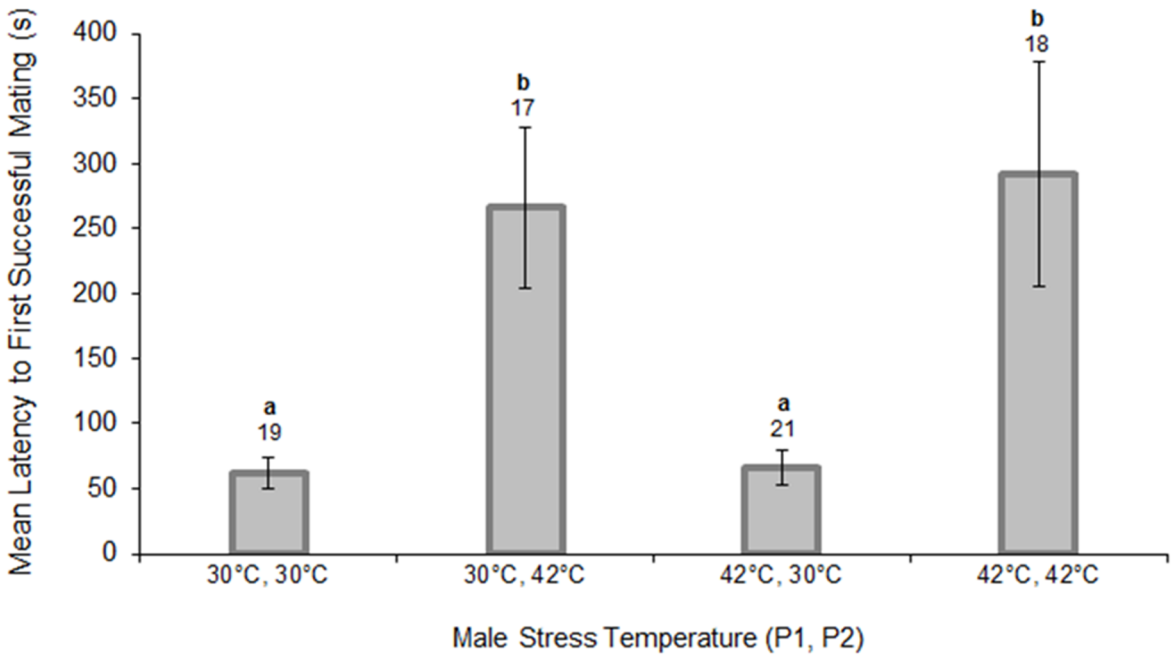

Figure 5.4 Latency for females to re-mate with a second male is not dependent on heat-wave condition of the first male, but depends on current (second) male treatment condition. Females mated to control or treated males for 48h, recorded with second male for 30 minutes. Error bars show standard error about the mean. Sample sizes and homogenous subsets are displayed above data points.

Duration of first successful mating

Data were non-normal (D = 0.185, p < 0.001) and transformed by Ln(x) (D = 0.099, p = 0.065), data were homoscedastic ($F_{3,71}$ = 1.099, p = 0.355). A two-way ANOVA with P1 and P2 temperatures as independent factors gave a significant effect of the second male's treatment (Figure 5.5), with duration increasing for females that received a heat-wave treated male following a control male ($F_{1,71}$ = 4.810, p < 0.001). There was no effect of first male temperature treatment on female remating behaviour ($F_{1,71}$ = 1.616, p = 0.208), with no significant interaction ($F_{1,71}$ = 3.610, p = 0.06). *Post-hoc* pair-wise TUKEY tests showed significant results only between the shortest mean duration, when females mated to stressed P1 males then control P2 males, versus the longest duration for the reciprocal cross (control followed by stressed males) (M = 0.732, SE = 0.258, p = 0.029), this is not significant if Bonferroni correction is applied.

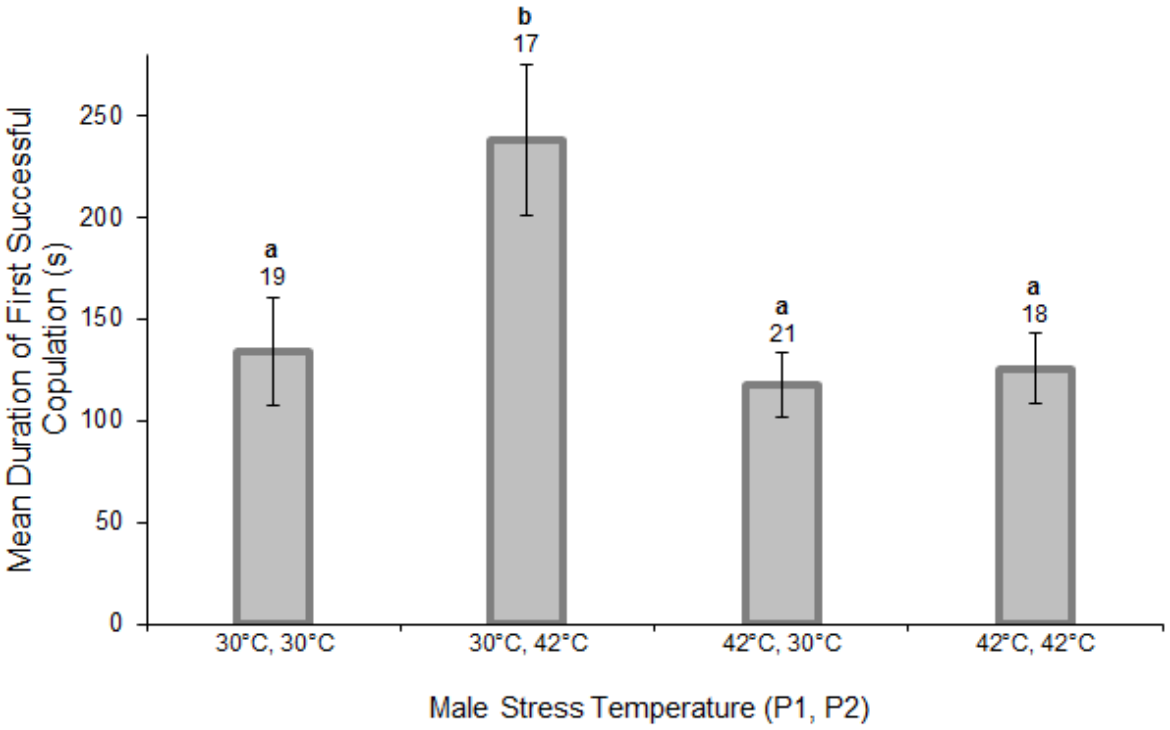

Figure 5.5 Females with prior exposure to control males remate for longer with second males that had been thermally treated. Those with previous exposure to heat stressed males do not have a lengthened copulation duration, indicating that females are less keen to mate with a stressed male a second time. Females mated to control or treated male for 48h, recorded with second male for 30 minutes. Error bars show standard error about the mean. Sample sizes and homogenous subsets are displayed above data points.

Number of mating attempts

Data were non-normal (D = 0.832, p < 0.001) and homoscedastic ($F_{3,81}$ = 0.120, p = 0.948), they were transformed by $\sqrt{x}$ (D = 0.078, p = 0.200) and a two-way ANOVA with P1 and P2 temperatures as independent factors was run. As shown in Figure 5.6, there was a significant decrease in the number of mating attempts if the second male had been exposed to heat-wave conditions ($F_{1,81}$ = 21.488, p < 0.001), but no change in mating behaviour due to the condition of the first male ($F_{1,81}$ = 0.180, p = 0.672) and no interaction ($F_{1,81}$ = 1.249, p = 0.267). Pairwise comparisons gave no significant difference between the two conditions with control P1 males (M = 1.215, SE = 0.497, p = 0.077), but otherwise showed significant differences only when the treatment status of the P2 males differed. When Bonferroni correction is applied the treatment with two control males is no longer significantly different from the treatment with two stressed males.

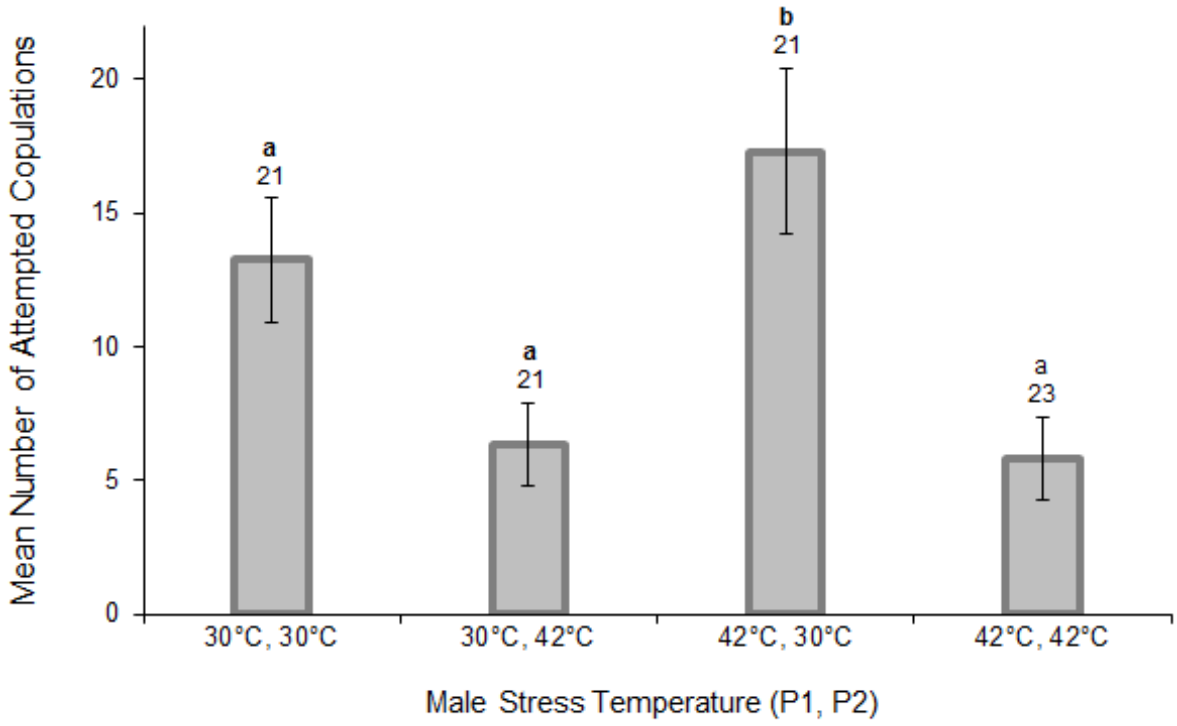

Figure 5.6 Male frequency of copulation attempts are not affected by the heat-wave condition of the first male, only by the current (second) male condition. Females were mated to control or heat-treated males for 48h, and then recorded with the second male for 30 minutes. Error bars show standard error about the mean. Sample sizes and homogenous subsets are displayed above data points.

Number of successful matings

Data were non-normal (D = 0.179, p < 0.001), and so were square root transformed (D = 0.096, p = 0.052); they were homoscedastic ($F_{3,81}$ = 0.132, p = 0.941). A two-way ANOVA with P1 and P2 temperatures as independent factors showed a similar pattern as for the mating attempt assay: a significant reduction in the number of matings long enough for sperm transfer if the second male had received heat-wave treatment ($F_{1,81}$ = 16.574, p < 0.001), but not if the first male had ($F_{1,81}$ = 1.390, p = 0.242), and no interaction between treatments ($F_{1,81}$ = 0.831, p = 0.319), Figure 5.7. Again, there were significant pairwise differences when the P2 males differed in heat-wave status, except between the two treatments with control P1 males (M = 0.607, SE = 0.284, p = 0.151). There were no pairwise differences when P1 males alone differed. There was no change in the significance level of the results when Bonferroni correction was applied.

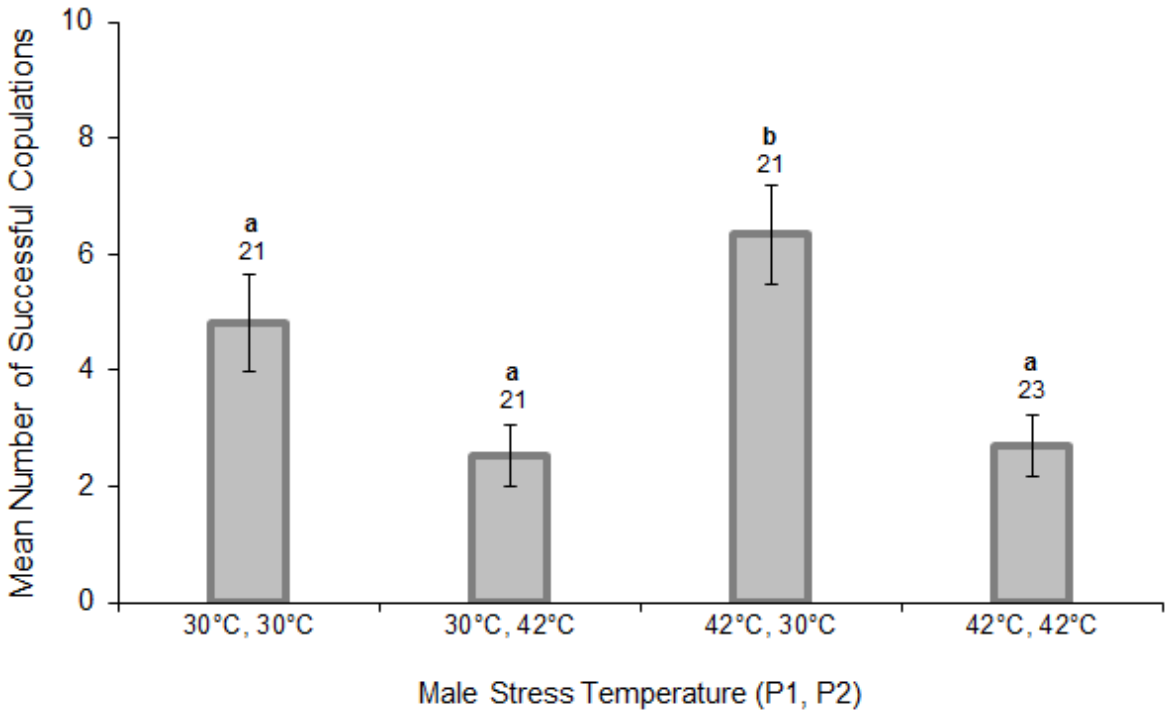

Figure 5.7 Female motivation to mate frequently is not affected by prior male heat-wave treatment, only by current (second) male condition. Females were mated to control or heat-treated males for 48h, and then recorded with the second male for 30 minutes. Error bars show standard error about the mean. Sample sizes and homogenous subsets are displayed above data points.

5.3.4 Remating: post-copulatory stress

Latency to first successful mating

Data were non-normally distributed (D = 0.328, p < 0.001) and attempts to transform them were unsuccessful. A KRUSKAL-WALLIS test gave no significant difference in the distributions ($X^2(3)$ = 0.767, p = 0.857) between any combination of female mating experience and female heat stress temperature, although thermally stressed females took longer to accept males with considerable variation in the latency (Figure 5.8). There was no change in the significance level of the results when Bonferroni correction was applied.

