Thermal adaptation in a model pest insect

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Thesis submitted for the degree of Doctor of Philosophy

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September 2020

Thesis abstract

Climate change is one of the key issues of our time, causing rapid changes to habitats worldwide and exerting novel pressures on natural populations. Central to understanding species responses to climate change is the process of thermal adaptation, whereby populations adapt to increased or changing temperatures. However, we do not understand how important thermal adaptation is in nature, nor the phenotypes that underpin it. Understanding thermal adaptation in insect species is of particular interest, as they provide many ecosystem services (e.g. pollination, pest control and recycling of organic matter), and are particularly vulnerable to temperature changes due to their inability to thermoregulate