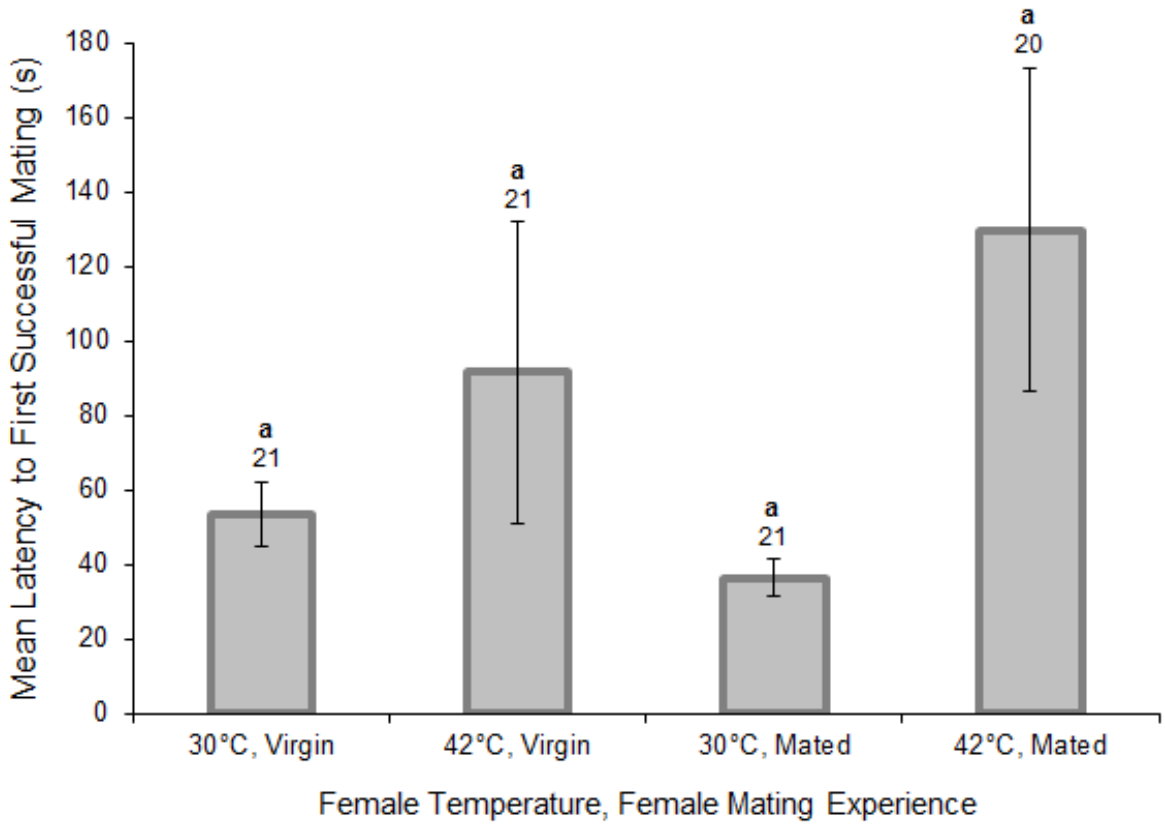

Figure 5.8 Female mated status and previous thermal treatment in relation to latency when mating with a focal male. Virgin or mated females were heat-wave stressed with or without sperm in storage (obtained from 48h mating with control male) and recorded with a control male for 30 minutes. Thermal treatment slowed down latency of the female, but there were no differences between mated versus virgin female status. Error bars show standard error about the mean. Sample sizes and homogenous subsets are displayed above data points.

Duration of first successful mating

Initially non-normal (D = 0.212, p < 0.001) data were transformed by $1/x$ (D = 0.088, p = 0.172) and were homoscedastic ($F_{3,79}$ = 2.144, p = 0.101). A two-way ANOVA with female temperature and prior mating experience as independent factors found a significant effect of temperature stress on mating duration ($F_{1,79}$ = 10.372, p < 0.001), but no effect of prior mating experience ($F_{1,79}$ = 2.897, p = 0.093), and there was no interaction ($F_{1,79}$ = 0.033, p = 0.857). *Post-hoc* pairwise TUKEY tests showed a difference in mating duration between control virgin females and premated, heat stressed females (M = 0.007, SE = 0.002, p = 0.005) only. Surprisingly, differences between heat stressed virgin and premated females are not significant; this is due to wide variation within heat stressed virgin female mating durations. After thermal stress, mated females remated for less time (Figure 5.9). There was no change in the significance level of the results when Bonferroni correction was applied.

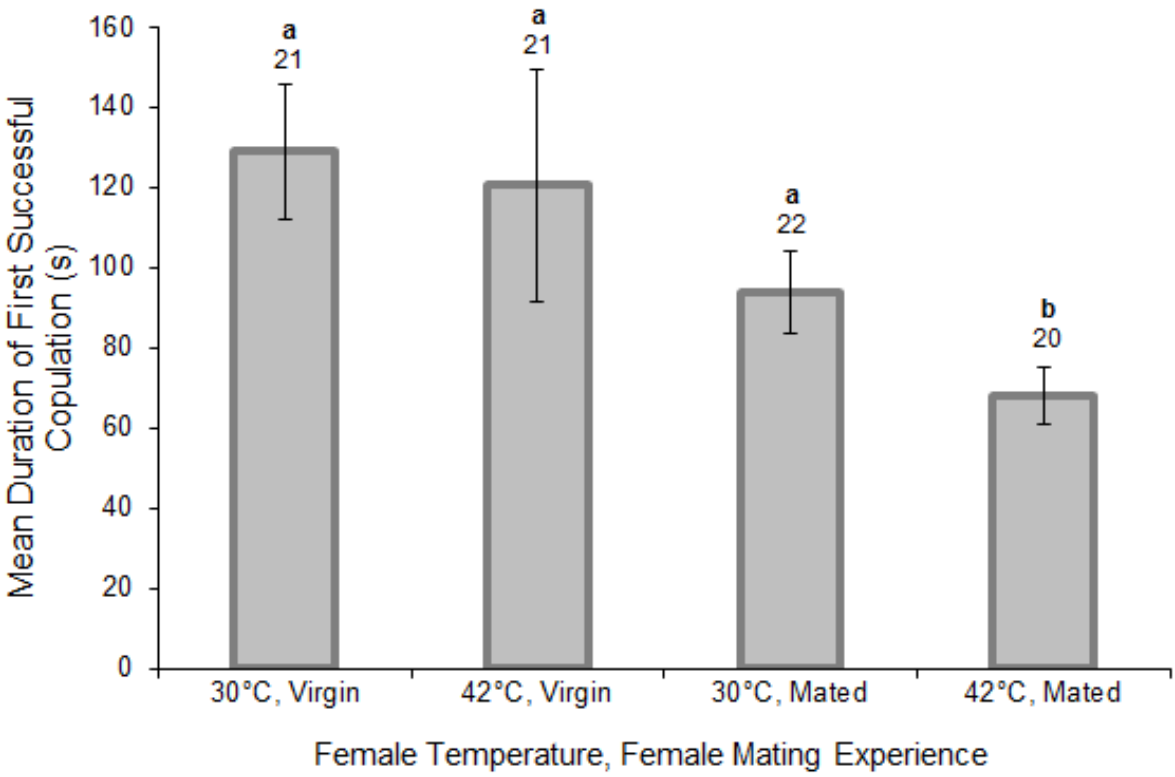

Figure 5.9 Females with prior mating experience mate for shorter durations if they have been previously thermally stressed. Females were heat stressed as virgin or mated status (i.e. with or without sperm in storage, and created following 48h mating periods with a control male) were recorded with a control male for 30 minutes. Error bars show standard error about the mean. Sample sizes and homogenous subsets are displayed above data points.

Number of mating attempts

Data were non-normal (D = 0.129, p < 0.001) and could not be transformed. A KRUSKAL-WALLIS test gave a significant difference between distributions ($X^2(3)$ = 8.536, p = 0.036). Pairwise DUNN'S tests showed a significant difference between heat stressed, virgin females and both control, virgin females (Z = 19.565, p = 0.012) and stressed, mated females (Z = 19.761, p = 0.011), with heat stressed virgin females receiving more mating attempts (Figure 5.10), the converse from when heat stressed males mated with virgin females (see Chapter 4.3.2). With Bonferroni correction none of these results are significant.

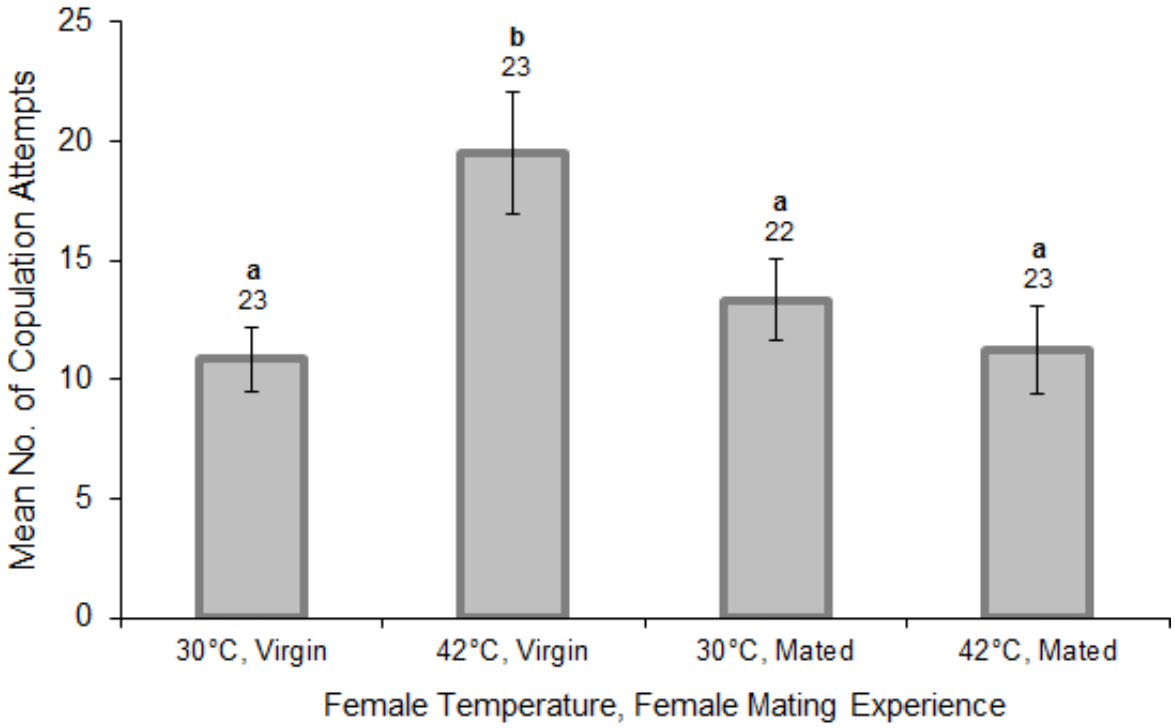

Figure 5.10 Virgin females accept more mating attempts when they have been heat-wave treated. Females stressed with or without sperm in storage (obtained from 48h mating with control male) were recorded with a control male for 30 minutes. Error bars show standard error about the mean. Sample sizes and homogenous subsets are displayed above data points.

Number of copulations with sperm transfer

Data were non-normal (D = 0.129, p < 0.001), and could not be transformed. A KRUSKAL-WALLIS test gave a significant difference between distributions ($X^2(3)$ = 16.33, p = 0.001). Pairwise *post-hoc* DUNN'S tests showed that thermally stressed, premated females engaged in significantly fewer full matings than control, virgin females (Z = 30.239, p < 0.001) and control, mated females (Z = 17.099, p = 0.028). Stressed, virgin females also engaged in significantly fewer matings than control, virgin females (Z = 20.152, p = 0.009). There is an overall trend for heat-wave treatment to decrease the number of successful copulations that were over 35 seconds in duration (Figure 5.11). When Bonferroni correction is applied the latter two pairwise comparisons are no longer significant.

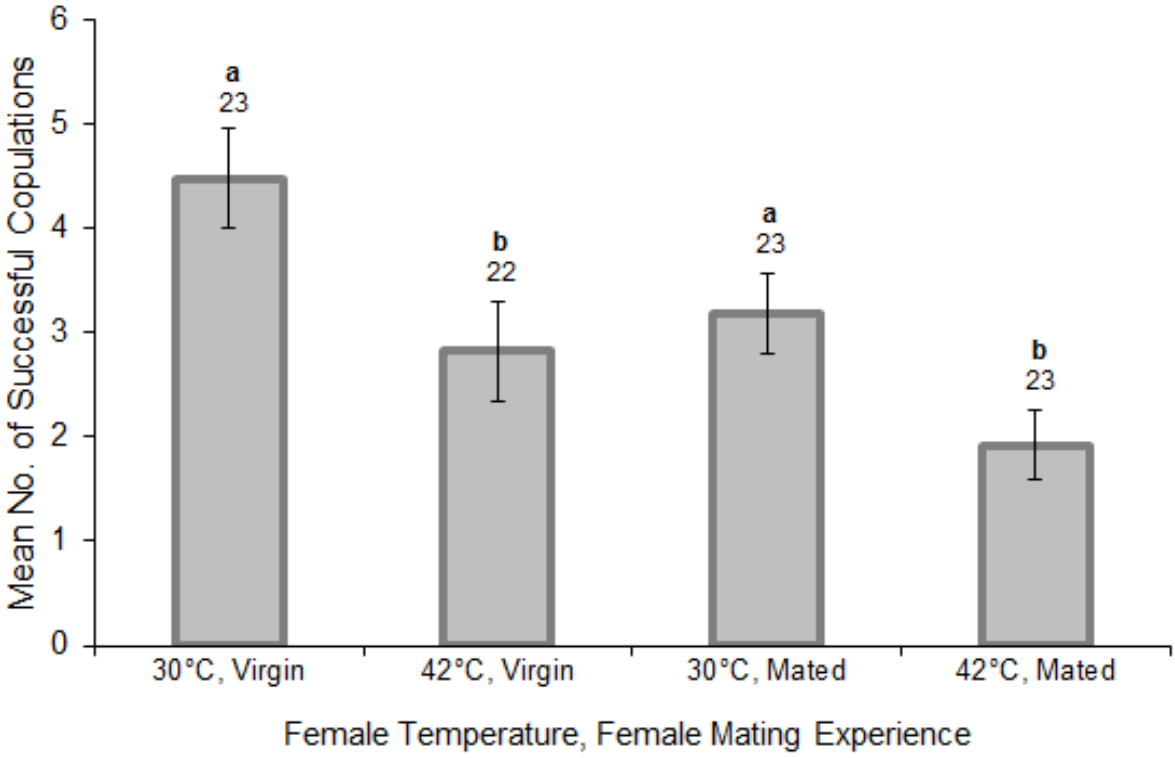

Figure 5.11 Females that have been exposed to heat-wave conditions mate less often, if they have also been previously mated. Females heat stressed with or without sperm in storage (obtained from 48h previous mating access to control males) were recorded with a control male for 30 minutes. Error bars show standard error about the mean. Sample sizes and homogenous subsets are displayed above data points.

5.4 Discussion

5.4.1 Fertility rescue: pre-copulatory stress

The results in this chapter demonstrate that females can use polyandry to protect their own reproductive fitness against male-specific infertility following heat-waves, but that this most likely operates via a mechanism before sperm enter storage, because the second rescue experiment with stress applied to females with sperm in storage showed that monandrous or polyandrous sperm stores showed equal damage from heat stress. Importantly, this second experiment also shows that heat-wave conditions can somehow damage sperm in female storage.

As in chapter 3, I find that males suffer reproductive damage following a heat-wave, with females showing reduced offspring production when mated with single, heat stressed males. However, when I provided opportunities to copulate with up to five heat stressed males, females were able to rescue their reproductive fitness up to

the same levels as females mating with control, non-stressed males. Under control conditions, there was no significant difference in female offspring production when they were given mating opportunities with either one or five males with only a slight (7.6%) drop in the polyandrous condition. This suggests there are few costs to polyandry under control conditions in *T. castaneum*, and under heat stress this small cost of polyandry is removed, indicating the benefits of mating with multiple males far outweigh the costs when females are in a population containing heat-stressed males.

In chapter 3.3.2, thermal stress was shown to reduce the reproductive output of individual males to varying degrees, with ~25% of the males producing no offspring. Fertility rescue could therefore occur because having the option to mate with multiple males will provide a female with an increased chance that one or more males in the 'mating pool' will be able to resist fertility loss (likely by better protecting sperm from damage) from high temperatures. An alternative scenario could be that all males have an equal fertility loss, but the pooling of sperm from multiple matings allows the female to improve the stored population in the spermatheca. That is, if heat stressed males inseminate females with only ~20% of viable sperm on average compared to control males, mating polyandrously could allow females to rescue their fertility either because (1) 20% of males have 100% functional sperm, and polyandry increases their chance of mating with these males or (2) all males have ~20% functional sperm, but mating with five such males allows females to accumulate functional sperm for full fertility. Distinguishing between these possible rescue mechanisms is beyond the scope of the present work, but further work relating to this is discussed in chapter 8. However, the results of the first polyandry assay do add some more, indirect evidence that males vary in the ability to endure heat stress, as previously discussed in chapter 3.1.3.

5.4.2 Impacts of post-copulatory thermal stress on sperm in storage

By contrast with the pre-copulatory assays, I found no evidence that females could protect their reproductive fitness by carrying a polyandrous sperm store. In other words, it appears that it is the action of mating or sperm transport into storage that allows the rescue, rather than a pool of sperm from multiple males already in storage. Interestingly, however, sperm appear to suffer damage within female

spermathecal storage, regardless of how many males' sperm she carries. This confirms further the suggestion from chapter 4.3.5 that part of the loss in male fertility can happen through damage to mature sperm, either in males or in females. Only ~4% of sperm reaches storage in *T. castaneum* (Bloch Qazi, Aprille & Lewis, 1998; Fedina & Lewis, 2008), and females may employ cryptic choice to select best quality sperm (Fedina & Lewis, 2008; Pitnick & Hosken, 2010), perhaps based on their perception of male copulatory courtship behaviour (Edvardsson & Arnqvist, 2000). Since female fertility protection does not take place with multiple males' sperm in storage, it appears that there is either no variation in the ability of sub-populations of sperm to resist heat damage, even within the 4% high quality sperm accepted into storage, or that the female criteria for 'quality' males or sperm does not overlap with qualities pertaining to resistance to damage from heat stress. This latter possibility, that female assessment of male/sperm quality is not based upon male/sperm resistance to heat stress matches the lack of facultative remating also seen in this chapter (see results sections 5.3.3 and 5.3.4 and discussion below). The difference in rescue between the two assays therefore suggests that rescue could either be due to differential male resilience to heat stress, and/or that allowing competition and choice of males and/or their sperm allows females to use polyandry to filter males or their sperm which have suffered less reproductive damage from heat-waves.

The previous chapter showed that far fewer sperm cells are inseminated into females when males have been heat stressed, so it is unlikely that the difference between the advent of rescue between the two assays is because males are able to protect sperm in their testes better than females can in spermathecal storage. The second rescue assay, featuring post-copulation stress, removes the possibility of spermatogenesis and the need for insemination of stressed sperm, and yet polyandrous treatments still have a heat-induced loss of fertility, so it is likely that rescue was possible in the first experiment because males were able to produce more sperm after stress during the 24-hour recovery and 48-hour mating periods, or that undamaged sperm were somehow filtered better into storage. This may mean some males do have impaired spermatogenesis after thermal stress, explaining why the average productivity drops in the monandrous condition, and could mean the advantage of polyandry is that it increases the chances of mating with a male with greater resistance to thermal stress. However, the evidence for the effects on spermatogenesis are mixed and indirect, and I discuss this in light of potential further work in chapter 8. It can be more clearly inferred that heat stress does not damage

haploid DNA or ability to fuse with the female pronucleus, otherwise rescue could not take place in either assay.

The damage to mature sperm in female storage is an important finding regarding the consequences of climate change on many ectotherm species, in which many store sperm. Rising temperatures could therefore impact on reproduction even after mating has taken place before any stressful heat-wave conditions, and could have major impacts on natural populations such as *T. castaneum* which probably colonise new habitats through females carrying sperm in storage.

5.4.3 Remating: pre-copulatory thermal stress

In general, I do not find evidence that females are able to assess the heat-wave status of either themselves or their previous mating male; females showed no clear indication that they increase mating effort to combat male-specific infertility. This finding is surprising given that initial tests clearly show that females can use polyandry and remating to protect themselves against male-specific infertility following thermal stress. In the first set of remating behaviour assays, with females premated to stressed or control males, and then remating recorded with either stressed or control males, female mating behaviour was unaffected by the male's heat-wave exposure history. There were no significant interactions between first and second male treatment, with most of the clear differences in mating behaviour able to be explained by the second focal male being impacted by heat stress, consistent with previous work for male mating behaviour in Chapter 4, with adjustments for the consideration of females having been premated rather than virgins. For example, the numbers of both attempted and successful matings with mated females were lower across all treatments compared to virgin females (compare Figures 5.5 and 5.6 with Figures 4.3 and 4.4).

There were some differences to the behavioural patterns seen by males in chapter 4, revealed by pairwise testing. When females were premated with heat stressed males, there were more attempted and successful copulations with a control P2 male, compared to when both P1 and P2 males were control (Figures 5.6 and 5.7). Furthermore, copulation duration decreased when both males had been heat stressed, breaking the pattern of increased duration seen with stressed males in chapter 4

(Figure 4.5), and when the P1 male was not exposed to a previous heat-wave (Figure 5.5), showing a possible influence of the first male's thermal and fertility status on the behaviour of the female or the second focal male. All other measures followed the same pattern as when male mating behaviour was recorded following heat-wave conditions in chapter 4.3, with latency increasing, and the number of attempts and successful matings decreasing when the second male is stressed, regardless of the condition of the first male.

It is not clear whether these deviations from the single male behavioural patterns are due to a female influence, but they could be advantageous to female reproductive fitness: if mated to a poor condition, stressed P1 male, a female would benefit from mating with a P2 control male more often and actively. However, the greater number of attempts by control P2 males when they followed a stressed P1 may be due to male-driven behaviour. If there is reduced sperm transfer from the P1 male (as seen in Chapter 4.3.5), the offensive male may then be treating the female as if she is a virgin. *T. castaneum* males have been shown to mate more frequently with females they detect as virgin (Arnaud & Haubruge, 1999), but males may not detect this when females have been mated to a heat-stressed male who did not transfer sperm.

In chapter 4 I found that heat-stressed males mated for longer. It is unclear whether this is due to male or female action but, if females do acquiesce to the first instance of a stressed male for longer, she is not so tolerant with a second stressed male. The fact that the duration when both males were stressed did not increase suggests that females are less willing to accommodate a second, poor quality male who takes a long time to transfer sperm (as in Chapter 4, Figure 4.5). If females are able to detect and assess male condition after heat-stress it could be through inefficient male mating behaviour or condition-dependent attractiveness, such as damage to pheromone production (Ming & Lewis, 2010).

At best, it can be concluded that it is not completely clear that females are keener to re-mate when previously mated to thermally treated males. This may be because they cannot detect the quality of a male and/or his previous ejaculate. Without further compelling evidence, it seems that female *T. castaneum* cannot detect changes in male reproductive quality following thermal stress.

5.4.4 Remating: post-copulatory stress

The second set of remating observations, when virgin or premated females were stressed prior to recording behaviour with a control male, provides even less clarity regarding any changes in female remating behaviour. I have shown that when females are exposed to heat-wave conditions with sperm already in storage, that her subsequent fertility declines, so there is an *a priori* expectation that females should be more motivated to remate with new, perhaps undamaged males, following potential damage to her sperm in storage. The duration and number of successful copulations with sperm transfer were subsequently lower when females with sperm in storage had been thermally stressed, which is the reverse of what would be expected if females compensate for damaged stored sperm. Although not significant, latency also increased when females had been stressed, which was again contrary to expectations of fertility rescue. Other factors do follow some expected patterns: mating durations are shorter when the female had been previously mated, and the number of successful copulations is reduced (regardless of thermal stress treatment). Both of these findings are what would be expected from male behaviour detecting virgin versus mated females, but there were no interactions with the female's heat-wave exposure history. The number of attempts increased when females were virgins and heat stressed, although number of mating successes was lowest within this treatment, and number of mating attempts was lowest when females were virgins and not exposed to heat-waves. If females were compensating for poor males or sperm quality, the stressed, mated treatment would be expected to generate more female remating activity leading to more copulations with sperm transfer so that already-stored and damaged sperm might be superseded by new functional spermatozoa.

Again, it appears that females are not strategic in their remating behaviour; they either cannot detect that stored sperm have been heat stressed, or perhaps the highly promiscuous mating pattern of *T. castaneum* creates a condition where frequent re-mating occurs, irrespective of previous heat-wave damage. Females may have lost pressure to be selective in their remating behaviour after prolonged periods of maintenance in the laboratory under conditions where sperm limitation is highly unusual, and promiscuity is high. Thus, females could have lost any discriminatory behaviour due to very low risks of infertility. The use of the Krakow Super Strain might have controlled for this, as it contains some wild-type genetic background, and it could be compared with the GA-1 strain, which has been in the lab even longer and

might demonstrate even less mating partner discrimination. Use of another species which has more selection pressure to be more discriminatory in its mating behaviour may be more appropriate, such as a butterfly model which does not routinely mate as frequently (Janowitz & Fischer, 2011).

Other studies have found similar results indicating lack of female mating discrimination. In parasitoid wasps (*Nasonia vitripennis*), females did not appear to distinguish heat-stressed from control males, and did not remate more frequently to compensate for the lack of sperm transferred (Chirault *et al.*, 2015). Zhang *et al.* (2016) found no increase in copulation duration in females of the predatory mite (*Neoseiulus barkeri*) following heat stress conditions. Bloch Qazi (2003) summarizes variation in sperm precedence as being explained by relative male body size, male olfactory attractiveness, and male leg rubbing rate during copulation (when perceived by the female) whilst '*no specific female attributes have been related to differences in sperm transfer, sperm storage or second male sperm precedence*' (Bloch Qazi, 2003). It is not perhaps surprising therefore that this chapter provides little evidence that female attributes drive mating patterns after thermal stress.

This chapter showed that when a population has suffered thermal stress, there is a polyandry benefit for female reproductive rescue, but that maintaining a more diverse sperm store does not protect against negative actions of heat-waves upon female reproductive fitness through damage to sperm in storage. There is clearly some beneficial selection through polyandry by filtering competent sperm into storage, or allowing males to compete for mating access. However, once sperm are in the spermatheca, there is no evidence that maintaining sperm stores from a variety of males is of benefit in protecting against reproductive damage from heat stress. The effect of heat on sperm in storage indicates damage can occur to mature sperm.

Rescue *can* take place (Figure 5.1) but since females do not facultatively alter their mating behaviour following their own, or their male mating partners' heat-wave histories, it is questionable whether rescue or protection actually would encourage polyandry to increase spontaneously in a natural environment outside of the forced placement of females with multiple males in the lab. Of course, they may still adapt to become more selective and more polyandrous over time: *T. castaneum* were recently shown to do this to avoid depression or extinction in inbred populations (Michalczyk *et al.*, 2011b). Further work could follow the same experimental design to determine

whether females have evolved heightened polyandry within populations routinely exposed to heat stress across multiple generations.

6 Are there transgenerational effects of heat stress on reproductive fitness?

CHAPTER SUMMARY

This chapter takes a wider view of responses to, and effects of, heat-wave conditions, exploring whether transgenerational impacts of thermal stress occur. Here, I examine whether the negative impacts of heat-wave conditions on male reproductive function are also somehow passed on to influence the reproductive function of sons. Such an effect might be possible if, for example, heat stress causes sperm DNA damage or DNA methylation.

Having exposed T. castaneum fathers to heat-wave conditions, I reared offspring sired by them, and compared their reproductive fitness with sons from control males. I found limited evidence for transgenerational thermal stress effects, with sons of heat stressed fathers producing similar numbers of offspring as sons of control fathers. Under these conditions, therefore, it appears that heat stress does not cause transgenerational reproductive damage.

Contributors: Alyson Lumley and David Hancock assisted with implementation and data collection.

6.1 Background

In this thesis, I have explored how male reproductive biology is impacted by heat-wave conditions, and how females react to the subsequent loss of male fertility following thermal stress. So far, these questions have been focused upon effects and responses within individuals, and within a single generation (their progeny were used as a proxy for fertility, but the f1 generation was not studied in further detail, beyond counting offspring numbers). In this chapter, I investigate transgenerational effects of heat-wave conditions.

I study whether negative impacts of heat stress on males in one generation also passes on to their sons as an additional constraint on reproductive fitness. In chapter 4, I showed that a large reduction in the number of complete sperm cells is

closely linked to temperature-induced male fertility loss (Figure 4.6), and the results of chapter 5 strengthen the importance of sperm function being a major factor by the fact that females stressed with sperm in storage have a reduction in fertility (Figure 5.2), whereas they have no significant loss in subsequent reproductive output when thermally stressed as unmated virgins (Figure 3.1).

Damage to sperm cells could reduce fertility for one or both of two reasons: 1) the cells lose function and are less able to reach and fertilise ova due to structural or biochemical damage, or 2) there is chromatin damage to spermatozoal haplotypic DNA, meaning that the cell can fertilise the egg, but the subsequent zygote or embryo is disrupted. In chapters 3.3.2 and 5.4.1 I discuss how male fertility loss and female fertility rescue via polyandry show variation in male resilience to heat stress. If the route of thermal impact on sperm cells is via DNA damage, it is possible that there may be wider transgenerational impacts of heat-waves on the fitness of offspring in the filial generation. These problems, if they exist, may be likely to show up in the reproductive fitness of a heat stressed male's sons. To test this possibility, I assay whether there is a transgenerational impact of heat stress on fathers that passes through to the reproductive potential of their sons, comparing against the sons of males that were reproduced without any exposure to heat-wave conditions.

From the rediscovery of Gregor Mendel's work at the turn of the last century, and the establishment of the importance of genes in evolution in the new synthesis in the 1920s and '30s, it was believed that environmental changes had only an indirect effect on subsequent generations through natural selection (Heard & Martienssen, 2014). Since the end of the Second World War, there has been mounting interest into whether environmental effects on one generation can influence later generations (Nakamura, 2006, Heard & Martienssen, 2014). Following the first usages of the atomic bomb, for example, especially the one dropped on the populations of Hiroshima and Nagasaki, studies on the children of survivors examined whether the ionizing radiation from nuclear fallout leads to any transgenerational increases in miscarriages, malformation or cancers caused by DNA damage (Nakamura, 2006). Contrary to popular belief, no increase in the incidence of birth problems or illness have been found in a large contingent of offspring studied over many years (Nakamura, 2006). Since then, a large body of literature has shown radiation can cause the kinds of problems across generations that caused concern in Japan (see below), although they seem to occur only at higher doses (Nakamura, 2006).

With the advent of therapies using radiation for cancer, interest in the effects on people and their children increased, and chemotherapies began to be researched for side-effects in the same way (Arnon *et al.*, 2001). Although radio- and chemotherapies used as cancer treatments cause sperm DNA damage (Arnon *et al.*, 2001; Morris, 2002), '*no increase in genetic defects or congenital malformations was detected among children conceived to parents who have previously undergone chemotherapy or radiotherapy*' (Arnon *et al.*, 2001); again, effects in their children have yet to be conclusively shown (Arnon *et al.*, 2001; Nakamura, 2006). In female cancer patients, miscarriage and congenital malformations in their children are not increased following chemotherapy: during the first two weeks after fertilization of the embryo, radiation is lethal but not teratogenic (Arnon *et al.*, 2001). High doses of radiation during pregnancy do affect offspring if exposed *in utero*, inducing anomalies, impairing growth and causing mental retardation, as well as possibly increasing risk of childhood leukaemia and other tumours in the offspring, but these are not seen if conception takes place after treatment (Arnon *et al.*, 2001).

Transgenerational effects of radiation in humans are therefore debateable (Kovalchuk & Baulch, 2008). They are, however, much clearer in non-human animal models, showing that the effects of parental radiation exposure are transmitted to the progeny of the irradiated parent through the germline, and can span several generations (Kovalchuk & Baulch, 2008). Irradiated f0 mice produce elevated mutation rates in the f1 and f2 generations (Barber *et al.*, 2002), and parental exposure of mice to radiation and chemicals causes a variety of adverse effects in the progeny, including increased incidence of tumours, congenital malformations, and embryonic deaths; these tumour-susceptibility phenotypes are also transmissible beyond the first post-radiation generation (Nomura, 2006).

In the half century since radiation became a topic of study, an array of other environmental effectors has been found to elicit transgenerational effects in various organisms, including starvation (Curley *et al.*, 2011), endocrine disruptors (Anway *et al.*, 2005), salt stress (Suter & Widmer, 2013), social isolation (Goerlich *et al.*, 2012), temperature (Lang-Mladek *et al.*, 2010) and exposure to pathogens (Sadd *et al.*, 2005). There is also evidence for a particular effect upon males, for example, developmental toxicology has provided evidence for an association between male exposure to various drugs/toxins and an increased occurrence of DNA damage and mutations, including

'*numerical and structural chromosomal abnormalities, point mutations, copy number variant (CNV) changes, and duplications/deletions of microsatellites*' (Curley *et al.*, 2011).

In this chapter I will look at whether transgenerational effects of thermal stress experienced under heat-wave conditions occur in *Tribolium castaneum* males. The understanding of transgenerational effects has evolved since the first investigations into radiation damage. Research has moved away from effects caused by damage to DNA sequences, with the discovery of non-sequence-based inheritance mechanisms, which can be influenced by environmental variation. Animal and cellular studies, for example, started to indicate that the irradiation of males was leading to observable effects in the somatic cells of their offspring over several generations that were not attributable to the inheritance of a simple mutation through the parental germ line (Bridges *et al.*, 2013).

These non-sequence-based inheritance mechanisms are described as 'epigenetic' effects; responses to environmental conditions that change gene expression, by the addition of regulatory molecules onto DNA sequences. The term epigenetics originates from Conrad Waddington's work integrating the new knowledge about genes and genetics to embryology in the 1940s (Skinner, Manikkam & Guerrero-Bosagna, 2010). The integration of the study of embryological growth and differentiation (commonly known as epigenesis) and genetics gave Waddington the term 'epigenetics' (Skinner, Manikkam & Guerrero-Bosagna, 2010). While focusing on gene-environment interactions, at first it was only concerned with non-genetic changes copied in mitosis within a single generation (Skinner, Manikkam & Guerrero-Bosagna, 2010), described as '*the perpetuation of gene expression and function across cell divisions without changes in DNA sequence*' (Heard & Martienssen, 2014). Since then, the definition has shifted towards the notion of heritability, with the discovery that these epigenetic factors can be passed through cell lineages (Heard & Martienssen, 2014). Thus, epigenetic mechanisms, modulated by environmental cues, can be said to allow a kind of "soft inheritance" permitting adaptation to fluctuating environments and other stresses (Whittle *et al.*, 2009; Heard & Martienssen, 2014), creating a feedback on the fitness of stressed populations (Hauser *et al*., 2011). Responses to environmental conditions in one generation can therefore potentially influence responses to similar conditions in future generations.

It is important to discriminate between copying of established epigenetic marks in mitosis within multicellular organisms, and the inheritance of such epigenetic marks across generations (Hauser *et al.*, 2011). These alterations in gene expression have been reported to persist throughout the life of various organisms (Daxinger & Whitelaw, 2010) and this information can be inherited and expressed in '*at least eight*' generations after initial addition of epigenetic markers, as seen in the model plant *Arabidopsis thaliana* (Johannes *et al.*, 2009).

These new mechanisms can help explain transgenerational effects that had previously remained obscure. Transgenerational carcinogenesis had linked to transgenerational genome instability, which manifests as elevated delayed and nontargeted mutation, but the mechanisms by which it arose were inexplicable (Koturbash *et al.*, 2006). Epigenetics allowed Koturbash *et al.*, (2006) to hypothesize that epigenetic dysregulation in the offspring could lead to genome destabilization, and possibly serve as a precursor for transgenerational carcinogenesis.

When transmitted to filial generations, these epigenetic mechanisms are technically a subset of transgenerational effects. However, as focus has switched to these mechanisms, the terms 'transgenerational' and 'epigenetic' have become linked, and are often used interchangeably. Heard & Martienssen (2014) note that '*The term transgenerational is often used rather broadly to describe all nonsequence-based effects that can be transmitted from one generation to the next*'. Donelson *et al.* (2001) also note that '*influences on offspring phenotype that are not solely due to offspring genotype'* are *'termed transgenerational effects*'. But Carey (2011) gives 'epigenetic' the almost same definition, stating that in its widest meaning 'epigenetic' refers '*to all the cases where the genetic code alone isn't enough to describe what's happening*'. Indeed Little *et al.* (2003) make special note of the fact that the term 'transgenerational' has been used in several differing senses in the literature, but the trend has been for those stable genetic alterations to be overlooked as the focus on epigenetics has meant that for many authors 'transgenerational' *is* 'epigenetics'.

It seems reasonable to consider whether the response is an evolved adaptation that enables rapid passing of environmental information between generations, or if it is simply a form of transgenerational damage that is inherited. Earlier definitions such as by Anway *et al.* (2006) still distinguished between transgenerational effects of the type investigated in early radiation studies, stable genetic alterations (i.e.

mutation, change in the DNA sequence), and those altered via an epigenetic mechanism. In the present work, I will use 'transgenerational' as defined by Little *et al.* (2003) in the broadest sense of inheritance of changes caused by environmental factors, including changes to genetic sequence and non-sequence-based changes, in offspring that were not exposed to the stimulus that caused the change(s) in the parental generation. I will use 'epigenetic' as that specific set of transgenerational effects expressed by Pembrey *et al.* (2006) as '*heritable changes in gene function that cannot be explained by changes in DNA sequence*'.

Transgenerational effects, then, can be genetic or non-genetic. In this chapter, I examine whether a damaging effect on male fertility also passes between generations following thermal stress in f0. Any such effect could be genetic or epigenetic, but establishing the mechanism for any effect is beyond the scope of this thesis, and at this stage my primary aim is to test whether such a transgenerational effect on phenotypes occurs. Indeed, it is not yet clear to what extent genetic and non-genetic/epigenetic transgenerational effects are linked or separate phenomena, and it is unknown if there is a causal relation between '*the persistent elevation of DNA damage and the persistent epigenetic alteration of gene expression*' (Little *et al.*, 2003).

In this experiment, I heat-stressed the f0 generation, and then examined whether any kind of negative (or potentially positive) transgenerational effects could be seen among f1 males, measured through their reproductive fitness in the production of f2 offspring. Below, I give an overview of the body of work that shows why it is worth searching for transgenerational effects of heat stress in *Tribolium*, focused more on the transgenerational effects, rather than any underlying mechanisms that might cause them.

If heat stress causes a transgenerational effect from one generation to affect sons (and, by extension, possibly grandsons and beyond), then effects of heat-waves may not be limited to the few days of higher temperature, but could affect a population into the future for much longer time periods. It is therefore important to determine if such lasting effects occur. Several studies in plants have indicated that stress enhances transgenerational epigenetic effects, increasing the frequency of transgenerational epigenetic effects in unstressed progeny (reviewed in Hauser *et al.*, 2011); although reports in plants are often conflicting (Hauser *et al.*, 2011), the

suggestion that biotic and abiotic stressors have this effect makes transgenerational effects of heat stress worth investigating.

The first to look at transgenerational effects of heat stress was the discoverer of epigenetics, C. H. Waddington (1953), who demonstrated '*genetic assimilation of an acquired character*', as he called it, although it is now known it was likely epigenetic in nature. Waddington's initial experiments were on *Drosophila*, and he found that a temperature shock of 4 hours at 40°C, 17–23 hours after puparium formation, induced a phenotype with cross veinless wings when they eclosed. After an initial reduction of the cross wingless phenotype in offspring, the frequency of the abnormal phenotype rose again after the sixteenth generation (Waddington, 1953).

Since Waddington, most transgenerational effects of heat stress have focused on plants. Being sessile, plants that can rapidly adapt their phenotype may be more likely to survive changing environmental conditions, and therefore there may be greater selection pressure for plants to evolve heritable epigenetic variation that provides a route to achieve rapid adaptation (Suter & Widmer, 2013). The effects of growing plants at a different temperature are also of great economic interest, as such the influence of temperature and season on flowering time is perhaps the most well-known epigenetic environmental cue in plants (Heard & Martienssen, 2014).

In *Arabidopsis thaliana*, exposure to heat stress in previous generations accelerated flowering in the f4 generation under control conditions, and these transgenerational effects were maternally and paternally inherited (Suter & Widmer, 2013). In genetically-identical *Arabidopsis thaliana* lines exposed to mild heat (30 °C) treatment in the parental and f1 generations, there was more than a fivefold improvement in fitness (measured by seed production per individual) when plants were exposed to heat in a later generation (f3), even though the intermediate f2 generation was grown at a control temperature (Whittle *et al.*, 2009). These heat-specific fitness improvements were observed among f3 plants. This temperature-induced adaptive epigenetic phenomenon was not detected for cold-treated plants (16°C), indicating different biological responses to heat and cold shock (Whittle *et al.*, 2009).

These transgenerational effects have been found to have a molecular basis: in *Antirrhinum majus*, growth at 25°C resulted in hypermethylation of Tam3 sequences

in the genome, compared to growth at 15°C which resulted in reduced methylation (Hashida *et al.*, 2003). These methylation states were reversible within a single generation in response to a change to the other growing conditions (Hashida *et al.*, 2003). The most common molecular mechanisms, however, involve heat shock proteins, specifically Hsp90 (see chapter 4.1). Hsp90 seems to have an important role in transgenerational epigenetic inheritance (Ruden & Lu, 2008), and may act as a capacitor for phenotypic variation (Queitsch, Sangster & Lindquist, 2002) and morphological evolution (Sollars *et al.*, 2003), suggesting that it may play a role in any transgenerational effects of heat stress studied in the current thesis.

The results of chapter 4 showed that the most probable cause of infertility in *T. castaneum* males exposed to heat stress is a reduction in sperm count, with a probable impact on sperm cell integrity and function. In the past fifteen years, there has been growing evidence that male infertility has an epigenetic component affecting sperm cells and their production, which strengthens the case for looking at transgenerational effects of heat stress in the current thesis: Pembrey *et al.* (2006) were among the first to study epigenetic responses down the male line, noting that the vast majority of studies before this had focused on the transgenerational effects of maternal exposure to environmental cues. As noted above, transgenerational genetic studies had previously shown sperm DNA is damaged by environmental factors such as radio- and chemotherapies used for cancer treatment (Arnon *et al.*, 2001; Morris, 2002), and work with rats suggested that the offspring of irradiated males showed an enhanced level of spontaneous chromosomal damage, with DNA strand breakage (Little *et al.*, 2003). High-dose pre-conceptional irradiation at the spermatozoa stage confers enhanced radio-sensitivity to cells in the offspring, which seems to be genetic rather than epigenetic (Little *et al.*, 2003).

Looking for sex-specific effects from paternal exposure to smoking and the paternal grandfather's food supply in humans, Pembrey *et al.* (2006) found that ancestral exposure information is found on the sex chromosomes carried by sperm cells. Paternal smoking caused greater body mass index in sons, but not daughters, and the diet of paternal grandfathers influenced the mortality of grandsons but not granddaughters. Later that year Anway *et al.* (2006) found that exposure of f0 rats to the endocrine disruptor vinclozolin during pregnancy affected f1 embryonic testes development, reducing spermotagenetic capacity, and this effect was passed on to generations f2-f4. In 2011, Hammoud *et al.* found that infertile men had differences in

the histone location and chromatin packaging in their sperm cells compared to fertile men, epigenetic modifications which may have a cumulative detrimental effect on reproduction. While Curley *et al.* (2011) discussed transgenerational sperm effects that are not genetic, surmising that *de novo* mutations in sperm DNA sequences '*may be induced by exposures or increased with age, and that these genetic effects account for the behavioural changes observed in offspring*'. But there may be a link between genetic and epigenetic changes with epigenetic changes in sperm promoting genetic mutations in later generations, '*observations suggest the environmental induction of the epigenetic transgenerational inheritance of sperm epimutations promote genome instability, such that genetic CNV mutations are acquired in later generations*' (Skinner, Guerrero-Bosagna & Haque, 2015).

The findings discussed above make it worth exploring transgenerational effects of exposures to heat stress in *T. castaneum*. My experiment will examine whether there is any sign of a transgenerational effect on non-stressed male offspring fertility when fathers are heat stressed. If there is an effect, I predict that sons will suffer reduced reproductive fitness, perhaps via fertility impacts, from thermally stressed fathers, as based on work by Eggert, Diddens-de Buhr, & Kurtz (2015). As well as 'true' transgenerational effects that are found in filial generations that were not exposed to the initial environmental signal that triggered the change, the impact of *in utero* exposure to particular nutritional, hormonal, or stressful environments can influence the developing embryo, and these parental or intergenerational effects passed on to its germline (Heard & Martienssen, 2014). As such, I will stress males before mating to ensure there is no embryonic effect, with mating, oviposition and development of offspring taking place under standardised control conditions for both treated and non-treated regimes.

In this transgenerational experiment, there are two obvious predictions of the outcomes: 1) sons from heat stressed fathers produce the same number of offspring as non-stressed fathers, in which case no transgenerational effect was evident; or 2) there may be a deleterious effect on the reproductive fitness of f1 males measured through their ability to sire offspring (with standard control females), perhaps equivalent to that seen when f0 males are heat stressed, indicating a transgenerational effect passed to the f1 male generation from their (stressed) fathers. This could be epigenetic in nature, or caused by DNA chromatin damage, and the

phenotypic results will determine whether further exploration of a mechanism is worthwhile.

There is an emerging understanding that a subset of epigenetic changes represents an adaptive result of non-genetic inheritance, i.e. a f0 stress response is passed to the next generation leading to improved stress response in f1. If there is no improvement in stress response, or even a decline, then the phenomenon is not interpreted as 'epigenetic' in this sense (Whittle *et al.*, 2009; Heard & Martienssen, 2014). Epigenetic inheritance in this sense of a cross-generation coping mechanism could be confirmed by stressing the f1 generation as well as the paternal generation and looking for an improvement in f1 fertility compared to their fathers. If the f1 males do not suffer as great a reduction in offspring production as the f0 males then an epigenetic adaptation has taken place (as in *Arabidopsis* under transgenerational salt stress (Suter & Widmer, 2013) or heat stress (Whittle *et al.*, 2009)).

6.2 Methods

F0 fathers were produced from the KSS stock population, and 80 virgin f0 males were heat-treated at 42°C following chapter 2.2.2, keeping thirty virgin f0 males at the control temperature of 30°C throughout. After a 24-hour recovery period, all males were 35 days old, and 50 males from the heat stress treatment were then mated to control, virgin f0 females from the same population for 48 hours in microcentrifuge tubes, approximately half full of standard flour mix. 30 control males who had not experienced heat-wave conditions were mated under similar conditions. Individual females were then allowed to lay for ten days in standard flour mix to produce f1 generation family lines, yielding fifty full-sib families sired by thermally stressed males and thirty full-sib families sired by control males. Females were then removed and discarded, and offspring allowed to develop. At the same time, an extra 300 adults of the f0 population were rotated to fresh flour mix to produce the source of control females for the f1 generation treatment males to mate with.

The eggs of the f1 generation lines were allowed to develop to pupal stage, and then ten male pupae were sexed from each of the 80 family lines. Two hundred female pupae from the f1 generation stock population were sexed at the same time. All pupae were kept separate in Petri-dishes by family and gender, and were left to develop to adults until they were 35 days old.

Three virgin f1 males ('sons') from each of the control and heat-treated family lines were then put individually in microcentrifuge tubes, representing sons in 30 lines fathered by control f0 males, and 30 of the lines fathered by heat stressed f0 males, the latter chosen at random out of those lines that produced f1 offspring (i.e. that were possible to then assay for f2 offspring). Added to the microcentrifuge tubes were virgin, marked control females from the f1 generation stock. Not all f1 males survived the mating period and their offspring were discarded from analysis. This therefore yielded N=1-3 breeding pairs per family, with N=30 families fathered by control 30°C males, and N=28 families fathered by 42°C heat-wave treated males. Each pair was then allowed to mate for 48 hours.

Having mated, females from each of the microcentrifuge tubes were then placed in fresh flour to lay eggs for 80 days, being rotated to fresh flour every ten days. The experiment therefore tested the ability of males fathered by either control or heat-wave treated males to sire offspring when given mating access to control females for 48h and the females then allowed to oviposit for 80 days. Thus, I was measuring the ability of males to pass viable sperm to females that could then yield offspring for 80 subsequent days of oviposition. Females were moved to new Petri dishes and fresh flour every 10 days, providing optimal conditions for females to reproduce, and minimising larval competition effects. Vacated Petri-dishes were left under control conditions for 40 days to allow all offspring to eclose into adults. Because any differences between treatments when studying transgenerational effects are expected to be less overt than single, within-generation effects, 80 days of f1 production were tracked to maximise the male's reproductive fitness, which would encompass ~100% of a female's offspring production following a 48h mating period under these conditions (Chapter 2.1.3). Reproductive fitness was then measured by counting the number of emergent offspring in each of the eight 10-day blocks.

6.3 Results

The distribution of number of adult offspring produced were normal for sons of both control and heat-wave treated males (D = 0.065, p = 0.200; D = 0.084, p = 0.200). Although sons of heat-treated males produced 6% fewer offspring than sons of control males (Figure 6.1), an ANOVA showed that there was no significant difference

between paternal treatments in offspring production over the 80-day oviposition period ($F_{1,57}$ = 0.978, p = 0.529). An ANOVA was used to allow family line to be controlled for as a random factor, there were no significant effects due to line identity ($F_{57,155}$ = 0.988, p = 0.512). Analysing the counts of the first 20 days of offspring production (as has been routinely used as a reproductive fitness measure in the rest of this thesis) also yielded similar results; sons of heat-wave treated males showed a consistent but non-significant 6% reduction in the number of offspring they sired, compared to sons of control males.

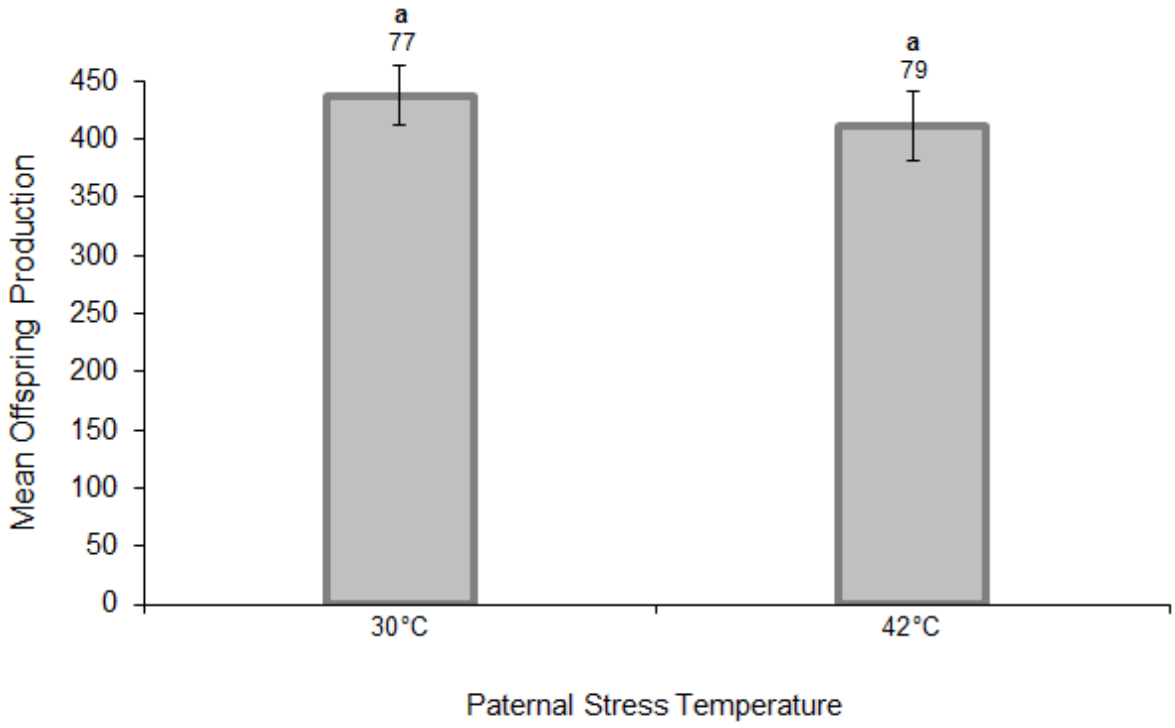

Figure 6.1 Heat induced loss of male fertility is not inherited. Offspring production over 80 days by f1 males is not affected by treatment of f0 male fathers. Error bars show standard error about the mean. Sample sizes and homogenous subsets are displayed above data points.

6.4 Discussion

In this experiment, I find no clear evidence that heat-wave exposure causes transgenerational effects upon male reproductive fitness in *T. castaneum*. Although there was a slight 6% decrease in offspring production by the sons of heat stressed males (Figure 6.1), the difference was not statistically significant.

The absence of any predicted transgenerational effect could occur for several reasons. First, such damage may simply not be passed to the next generation, possibly

because any thermal damage has been repaired: cells have 'surveillance mechanisms' that repair DNA mutations, and erase epigenetic marks (Hauser *et al.*, 2011). The latter of these processes is known as germline reprogramming, occurring in both the germline and in the zygote immediately after fertilization, resetting histone variants and their modifications, as well as small RNAs and DNA methylation (Heard & Martienssen, 2014). This reprogramming of cells to a 'default state' ensures their totipotency in the early embryo, enabling them to differentiate down any pathway, but this means that for transgenerational epigenetic inheritance to occur at a particular locus, this reprogramming must be bypassed (Daxinger & Whitelaw, 2010). Epigenetic responses are far more common in plants (Heard & Martienssen, 2014). As they cannot migrate or behave in the same way as mobile animals, such cross-generational adaptability may be more vital for sessile organisms facing changing environmental conditions (Suter & Widmer, 2013). Presumably this would mean plants have less stringent cellular surveillance mechanisms. *T. castaneum* and other mobile animals may therefore not face the same selection pressure to allow transgenerational effects.

The lack of epigenetic effects corresponds to other work on *T. castaneum*, which has begun to be used as a model to study paternal transgenerational inheritance over the past five years. Looking at transgenerational immune priming, Eggert, Kurtz & Diddens-de Buhr, (2014) mated females to two males, which had either received immune priming or had not, in a full factorial design, separating genetic from non-genetic transgenerational effects. If non-genetic information was inherited in other substances contributed by primed males, i.e. seminal fluid in the spermatophore, it would be present in the spermathecae of females that had been mated to a primed male and then be transferred to zygotes fertilised by non-primed males those females had also mated with. If seminal fluids influenced transgenerational inheritance, it would be apparent in a primed male's step-offspring; i.e. those offspring sired by a non-primed male who mated with the same female. Using this method, the study demonstrated immune priming is only transferred transgenerationally to genetic offspring, suggesting epigenetic inheritance from fathers in *T. castaneum* is via sperm alone. This may mean the post-zygote effects seen in chapter 3, in particular the reduction in pupal eclosion rates (but possible also post-zygotic egg hatch failure), are due to genetic effects such as DNA damage, rather than other transgenerational effects.

There are also a number of potentially confounding transgenerational mechanisms which may account for the effects seen in some of the studies referenced in the introduction, above. These studies may have resulted in transgenerational effects due to transmission of information via intestinal flora or metabolites that can be propagated across generations, and these may '*act as co-factors for chromatin modification or RNA processing for example*' (Heard & Martienssen, 2014). Furthermore, apparent transgenerational effects of heat shock proteins may be confounded by their role in immune priming (Eggert, Diddens-de Buhr, & Kurtz, 2015).

Epigenetic effects are still poorly understood phenomena, and attempts to produce and replicate them yield inconsistent results (Pecinka *et al.*, 2009). In 2009, Pecinka *et al*. undertook a replication study in *Arabidopsis*, exposing different lines to ten different physical and chemical stress treatments, scoring them for the number of somatic homologous recombination (SHR) events in the treated generation as well as in the two subsequent untreated generations. This study concluded: '*In spite of the significant stimulation in the stressed generations, the two subsequent non-treated generations only showed a low and stochastic increase in SHR that did not correlate with the degree of stimulation in the parental plants*' (Pecinka *et al.*, 2009). Indeed, in reviewing the literature, Hauser *et al.* (2011) note that the likelihood of epigenetic alterations appears to be influenced not only by the growth conditions, time, and duration of stress application, but also by the experimental setup: the methods of material sampling and scoring seem to affect the outcome of experiments. It is interesting to compare the inconsistency of transgenerational effects with the regularity of Mendelian ratios when looking at genetic characteristics, the former are clearly much more complex and unpredictable, requiring further research.

Finally, the failure to observe transgenerational effects may simply be that my assay did not place sufficient demands upon male fitness to tease out differences between the two treatments. There was a 6% reduction in offspring production between the sons of heat-wave treated and control males, but *T. castaneum* males have very high reproductive potential (Lumley *et al*., 2015), so a single mating with one female for 48-hours may not be sufficient to uncover any reproductive differences. Future experiments could look at offspring production when sons are stretched to reproduce with multiple females, or perhaps placed into competition. These conditions will be more relevant to reproduction in the natural environment, where multiple

mating and competition for reproduction will be prevalent. Regardless of the reason for the negative result the finding that the impacts of heat-wave conditions on male fitness are not transgenerationally inherited in *Tribolium castaneum* and, by extension, other wild insect populations, is a positive result for population viability and biodiversity in the face of climate change and weather extremes. My results suggest that the impact of increasing frequency, duration and intensity of heat-waves appears limited to one generation only. The next chapter looks at whether male resilience to heat stress can adapt across generations exposed to raised temperatures.

7 Can adaptation to higher ambient temperatures improve male reproductive resilience to heat-wave conditions?

CHAPTER SUMMARY

This final data chapter explores whether multi-generational selection from higher average temperatures reduces the sensitivity of male reproductive function to thermal stress. Replicate independent selection lines were maintained at 38°C, simulating a rise in average ambient temperature due to climate change. After eight generations of selection, these warm-temperature lines were compared to equivalent control lines maintained at 30°C, to determine whether development and/or selection under higher average temperatures can increase male reproductive resistance to heat-wave conditions. Males from lines raised at higher ambient temperatures for eight generations showed no greater reproductive fitness following heat-wave conditions compared to males raised in control 30°C conditions. I therefore find no evidence that *T. castaneum* populations can adapt to heat-wave conditions following eight generations of selection at 38°C.

Contributors: Alyson Lumley assisted with line maintenance.

7.1 Background

In chapter 1 I introduced the predictions that climate change will cause both an increase in average environmental temperatures, and an increase in the frequency and extent of temperature extremes. Through my thesis so far, I have demonstrated that extreme heat-wave conditions can have significant and detrimental effects on male reproductive fitness, damaging sperm function and causing reductions in male fertility. In this final data chapter, I examine whether populations may be able to adapt over a few generations of increased average temperatures, as predicted under climate change, and that this will lead to an improvement in male resistance to thermal stress and a maintenance of fertility following heat-waves. I therefore reared *Tribolium castaneum* populations at a higher average temperature across their entire life cycle, and then explored whether adaptation through genetic selection and/or

acclimation had occurred in a male's ability to tolerate the stress on reproduction generated by heat-wave conditions. After eight generations of maintenance at an average temperature regime which was 8°C above the ancestral condition, I then subjected individual males from these lines to 42°C heat-wave conditions to establish whether an increase in average temperature stimulates these selection lines to be more resilient than control 30°C populations.

Measuring the capacity of populations to adapt to climate change is a relevant question, as Visser proposed: '*The pivotal question in the debate on the ecological effects of climate change is whether species will be able to adapt fast enough to keep up with their changing environment*' (Visser, 2008). The capacity of populations to cope with higher temperatures is '*critical for making predictions about the biological impacts of global warming*', but remains one of the most poorly understood aspects of climate change science (Donelson *et al.*, 2001). This test using a relatively brief period of experimental evolution will measure whether populations of a model insect can adapt one thermally sensitive trait to climate change across a relatively short period of selection.

In natural populations, local adaptation to temperature is well known; it has been demonstrated in, for example, *Caenorhabditis elegans*, in which isolates vary in thermoresistance of fecundity (Harvey & Viney, 2007), *C. briggsae* shows local adaptation in reproductive fecundity to temperature according to a population's global latitude (Presad *et al.*, 2011), and in *Drosophila melanogaster* tropical strains are more heat-tolerant than temperate strains (Rohmer *et al.*, 2004). These local adaptations could have occurred genetically, over periods of evolutionary time since the different populations have established themselves in different thermal regimes, or plastically through acclimation. Responses to rapid climate change might occur through phenotypic plasticity, allowing spatial and temporal changes in important phenotypic traits such as phenology, observed over the past 40 years (Bradshaw & Hozapfel, 2006). More recently, evidence is starting to build that genetic responses could also occur through relatively short periods of climate change, including '*heritable, genetic changes in populations of animals as diverse as birds, squirrels, and mosquitoes*' (Bradshaw & Hozapfel, 2006). The majority of adaptations to climate change in wild populations have been linked to traits under selection from changes in average thermal regimes; studies exploring adaptation to extreme conditions have

received far less attention (Hoffman, Sørensen, & Loeschcke, 2003; Bradshaw & Hozapfel, 2006).

Studies of adaptation in the natural environment are most relevant as they '*reflect the complexity and richness of the real world*' (Bennett & Lenski, 1999). However, they are logistically demanding, with genetic and physiological characters being difficult to measure on a large scale in the field, as well as being time and labour intensive. Complexity can also create disadvantages, making studies problematic to replicate or to adequately control, and disentangling genetic and environmental influences on phenotypes in the field can also be difficult (Gienapp *et al.*, 2008), and the number of generations which can be feasibly studied being limited when studying many natural populations (Bennett & Lenski, 1999). Measures of thermoresistance in the laboratory have also provided important insights, with quantitative genetic analyses revealing genetic variation for thermoresistance and '*in a few cases selection responses within laboratory populations have been linked to specific candidate genes and physiological mechanisms*' (Hoffman, Sørensen, & Loeschcke, 2003). Although, these studies could be controlled and replicated effectively under laboratory conditions, the relevance of these measures to selection pressures in nature remains open to question (Hoffman, Sørensen, & Loeschcke, 2003). Despite this disadvantage, most studies exploring genetic changes to thermal reaction norms have been conducted in laboratory studies using experimental evolution (Hoffman, Sørensen, & Loeschcke, 2003).

Experimental evolution, or 'controlled natural selection', is a powerful tool (Conner, 2003) which provides a valuable approach to testing *a priori* hypotheses and assumptions about the evolution of functional characters (Bennett & Lenski, 1999). This approach allows direct observation and manipulation of characters under selection, allowing research to '*watch evolution in action, not just to speculate on its operation through its consequences*' (Bennett & Lenski, 1999). While lacking the complexity of the natural world, a laboratory environment allows the ability to control experimental conditions, to replicate experiments, and maintain large population sizes, minimizing the effects of random genetic drift on experimental outcome (Bennett & Lenski, 1999).

Here, I use a short period of experimental evolution to track and measure any changes in the resistance of male fertility to thermal stress, i.e. the thermal reaction

norm, when 'evolved' at an elevated temperature regime. This adapted thermotolerance is known to occur in natural populations (Krebs & Loeschcke, 1995; Zatsepina *et al*., 2001) and lab strains of Drosophilids (Kilias & Alahiotis, 1984; Krebs & Loeschcke, 1996; Rohmer *et al.*, 2004). However, these populations were either selected for resistance to heat-waves, being given a short acclimation period and exposed to heat stress within every generation, and then survivors producing the next generation (Krebs & Loeschcke, 1996), or they involved individuals taken to the lab from wild populations where they were exposed to different average and extreme temperature (Rohmer *et al.*, 2004). My approach is to divide *T. castaneum* lineages into two thermal regimes, all taken from a common ancestor adapted to 30°C, and exposing them to eight generations throughout their entire life cycle to either 30°C or 38°C. Following this period of continuous selection, individual males were exposed to heat-wave conditions and responses compared. Rather than the 'laboratory culling' used in studies such as Krebs & Loeschcke (1996), which directly selects on a specific character, this is 'laboratory natural selection', which aims to simulate exposure of natural populations to climate change, and it therefore does not '*constrain the pathways along which evolution could proceed to solve the more general problem*' (Bennett & Lenski, 1999).

Changes to thermoresistance over short periods of laboratory selection could occur via acclimation, through adaption and microevolution, or via the evolution of plasticity (Krebs and Loeschcke, 1996; Gienapp *et al.*, 2008; Hauser *et al.*, 2011 & see Chapter 1). Epigenetic change may also assist adaptation to temperature shifts across generations (Whittle *et al.*, 2009; Suter & Widmer, 2013), and other transgenerational factors such as parental effects may also be influential (Donelson *et al.*, 2001). Distinguishing which mechanism (plastic, genetic or epigenetic), or combination thereof, occurred within the regimes is beyond the scope of the current thesis. Identifying the specific cause of any responses between regimes can be a challenge because mechanisms may mask one another. For example, Crill Huey & Gilchrist (1995) identified that '*marked phenotypic plasticity complicates attempts to predict responses to selection*' (Crill, Huey & Gilchrist, 1995). For this initial experiment, my primary aim is to establish whether changes in average temperature across eight generations of selection (about one year of experimental evolution) can lead to shifts in male thermal reaction norms and the impact of heat-wave conditions, and therefore a capacity to cope with climate change. Having established if such a response can

occur, future research can establish whether adaptive responses occur through plastic acclimation or genetic selection.

For selection on the response of male *T. castaneum* to heat stress to work, there must be individual variation for selection to act upon. Throughout this thesis I have established that between-male variation in thermotolerance exists, from the difference in fertility between the GA-1 and Krakow Super Strain males at the highest temperatures (Chapter 3.4.1), the variation within the Krakow Super Strain at 42°C, with some individuals exhibiting complete sterility following heat-wave conditions, while others maintaining normal levels of reproductive fitness (Chapter 3.3.2) and the fact that mating polyandrously with stressed males allows rescue of female fertility (Chapter 5.4.1). This variation could be the result of genetic variation for thermal tolerance, or it could derive from variation in behaviour during the heat-wave that makes males more or less sensitive to thermal stress and infertility. To avoid limitations of inference by using inbred lab strains, such as GA-1, I derived selection lines from the more genetically diverse Krakow Super Strain in this experiment. The results for stressed Super Strain males, showing a significant (~50%) but not complete loss of fertility (adult offspring production) at 42°C on average, whereas GA-1 showed complete sterility at 42.5°C.

The temperature used to represent the new, increased average from climate change should be sufficiently high to exert selection pressure on *Tribolium* males, but not so severe that long-term exposures are lethal. In chapter 3, the lowest temperature at which males of the Krakow Super Strain started to show a significant decline in reproductive fitness was 38°C. Although the effect was not significantly different to production by control males at 30°C, it was when compared to their optimum production at 35°C (see Chapter 2.2.2). The results for female reproductive fitness in the same experiment gave an optimum at or around 38°C. I therefore selected 38°C as a temperature that was 3°C above the 35°C 'optimum' for both males and females, representing a reasonable approximation to climate change, while ensuring males received a high, but not lethal, selection pressure.

Previous work has applied experimental evolution under thermally-different conditions across dozens of generations over many years; for example, James & Partridge (1995) selected *D. melanogaster* populations for 9 years, and bacterial studies such as Bennett & Lenski (1999) have achieved as many as 2000 generations

of experimental selection. However, time and resources available meant that my experiment was limited to a handful of generations lasting about a year. Importantly, if climate change occurs rapidly, then the capacity to adapt over shorter selection times will be relevant to study. In longer-lived fish systems, Donelson *et al.* (2001) found a degree of acclimation to higher temperatures after just two generations when both parents and offspring were reared throughout their lives at elevated temperatures. Kilias & Alahiotis (1984) noted that selection, '*even of a very short duration can induce significant adaptive and evolutionary changes*'. They used ten generations to develop heat sensitive and resistant strains of *Drosophila melanogaster,* after which differences were revealed between strains. However, and like many studies of this nature, the design did not allow conclusions that changes had taken place very quickly via plastic adaptation, or via genetic selection. In an equivalent study into resistance to heat shock after direct selection, measurements of divergences in survival rate every generation from the start of the experiment were able to conclude that responses started to occur in the ninth generation of selection (Krebs & Loeschcke, 1996). In this experiment, therefore, I raised multiple lines of the *T. castaneum* Krakow Super Strain in two regimes 'warm' (38°C) and 'control' (30°C) to determine whether responses to elevated thermal regime could occur over eight generations.

7.2 Methods

Regime protocol

Two thermal regimes were established which maintained populations throughout their entire life cycle at either 30°C or 38°C for up to eight generations. Initially, twenty separate lines were established for either thermal regime, using 100 adults in each. The regimes were raised at their respective temperatures in A.B. Newlife 75 Mark 4 (44cm x 44cm) incubators. Each line was cultured in a 300ml pot containing 150ml of standard flour mix (see Chapter 2.2.1). For the first six generations, pupae were sexed in each generation to check that equal adult sex ratios were being produced to reproduce the next generation, and therefore to attempt to control effective population size between regimes. Having established that no sex ratio skews were occurring under different thermal regimes, each adult generation was then established after generation 6 by selecting 100 random pupae to be rotated to fresh flour without checking for sex (but see problems below). The 30°C lines produced about twice as many offspring as those of the 38°C lines, indicating general

impacts on fertility and reproduction as previously recognised. Therefore, to control for larval density effects once the adult mating and oviposition stages had been completed, half the flour and eggs were removed in each of the 30°C lines, replaced with an equal amount of fresh flour fodder.

Higher temperatures can accelerate development time in insects (Ragland & Kingsolver, 2008; Damos & Savopoulou-Soultani, 2011), including in *Tribolium castaneum* (Hagstrum & Milliken, 1991). Therefore, across the first six generations of this selection experiment I checked for a divergence in development time to pupation, but found no evidence that the lines from the warm regime consistently produced pupae first. Similar results were found in *D. melanogaster* (Kilias & Alahiotis, 1984; James & Partridge, 1995), and in *T. castaneum* where heat shock of parents lengthened development time of offspring (Eggert, Diddens-de Buhr & Kurtz, 2015). Anecdotally, it was observed that Krakow Super Strain beetles developed faster than the GA-1 wild type strain, pupating 2-4 days earlier. It may be that in the outbred strain these beetles had already reached the lower limits of their possible development speed and this is why temperature did not affect time to pupation further. Accordingly, the 30°C and 38°C line were rotated simultaneously, and did not fall out of synchrony.

Between the f5 and f6 generation, the 38°C lines showed major declines in offspring production. The cause of this decline was not clear, but could be a consequence of 'harsh' selection at 38°C if this is close to the population tolerance, combined with an additional consequence of inbreeding if, for example, the lines were being sired by a small subset of reproductively-competent males. To avoid confounding effects of inbreeding (Folk *et al.*, 2006), I restored populations to larger adult numbers by increasing yeast concentration to 20% in both regimes (allowing greater egg production and larval development), and combining adults from two independent lines to make new single lines. Following Sørensen *et al.* (1999), who re-founded lines from thermal regimes using a mixture of lines, I took the remaining sixteen 38°C lines, and combined pairs of these to create eight lines. These new lines had 33-40 adults each to produce offspring for the f7 generation. These new populations consisted of twenty individuals from each of the two previous lines where possible, but the losses in some lines did not make this always possible. 30°C lines were also combined to give ten populations of 40 adults each to equalise effective population size and treatment between the 30°C and 38°C regimes.

Heat-wave assays

At the f8 generation, 20 virgin males from each of the lines were exposed to heat-wave conditions at 42°C for five days, following protocols established in Chapters 2 and 3. Sixteen virgin males from each line were also kept at control conditions of 30°C for the duration. Following the treatment two of the 30°C lines and one of the 38°C lines that had been heat-wave treated had only one or no males alive, and were therefore discarded. For the remaining lines, and after a 24-hour recovery period, 3-10 treated males from each line were mated to control virgin females from the Krakow Super Strain stock population for 48 hours. After males were removed, females were left to oviposit for 10 days to measure male reproductive fitness.

7.3 Results

Data were non-normal (D = 0.177, p < 0.001) and could not be transformed, but N-values were high (46-64 across four treatments), so a two-way ANOVA was run with regime temperature and stress temperature as independent factors, and independent line was entered as a random factor. Additionally, a one-way ANOVA was run to allow pairwise comparisons using TUKEY testing (see Chapter 6.3).

Although there was a significant effect of heat-wave conditions on offspring production ($F_{1,18.002}$ = 96.232, p < 0.001), again causing a ~46% reduction in offspring, compared to control regimes (replicating the findings of section 3.1), I found no evidence for adaptation in the warm selection regime males. In fact, the 38°C temperature selection regime showed a greater 69% reduction in reproductive fitness following a heat-wave (Figure 7.1). The differences in reproductive fitness between selection regimes were not statistically significant: there was no effect of selection regime ($F_{1,3.162}$ = 1.411, p = 0.316) and no interaction between regime and the two treatment temperatures ($F_{1,3.225}$ = 0.859, p = 0.418). There were also no significant effects of line identity, indicating consistent responses ($F_{11,.869}$ = 0.947, p = 0.686). Pair-wise testing of how lines responded also confirmed these findings, giving significant differences according to heat-wave versus control temperature exposures only. There was no change in the significance level of the results when Bonferroni correction was applied (α-level = 0.05/8 = 0.00625).

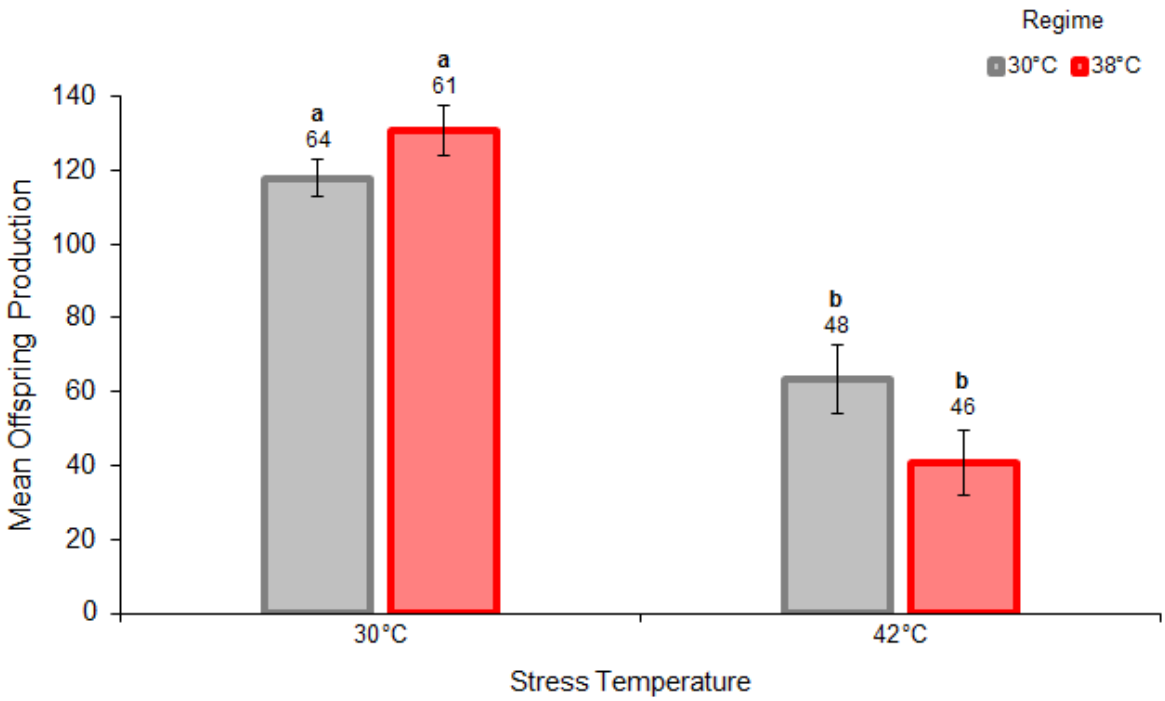

Figure 7.1 Selection regimes maintained for eight generations at higher temperatures are not more resistant to heat-wave induced loss of male fertility. Males from populations exposed to constant control or raised temperature for entire lifespan and offspring production measured from control females across 10 days of oviposition. Error bars show standard error about the mean. Sample sizes and homogenous subsets are displayed above data points.

7.4 Discussion

After applying selection across eight generations where the complete beetle life-cycle was maintained at 38°C, I found no evidence that male thermoresistance to heat-wave conditions had diverged, compared to the control 30°C regime. If anything, the 38°C temperature regime males showed an even greater decline in reproductive fitness following a heat-wave (Figure 7.1).

Past studies using laboratory selection have shown that selection at moderately increased temperatures and subsequent tolerance of survival to extreme high temperature occurs. Gilchrist, Huey & Partridge (1997), for example, raised lines of *Drosophila melanogaster* at 16.5°C, 25°C, or 29°C for 4-10 years, and found that adult survival under acute heat shock positively correlated with selection temperature. More recent studies that measured reproductive traits have found no greater tolerance of heat shock after thermal selection. In *D. melanogaster,* similar egg-to-adult viability and fecundity was found in replicate populations from two thermal regimes following higher thermal stress (Schou *et al.*, 2014), and in the dung

fly *Sepsis punctum*, there were no significant differences in fertility (measured here as the proportion of fertile females) and fecundity (number of f1 adults that had at least one adult offspring) between selection regimes after exposed to further heat shock (Esperk *et al.*, 2016). It is clear then that '*laboratory natural selection can lead to divergence in thermal sensitivity*' (Gilchrist, Huey & Partridge, 1997), but there is mixed evidence as to whether it leads to an increase in tolerance to greater thermal stress.

A number of other systems have shown either no adaptation to heat stress, or a diminished response following selection under heightened temperatures in a range of conditions. In a major survey of marine species living on the Great Barrier Reef, which is suffering bleaching through thermal stress, '*no evidence was found for a protective effect of past bleaching (for example, from acclimation or adaptation): reefs with higher bleaching scores in 1998 or 2002 did not experience less severe bleaching in 2016*' (Hughes *et al.*, 2017). This finding is surprising, because the results come from a major study of wild populations over a larger scale and longer time period than is typical for laboratory studies. In *Caenorhabditis remanei*, parents subjected to prior heat stress produced offspring that were less able to survive a severe heat shock (Sikkink *et al.*, 2014), indicating some sort of transgenerational damage from thermal selection. In fruit flies, Ohtsu *et al.* (1999) found no effect of rearing temperature (15°C and 21°C) on resistance to heat stress in the temperate *D. curviceps*, or the sub-tropical *D. immigrans* and *D. albomicans*. Worthen and Haney (1999) found only one out of three species (*D. putrida*, *D. falleni* and *D. tripunctata*), displayed signs of acclimation after exposure for five days at a heightened 30°C. Finally, Schou *et al.* (2014) found that, after thermal regime selection for 20 generations, replicate populations of *D. melanogaster* had similar egg-to-adult viability and fecundity when assessed at higher stressful temperatures. This last study is particularly relevant to my own findings, because it employed a similar experimental design to the one used in this chapter (although temperature in their 'hot' regime was increased gradually across the generations). Either the study design is not appropriate for detecting changes in thermotolerance or, simply, the result shows that selection across generations at raised temperatures does not increase the capacity to cope with heat-wave conditions. Overall, these studies show that adaption and acclimation are not a typical response, and therefore hard to predict and replicate, and there may be a number of reasons why this is the case.

One reason that I found no adaptation or acclimation could be due to costs associated with the process. Hardening responses have costs associated with the expression of a heat shock protein (Hsp70) (Hoffman, Sørensen & Loeschcke, 2003). In *D. melanogaster*, Hsp70 induction is implicated in reduced fecundity, and Hsp70 over-expression is associated with increased larval mortality, retarded growth, and reduced egg hatching (Folk *et al.*, 2006), any of which could explain the decrease in egg-to-adult viability in *T. castaneum*. Estay *et al*. (2011) maintained two populations of *Tribolium confusum* at the same average temperature (24°C), but under different levels of variability: ±0 and ±8°C, the latter corresponding to the difference between the day and night temperatures, but only the daytime being within the optimal range for the beetles at 32°C. Although it might be expected that populations living at least half the time at an optimal temperature have a higher reproductive performance than those living in a constant lower temperature, outside the optimum, the maximum per capita growth rate was significantly lower in the treatment with variable temperature than in the treatment with constant temperature. This finding showed that the costs of acclimation can be greater than those of developing at sub-optimal temperatures. With trade-offs such as these, which may not make acclimation the optimal strategy in certain circumstances, a number of conflicts within the costs and benefits of acclimation can be expected.

One such trade-off seems to exist between acclimation (plastic) and basal (non-plastic) resistance levels of thermoresistance (Hoffman, Sørensen & Loeschcke, 2003). Selection for heat resistance could either increase heat shock protein expression as the organism uses it to increase its thermotolerance, or decrease heat shock protein expression as improved resistance reduces the need to trigger a stress response, i.e. the organism is no longer stressed under the same conditions. Sørensen *et al.* (1999) tested to see if selection for high temperature resistance leads to an increased level of Hsp70 expression. Lines of *D. buzzatii* were selected for up to 64 generations, those with increased heat resistance, had reduced hardening and Hsp70 responses (Sørensen *et al.*, 1999). It therefore seems that if exposures inducing a stress response are too frequent, the fitness cost of hardening may outweigh the benefits of increased thermoresistance, therefore a fixed basal level of resistance is favoured through evolution and stress response plasticity is reduced in order to limit the costs of repeated heat exposure, (Sørensen *et al.*, 1999; Hoffman, Sørensen & Loeschcke, 2003). This would make the Hsp70 response '*an emergency system for populations*' (Hoffman, Sørensen & Loeschcke, 2003), used by those only rarely exposed to

temperature stress. In populations regularly exposed to thermal stress, the response becomes too costly and basal resistance is selected for. Thus, it would be expected that populations with relatively high basal resistance show relatively small acclimation and hardening responses, and this is supported by data from natural populations of *Drosophila* (Hoffman, Sørensen & Loeschcke, 2003). Whether acclimation or adaptation takes place should therefore depend on the frequency and severity of thermal stress that a population is exposed to. The duration or intensity of the thermal stress which I applied in the 38°C regimes may therefore have been too demanding (discussed further below), making acclimation too costly to be a viable option. However, this does not explain why genetic adaptation would not have occurred to alter basal resistance.

Although every effort was made to reduce confounds in the thermal regime experiment, the nature of the design may have introduced a number of factors which may have influenced the selection and post-heat-wave performance of males from the 'warm' 38°C lines that were difficult to control for. If selection was too harsh, and effective population size was lower than the adult population, which may have been possible if male fertility had been severely constrained, inbreeding could have created confounding problems. In order for a selection pressure to lead to microevolution there has to be differential survival, in the early generations there would have been higher mortality in the warm regime, and this was clear from population densities between the regimes and from sexing males and females at the pupal stages. This higher mortality, however, would also reduce the size of the gene pool and, after the population collapse in f6 where the population sizes were reduced to 40 or less, it is likely that there could have been a genetic bottleneck, which may also have led to inbreeding depression. Such a bottleneck would have been further tightened if only a small subset of males were able to reproduce after spending their total life cycle at 38°C. Thus, adaptation could have occurred, but the adapted males may also have exhibited generally reduced fertility due to inbreeding, countering the effect of beneficial selection on male thermoresistance from a warmer regime. Microsatellite testing for heterozygosity in the 30°C versus 38°C lines could reveal whether such inbreeding has occurred.

If inbreeding has occurred in my selection lines, this may still be relevant for natural populations. Climate change itself may lead to genetic bottlenecking, as populations experience rapid environmental change and suffer harsh selection and

reduced viability (David *et al.*, 2005), then become limited in size and distribution (Shoo, Williams & Hero, 2005). Increased mutational load expressed under inbreeding may also cause populations to become more vulnerable to the negative effects of thermal stress. This could partly result from the fact that a change in the environment may lead to the exposure of conditionally expressed detrimental alleles, which do not have adverse effects on fitness under benign environmental conditions (Bijlsma, Bundgaard & Van Putten, 1999). Inbreeding has been shown to reduce survival (Dahlgaard, Krebs & Loeschcke, 1995) and reproductive output (Dahlgaard & Hoffman, 2000; Pedersen, Kristensen & Loeschcke, 2005) in synergism with thermal stress. In *D. melanogaster*, for example, inbred individuals express novel deleterious alleles under thermal stress, even after attempted purging of known deleterious alleles (Bijlsma, Bundgaard & Van Putten, 2001). In the same species, the interaction of inbreeding and thermal stress on male fertility showed that relative sterility after thermal stress increased nearly ten-fold when flies were inbred, and sterility was more likely to be permanent in inbred lines (Pedersen *et al.*, 2011). Environmental stress and inbreeding could therefore function together to damage population viability, as exemplified in the extinction vortex (Gilpin & Soulé, 1986). Furthermore, inbreeding can influence thermal resistance in another way as high levels of inbreeding induce Hsp70 expression, even at low temperatures, having negligible effects on outbred lines, '*inbreeding itself may have a small but significant hardening effect*' (Hoffman, Sørensen & Loeschcke, 2003).

In a review of *Drosophila* studies, Hoffman *et al.* (2003) reported inconsistent results for the interaction between inbreeding and environmental stress, both within and between studies, concluding that '*inbreeding depression is environment dependent and population specific*'. Similarly, a 2005 review of inbreeding and environmental stress, of any kind, showed that inbreeding depression increases under stress in 76% of cases, although this increase is only significant in 48% of the 34 studies considered (Armbruster & Reed, 2005). Although it appears that an interaction between inbreeding and environmental stress cannot be predicted *a priori*, perhaps because a population bottleneck can also lead to purging of deleterious alleles (Bijlsma, Bundgaard & Van Putten, 1999), it is clear that in some cases the two can work together. If my 38°C warm selection lines had suffered inbreeding as a consequence of male infertility or some other effect of harsh selection across different life stages at higher 38°C temperature regimes, this may explain why my selection experiment did

not lead to superior reproductive performance by males following heat-wave conditions.

An alternative explanation is that the selection pressures were not strong enough in the 38°C temperature regime to cause microevolution. However, the differences in larval densities and the population crashes between the 5th and 6th generation suggest that the temperature in the warm regime did apply significant selection pressure, and that any higher temperatures would have been too harsh for populations to have remained viable throughout their whole life history across multiple generations. That is assuming that the population crashes were not due to external factors, such random variation in incubator temperature or disease. One solution, which was unfortunately not available, would have been to allow a few more generations of selection beyond the f6 population crashes and for populations to recover from any transient effects.

Alternatively, changing thermal reaction norms simply may not be the best way to test whether populations can adapt and cope with a changing climate, and some studies suggest that these conditions in the laboratory are not representative of what happens in the wild (Klepsatel *et al.*, 2013). Rohmer *et al.* (2004) found that the threshold temperature at which male sterility is induced appears to be fairly stable, insensitive to genetic drift or laboratory adaptation. Klepsatel *et al.* (2013) found no changes in thermal reaction norms for a number of traits examined in *D. melanogaster* under divergent temperature selection, which they suggest means 'thermal adaptation predominantly involves evolutionary changes in absolute trait values rather than in aspects of thermal reaction norms'. This may mean that expecting changes to thermal reaction norms in laboratory selection is unrealistic, and that upper limits on thermal tolerance within male fertility may carry little genetic variation for short-term selection to act upon.

Sub-tropical species such as *T. castaneum* may have less capacity to adjust or adapt to changes in temperatures compared to temperate species, because they have evolved in a more stable thermal environment, and they live close to their thermal maximum (Donelson *et al.*, 2001). The results of chapter 3, where I found populations did not survive heat stress above 43°C may mean that *T. castaneum* is at its physiological thermal maximum at the stress temperature used throughout this thesis. This would indicate that *T. castaneum* has a limited ability to adapt and,

despite the fact that the Krakow Super Strain was used as a basis for the thermal regime lines, which had been cultured to add in more genetic variation, the strain may still have had insufficient genetic variation for adaptation to heat-wave conditions at 42°C. It may therefore require many more generations of selection to affect an adaptive response if genetic variation is low, and the risk of inbreeding depression under harsh selection is high.

Finally, it is worth considering whether it is relevant to simply change the environment to a new, higher constant temperature through laboratory selection. Expecting populations to evolve in a stable thermal environment may be ecologically unrealistic (Ragland & Kingsolver, 2008), and a number of studies have considered whether it is more suitable to use variable temperatures in the laboratory, to more closely match the scenario populations would experience in the wild. Ragland & Kingsolver (2008) established laboratory colonies from three geographic populations of the mosquito *Wyeomyia smithii,* spanning a latitudinal and altitudinal gradient in eastern North America. They compared the effects on survival, development time, and mass at pupation for larvae reared at 16, 20, and 27°C constant temperatures, and in two fluctuating temperature treatments with means of 20 and 27°C. However, Ragland & Kingsolver (2008) found no significant effects of temperature fluctuation on survival at mean temperatures of 20 and 27°C for any population, and the effects of temperature variation on both development time and pupal mass depended on mean temperature. Bennett & Lenski (1999) replicated *E. coli* from a constant thermal environment of 37°C into a new environment that alternated between 32 and 42°C for 2000 generations. While fitness and efficiency of resource use increased in the fluctuating regime, fitness also increased in the ancestral environment of 37°C, and efficiency did not decrease. Therefore, these two studies do not provide strong evidence that use of a constant temperature for selection yields significantly different results from more ecologically realistic simulations.

According to models constructed by Gilchrist (1995) in constant environments, temperature specialists with narrow preference ranges are the favoured phenotype, and in environments in which there is considerable among-generation but little within-generation variation, temperature generalists with broad preference ranges are favoured. These are to be expected, however, in the same models, specialists are also favoured in environments in which there is significant *within*-generation variation. Because specialists in a constant environment have a mean fitness an order

of magnitude higher than any phenotype in more variable environments, natural selection '*favours increased efficiency during even limited times of optimal conditions rather than extending the range of conditions that support fitness-enhancing activity*' (Gilchrist, 1995). Therefore, as long as the temperature to which an organism is adapted is within the range of a fluctuating thermal environment, selection could discourage it from adapting to other temperatures, or from increasing its temperature generalisation. If the Krakow Super Strain is at its thermal maximum, with limited capacity to adapt, this factor identified by Gilchrist (1995) could further explain the lack of adaptation.

Negative results can, in many cases, provide information; this selection experiment appears to indicate that an increase in average temperature does not improve thermoresistance to heat-waves and that, in these specific conditions at least, populations cannot adapt to climate change. However, it is not clear that this study allows such a conclusion to be drawn, or whether the negative findings are a result of the experimental design, conditions and animal model. It may simply be that, overall, this study merely strengthens the concept that '*attempts to predict short-term evolutionary responses to selection are likely to be difficult*' (Crill, Huey & Gilchrist, 1995). Replication in other populations and species will be the key test of whether the results in this chapter apply generally, and that tropical insects may have limited capacity to adapt their thermal resistance over a short period of selection.

8 Summary, conclusions and further work

CHAPTER SUMMARY

In this final chapter I present a summary of the findings of this thesis. I briefly discuss their importance and their placement in the context of current research, and consider directions for future research. I make predictions how my results from laboratory experiments on a model organism will translate to natural populations in the wild, and how they can inform planning for climate change mitigation and conservation.

8.1 Summary of findings and directions for future research

The aim of this thesis has been to improve our understanding of how any thermal stress acting under heat-wave conditions impacts on reproduction in a model insect system. Using this research, I hoped to understand more about the proximate drivers of biodiversity loss under increasingly extreme weather events caused by climate change, and how traits, individuals and populations respond to heat stress and fertility loss. This understanding is important because climate change will lead to both an increased average temperature, and more extreme events such as heat-waves, which are set to become more frequent, more intense and longer-lasting (Meehl & Tebaldi, 2004; IPCC, 2012). Although most focus has been on biodiversity impacts of increasing average temperature scenarios, it may be the extreme events such as heat-waves that have the most profound effects on populations.

Concern for the sensitivity of populations exposed to heat-wave conditions is based on a long history of research showing temperature-induced male sterility in homeotherms (see reviews in Setchell, 1998 and Chapter 1.2), with only more recent studies showing the effect in ectotherms (David *et al.*, 2005). Ectotherms will be more sensitive to temperature change, and are also more abundant, speciose, and integral to most ecosystem services. I used *Tribolium castaneum* as a model organism to study the impacts of heat stress on reproduction. This species has a worldwide sub-tropical distribution, is classified as an insect and beetle, meaning that its generalised

physiology and phylogenetic position make it ecologically, physiologically and evolutionarily representative of a large proportion of species and geographic zones, many of which are sensitive to climate change. Furthermore, prior to the current work, *T. castaneum* had already been established as showing male-specific fertility loss under heat treatment, using the lab-adapted *Georgia-1* (GA-1) strain (Godwin, 2010), with males showing a complete loss of fertility in males at high temperatures (Chapter 1.5). I employed a genetically-more-diverse population in order to make it more applicable to non-inbred populations in the natural environment: the 'Krakow Super Strain' (KSS) was created by forcing a number of different strains together, with the aim to understand resilience to heat stress in natural populations.

The KSS was created from eleven interbred strains that originated from various different populations of *T. castaneum* and it, or lines derived from it, were used throughout the thesis (see Chapter 2.1.3). The other major tool I employed throughout were heat-wave conditions, which were developed from my own pilot work, as well as using the definition that a heat-wave occurs when environmental temperatures are 5˚C or more above the ambient average for at least 5 days (adapted from Frich *et al*., 2002, see Chapter 2.2.2). In *T. castaneum*, population fitness and productivity is greatest at 35˚C (Sokoloff, 1972-78), so the conditions used throughout this thesis were five days at 7˚C above the optimum for *T. castaneum*. I found that the upper limits for the duration and temperature of a heat-wave for a KSS population under heat stress, while still allowing significant survival for use in assays, was also five days at 42°C, with treatment for a longer time or at a higher temperature resulting in higher mortality. Following this determination, all experiments used a five-day treatment followed by a 24-hour 'cooling off' period at 30˚C. The first experiment presented in this thesis demonstrates that male fertility declines up to 42˚C, whereas female fertility is largely unaffected (Figure 3.1). Using these two main tools, my thesis investigated how and why male fertility is impacted by heat-wave conditions, and what responses to this male-specific fertility loss are possible.

My experimental results can be divided into three main categories: 1) the effects of heat-waves on fertility and reproduction, including offspring development and viability; 2) the underlying causes of these effects; and 3) possible responses to them. In this final chapter, I summarise my findings arranged by these categories and place them in context of other research. I then consider how my results have implications for the wider impact of climate change, and how they may influence

predictions of its effects on populations and ecosystems. Finally, I suggest some directions for further work to explore the effects of heat stress on reproduction, based on my findings in this thesis.

First, I established the effects of heat stress on male and female *T. castaneum* from the new KSS population, using offspring production as a measure of reproductive fitness. Matching previous work with the standard GA-1 'wild-type' strain of the species, only males of the KSS showed a reduction in fertility, with no significant reduction in female offspring production at the same temperature exposures. Furthermore, males of the more genetically diverse KSS were also more resilient to heat stress, retaining ~50% fertility at 42°C compared to the complete sterility of GA-1 males at this temperature. This 50% reduction at 42°C struck a good balance between impacting fertility sufficiently to see strong effects, while leaving enough offspring to be tested in other assays. This experiment clearly agreed with previous work on the sex-specific effects of heat stress in other models (David *et al.*, 2005; Dolgin, Whitlock & Agrawal, 2006; Ellers *et al.*, 2008), while also characterising the performance of the new strain and determining the best temperatures to use as heat-wave conditions throughout the rest of the thesis. It also demonstrated that there is some variation in resistance to heat stress among males, and that genetic diversity might increase resistance a population's resistance to this environmental stress.

It would be useful to track the longer-term effects of heat-waves. For example, is the drop in male fertility temporary or permanent? If recovery can occur, how quickly does it start and how long does it take? Future studies could investigate by allowing males longer recovery times after stress and measuring their reproductive fitness as it changes through a longer timeframe, for example. It would also be important to determine the longer-term effects of such a considerable drop in fertility for the survival of a population. Future work could employ techniques such as population viability analysis, which is effective for predicting population trajectories and estimating extinction risk (Brook *et al*., 2000), especially after exposing entire populations to heat-wave conditions.

With an effect of heat-waves on the ability of males to produce offspring established, I investigated the impact across the beetle life-cycle to determine how heat treatment curtailed offspring production. I measured at three key stages through

offspring production and development: 1) egg production, 2) egg hatch and 3) pupal eclosion rates, to ascertain when and where offspring production was limited. I found that females produced similar numbers of eggs with both control and heat stressed males, so fecundity is unaffected (Figure 3.3). However, fewer of those eggs hatched, and fewer pupae eclosed successfully (Figures 3.4 and 3.5) if the offspring were sired by thermally stressed males. These match findings for a paternal effect on larval development found in other insect models after heat stress (Huey *et al.*, 1995; Silberman & Tater, 2000; Montllor, Maxmen & Purcell, 2002; Cui *et al.*, 2008; Zhang *et al.*, 2013). Cui *et al.* (2008) found an effect on offspring eclosion when both sexes were stressed together, but my results isolate a paternal effect. The reductions in offspring numbers mapped onto the reductions I found in hatch rate and pupal eclosion suggest that the reduction in male reproductive fitness following heat-wave conditions can be explained by a combination of fewer eggs being fertilised or developing to hatch, as well as a next-generation effect on successful pupal metamorphosis.

With effects on offspring pupal eclosion, I explored further to see if there were any longer-lasting impacts on the reproductive fitness of adult offspring. I measured whether f1 sons from heat stressed fathers showed any reductions in reproductive fitness themselves. There was no obvious indication of a transgenerational effect: sons of heat stressed males produced 6% fewer offspring than the sons of control males, but this was not statistically significant (Figure 6.1), suggesting that any transgenerational effect on pupal eclosion in the f1 generation is not passed onto later life history stages or generations. This finding suggests that the post-zygotic effects on offspring production via reductions in eclosion rate could be somehow due to DNA damage from heat-waves, which would be corrected by DNA repair mechanisms in the adult offspring, rather than other transgenerational effects that would be inherited by later generations.

The fact that my results show problems at key points of development may be a consequence of choosing these specific points to take measurements. Future studies of life history and transgenerational effects could look at whether fewer eggs hatch because of developmental problems, or because fewer eggs are fertilised. Similarly, is the reduction in eclosion due to transgenerational effects causing problems in metamorphosis, or simply a factor of mortality over time? The latter would still be a

transgenerational effect but could be measured throughout larval development from hatch until adult eclosion and beyond.

Having established the extent of the impacts of heat stress on offspring production, I looked at possible causes behind male-specific heat-induced fertility loss. These could be driven by pre-copulatory issues relating to male mating behaviour and female mate choice, or post-copulatory mechanisms relating to sperm or seminal fluid function and/or cryptic female choice. Results showed that both pre- and post-copulatory factors were altered in adult males by heat-wave conditions. Observations of male mating behaviour showed that heat stress reduced male mating activity, with decreasing levels of mounting attempts and successful matings (Figures 4.3 and 4.4). However, the differences in mating behaviour are unlikely to explain the halving in male reproductive fitness following a heat-wave, because thermally stressed males were all still observed to mate successfully, albeit with a greater latency (Figure 4.2), reduced frequency, and longer copula duration (Figure 4.5). The changes in male mating behaviour I observed match research which shows heat stress reduces mating frequency (Fasolo & Krebs, 2004; Liao, Qian & Liu, 2014) and mating success (Janowitz & Fischer, 2011; Jerbi-Elayed *et al.*, 2015). These measures may also be affected by females rejecting males that have sustained thermal stress (see below and Chapter 5). The behavioural assay also seemed to show an effect on male condition, beyond the specific behaviours I measured: heat stressed males were lethargic and less aware of both females and their surroundings compared to control males (only control males escaped their mating arenas for example). Follow-up studies could look at the extent of the effect of heat stress on male condition to examine whether it affects the ability to mate with multiple females, dispersal and activity levels in general, which will contribute to finding sexual partners, and whether any sensory apparatus is impaired. It would also be informative to investigate why copulation duration is longer for stressed males. For example, the experiment reported in Edvardsson & Arnqvist (2000) could be repeated to explore whether heat stressed male sperm reaches female storage after longer copulations, i.e. whether the minimum duration required for successful sperm transfer increases.

To confirm whether insemination success was affected by heat-wave conditions, I also examined the rate of successful sperm transfer into females across a sequence, as well as estimating the number of sperm cells transferred by males in the spermatophore to the female bursa. Fewer males exposed to 42°C heat-waves

inseminated the first female they mounted compared with control males (31.8% vs. 79.2%), and these heat-wave exposed males also had a more than five-fold reduction in the number of sperm cells they transferred in their spermatophores (Figure 4.6). In addition, a number of spermatophores transferred by heat-stressed males contained significant amounts of globular material within the spermatophore sperm mass, where control male spermatophores contained only dense masses of elongate sperm cells. The reduction in insemination success rate among stressed males correlates with the increase in the number of males that produced no offspring following five days of exposure to 42°C heat-waves (Figure 3.2).

Heat stress is known to impact the viability and quality of sperm cells (Karaca *et al.*, 2002) as well as their production and function (Ayo, Obidi & Rekwot, 2011). My results provide strong evidence that declines in sperm number and disruption to spermatophore formation contribute to a reduction in male fertility following heat-wave conditions in *Tribolium castaneum*. Furthermore, male mating behaviour may be an additional factor in limiting the successful transfer of sperm to females.

An important question arising from the sperm count findings is what causes the reduction in sperm counts, insemination success and intact sperm cells, and therefore fertilisation success? Setting aside male mating behaviour and female choice, it could be that heat damages the sperm cells directly, reducing their integrity or limiting their function and therefore their ability to reach female storage and oocytes; it could damage haplotypic DNA within sperm cell nuclei, or seminal fluid proteins, any one of which could reduce a male's ability to fertilise eggs. Alternatively, thermal stress could disrupt spermatogenesis. While the experiments presented in this thesis were not designed to directly test for these possibilities, some of my results allow some inferences to be made about the mechanisms, and may provide directions for further work.

Disruption to spermatogenesis is unlikely to be the sole reason for male fertility loss, because offspring production was also reduced when mature sperm in female storage were thermally stressed (see Chapter 5). Furthermore, the difference between the decrease in sperm counts in chapter 4 (82.5%) and the reduction in offspring production in chapter 3 (47.4%) may be due to the difference in time allowed for mating. The former had 48 hours in which to mate after the cooling off period whilst in the latter males were removed after their first mount of a female. This may

indicate spermatogenesis restores some of the male's sperm count (from 17.5% to 52.6%) during the extra two days. The effect (or lack thereof) on spermatogenesis could be confirmed by treating males after the first two days of adult emergence, when sperm production begins (Fedina & Lewis, 2008), and comparing to males at ten days post-emergence, when mature sperm have developed (Sokoloff, 1972-78). However, there may be other factors making males more susceptible to heat-wave damage at the early adult stage, such as the lack a hardened exoskeleton, making new imagos more susceptible to damage from heat treatment with higher mortality rates (e.g. Godwin, 2010).

Although I found evidence for a reduction in sperm count, further work could explore sperm form and function following heat-wave conditions, including qualitative measures of any damaged sperm such as the number of deformed or damaged sperm by measuring the disruption of sperm cell membranes and the breakage of the axoneme of the cell tails. This could be done using tools such as the Live/Dead® Sperm Viability Kit L-7011 (Molecular Probes, 2001), which labels live sperm with green fluorescence and membrane-compromised sperm with red fluorescence, and has been used successfully with *Tribolium* (e.g. Fedina & Lewis, 2006).

In addition to disrupted form, function and number, damage to sperm cell DNA may also be a route for reducing male reproductive fitness, perhaps suggested by the transgenerational effect on pupal eclosion rates shown in chapter 3. However, I did not find additional transgenerational effects on the reproductive fitness of sons from heated fathers. In addition, the evidence that female polyandry can affect fertility rescue suggests that it is sperm number and function disrupted by heat, rather than chromatin DNA damage, as otherwise fertility levels under polyandry should not improve above that of monandry. Further work could measure chromatin DNA integrity directly using techniques such as the comet assay (e.g. Azqueta & Collins, 2013). My experiments do not isolate effects of seminal fluid proteins in the loss of male fertility, but the numbers and types of proteins produced by stressed and control males would be worth investigating and techniques to identify them have been developed but do require considerable effort to characterise (Avila *et al.*, 2011).

With the causes behind male-specific fertility loss following heat-wave conditions at least partially resolved, I next examined whether female beetles can mount a response to the risk of male infertility. Following Michalczyk *et al*. (2011b),

who showed that female *T. castaneum* can rescue their fertility from the genetic stress of inbreeding depression by mating promiscuously, I ran a similar experiment to determine if the same phenomenon can apply to protect female reproductive fitness from male-specific infertility following environmental heat-wave conditions. This experiment provided one (monandry) versus five (polyandry) males to a female simultaneously, and the males had either been heat stressed or maintained at control temperatures. Additionally, I ran a second experiment in which females were mated to one or five males before exposing females to heat-wave conditions after they had sperm in storage from one versus five males. These two experiments explored whether any polyandry benefits might come from the process of mating and sperm storage, or whether females can protect themselves against infertility under a heat-wave by maintaining a more diverse polyandrous sperm store. Both experiments measured offspring production as a proxy for female fertility and reproductive fitness.

I found that females were able to recover their fertility to pre-heat-stress levels that equalled the control treatments if they mated polyandrously with heat stressed males (Figure 5.1). This finding indicates that females can use polyandry to somehow filter fertile males and/or their sperm into storage following damage by a heat-wave, supporting fertility-assurance as a reason for polyandry under extreme weather conditions.

However, once females had mated and stored sperm, there was no polyandry benefit, with females showing a ~40% drop in offspring production if sperm were heat stressed in storage, irrespective of previous mating opportunities with one versus five males (Figure 5.2). This result confirms the important impact of heat-wave conditions on mature sperm cells, including within female storage, as a key factor behind fertility loss. It would be interesting and informative to explore whether higher risks of heat-wave conditions (and, consequently, infertility) promotes polyandry within populations.

Knowing that sperm were damaged in storage, and that female fertility rescue is possible, I carried out two further experiments on female mating behaviour to measure whether females can mate facultatively to guard against fertility loss under heat-wave conditions. In the first experiment, females were given two mating partners sequentially, either stressed or control or both, in a fully factorial design. The only deviation in male mating behaviour observed after stress (see above and

chapter 4) was that females who received two stressed males copulated for less time than if only the second male was stressed (Figure 5.5). In the second experiment, females were stressed either as virgins or with sperm in storage, before being given an opportunity to remate with a control male. In both scenarios, I predicted that female motivation to remate would be higher if they had previously mated with a heat stressed male, or had been exposed to fertility-damaging thermal stress themselves. Results showed that copulation duration decreased significantly if a female had been stressed with sperm in storage (Figure 5.9) and, while number of attempted matings increased if a virgin female was stressed, this was not the case if a female was stressed with sperm in storage (Figure 5.10). These results suggest that females cannot detect the damage to sperm in storage after stress, but they may be able to detect their own condition and male condition after stress. If they did detect male or their own stress condition, females were more resistant to further mating than they were compensatory. These results mean that in a natural population, where both sexes experience a heat-wave and females are exposed to multiple males, female mate choice and resistance to poor condition males may reduce mating success and sperm transfer. Given the already-high levels of promiscuity in *T. castaneum* (Lumley *et al*., 2015), perhaps facultative responses have not been exposed to selection.

My final experiment provided less support for the hypothesis that populations or lineages can adapt across short periods of selection to heat stress and overcome male fertility loss. Using experimental evolution, sub-populations (or thermal selection lines) of *T. castaneum* were exposed across their full life-cycle to a higher ambient temperature of 38°C, approximating to an increase in average temperatures that climate change may bring about. After eight generations, male beetles exposed to further heat-wave conditions at 42°C showed no improvement in adult offspring production (Figure 7.1). This experiment therefore provided no evidence for acclimation or adaptation by males to heat-wave fertility damage across generations. This was unexpected, not only because other studies have shown adaptation to heat-based selection pressures with higher temperatures leading to the evolution of greater resistance to extremes (e.g. Gilchrist, Huey & Partridge, 1997; Schou *et al.*, 2014; Esperk *et al.*, 2016), but also because my own earlier experiments throughout the thesis have provided direct or indirect evidence for variation in thermoresistance in males, and differential fertility as a result. The clearest evidence for variation in thermoresistance is simply the fact that males of the standard GA-1 strain are much more sensitive at the upper thermal limits, with all suffering complete loss of fertility

at 42.5°C (Godwin, 2010; see Figure 1.4), whereas in the KSS strain males retained about half their reproductive fitness following similar 42°C heat-wave conditions, suggesting a genetic capacity for variation in thermoresistance for which the out-bred strain provides more raw material. Furthermore, almost 40% of KSS males were completely infertile at 42°C whilst 60% of males retained some fertility, suggesting a variation in thermoresistance within the strain.

It is not clear why, despite evidence for variation in thermoresistance, that *T. castaneum* did not evolve a change in thermal reaction norms, even when using the genetically more diverse Krakow Super Strain. One obvious possibility is that the warmer lines had adapted, but also become inbred through genetic bottlenecking as a consequence of harsh selection and/or a skew in male reproductive success. If this is true, inbreeding depression may have masked the adaptation in males, and molecular measures of heterozygosity between the selection regimes would provide relevant information regarding this.

Overall, the results from this thesis suggest that populations have the *capacity* to adapt to climate change through fertility rescue and variation in thermoresistance, but when given opportunities to make use of this capacity, in the female remating and thermal selection line experiments, they did not capitalise on this capacity. However, the capacity may be more important than the results, as in a natural setting the conditions will be different, probably with more severe selection pressures forcing individuals to deliver reproductive success on that capacity, and to adjust plastically (using behavioural or physiological mechanisms) or adapt genetically. My work provides some evidence that this is possible, even if it requires further work to establish that it takes place and in what conditions.

Although I found no adaptive changes in males of the thermal selection lines, one of the advantages of laboratory selection studies is that they can be used *'as a means of generating new biological material for study'* (Bennett & Lenski, 1999). With the thermal lines already established and continuing, material has been produced for over 75 generations at the time of writing, which can be capitalised upon for a number of further research programs. Indeed, early work that has continued to make use of these thermal selection lines is showing that after ~50 generations, males in the warmer thermal lines have developed shorter sperm cells, and a greater ability to handle heat-waves (R. Vasudeva & K. Sales, unpublished). It would be interesting to run sperm competition experiments that compare success of the two different thermal

lines within females at different temperatures in order to measure whether sperm cells themselves can become locally adapted to thermal regimes. *Tribolium* females are thought to make mating choices post-copulation (Wade *et al.*, 1993; Lewis & Austad, 1994; Fedina & Lewis, 2006) and whether a male is in a 'defensive' or 'offensive' competition position (Bjork *et al.*, 2007), i.e. proceeding or following a competitor in copulation with a female, makes a significant difference regarding the precedence of his sperm (Arnaud, Gage, & Haubruge, 2001) and therefore the number of offspring he sires. Therefore, any change in a male's relative reproductive success would be strongly selected for and highly advantageous. Genetic markers such as the 'reindeer' marker, an easily distinguished, antler-shaped phenotype from the dominant *Rd* allele, which is selectively neutral, could be used to assign paternity (e.g. Michalczyk, 2008; Tregenza, Attia & Bushaiba, 2009). The ability of different males' sperm to 'win' fertilisations could then be assessed indirectly by placing a male of one of the regimes in sperm competition with reindeer marker males for control stock females, using this to determine paternity of offspring produced by the female. Replicating this for each regime and in 'defensive' (P1) and 'offensive' (P2) mating order would reveal each male's relative reproductive success according to the local thermal regime and his temperature selection background, and therefore whether sperm can be thermally adapted. Additionally, differential sperm precedence is an important post-copulatory, pre-zygotic isolating mechanism (Robinson, Johnson & Wade, 1994), so if there is an adaptive advantage in sperm competition it could indicate that the thermal selection lines are beginning to show reproductive isolation, through differential fertilization, and this would be worth investigating further to shed light on evolutionary responses to climate change. Finally, genomic and molecular analyses could be applied to investigate if sequence changes or expression profiles have occurred under laboratory selection, especially for heat shock proteins or other plastic metabolic responses.

8.2 Predictions and conclusions

If the findings of this thesis using the *T. castaneum* model can be translated to wild populations in the natural environment, it is possible to make some short- and longer-term predictions about the consequences of climate change for reproduction within relevant taxa, and the viability of their populations. As heat-waves increase in number, intensity and duration, the following sequence of events will occur:

Individual male mating behaviour will be affected, with a decrease in the number of matings achieving successful sperm transfer, causing a reduction in insemination success for the females mated with. Combined with a reduction in and damage to sperm cells, far fewer sperm will reach female spermathecal storage. In the next generation, fewer eggs fertilised by that male will hatch, fewer of those that do will eclose from pupae, and fewer offspring fathered by him will reach adulthood. The impacts will end there, with no diminishing of f2 offspring production from the male's sons.

From an individual female's perspective, she may detect that she has been stressed and initially compensate for this, but after mating with stressed males she will be more resistant to consecutive stressed males, although she may more readily mate with males who survived the heat stress in better condition due to variation in thermoresistance. If she is able to detect this variation in male condition she may be able to rescue her fertility via polyandry. Responses to compensate for or resist the changes in heat-waves would come from this mechanism, rather than from male potential for adaptation across generations, which may be limited.

In conclusion, the work contained in this thesis adds to the literature on the connection between heat stress, sperm and infertility, but it further suggests that heat stress affects multiple aspects of reproduction and life history, any of which can influence offspring production after heat stress. As covered above and in chapters 3 and 4, previous research has shown some of the same effects of heat stress on fertility that I found, including life history development and adult offspring viability, as well as implicating both mating behaviour and sperm damage as causes. However, by tracking the impacts across the whole reproductive cycle in a single model, measuring the effects before, during and after copulation, across f1 development and into the f2 generation, my thesis provides more a comprehensive picture of the heat-induced fertility loss phenomenon than is found in previous research. I also provide the first evidence that the negative effects of an environmentally-relevant thermal stress on fertility can be alleviated by polyandry. Although further work is needed, my thesis shows that one key trait of particular sensitivity to climate change and extreme weather is male fertility, and this trait can be key for population viability. If this knowledge is applied to systems where natural range shifts and/or extinctions have taken place in correlation with climate change, such as some butterfly species

(Parmesan *et al.*, 1999; Thomas, Franco & Hill, 2006; Chen *et al.*, 2011), it could be determined whether male-specific infertility is behind these impacts of climate change.

As a model organism, the findings of this thesis pertaining to *T. castaneum* can be generalised to other insect and invertebrate species, but perhaps up to a limit. The impact of thermal stress on sperm is likely to apply across species and taxa, as all dioecious invertebrate species have broadly similar reproductive and gamete biology, and are likely to have reduced sperm counts and damaged sperm cells under thermal stress. Male mating behaviour and offspring development will be more species-specific: not all invertebrate species have comparable life cycles, only holometabolous species will have reduced pupal eclosion for instance, and mating behaviour differs between ectotherms, e.g. sessile groups like coral and barnacles compared to much more mobile forms. However, in species where mating behaviour involves an interaction between male pursuit and female acquiescence it can be predicted that heat stress will curtail male persistence, increase female resistance, and decrease insemination success rate. The employment of polyandry as a mechanism to rescue fertility and limit the impacts of thermal stress would be expected to be available to those species for which polyandry is possible. However, if the selection pressure is strong enough, which is probable if male fertility reduces by 50% or more, and females have access to 80% fewer viable sperm cells, then polyandry may be selected for, even in species tending toward monogamy.

Beyond that, there is no indication that acclimation or adaptation by males will take place, even in more genetically diverse populations. Conversely, impacts on fertility are limited to the generation in which stress took place and in invertebrate species, many of which have multiple generations within a year, the fertility of generations between heat-waves will be able to recover. Therefore, while on average it can be predicted that the impacts of more frequent and intense heat-waves brought about by climate change, will be to cause populations and ecosystems to suffer rather than flourish, there are some ways in which the impacts of thermal stress will be limited or even mitigated.

Planning for climate change should take the findings of this thesis into account, for example where possible conservation efforts should provide the opportunity to encourage polyandry to aid population resilience to heat stress.

Similarly, time between heat-waves could be capitalised upon to restore population sizes. However, according to my selection experiment of a few generations, which indicated limited ability for further adaptation to warmer climates and more severe heat-wave conditions, the assumption that organisms will adapt their thermal reaction norms to cope with climate change cannot be relied upon. The prognosis for the impacts of heat-wave conditions on reproduction, then, is mixed. Hopefully, my thesis contributes knowledge that will reduce the uncertainties, and provide avenues for the future study of, and preparation for, climate change and the heat-waves it causes.

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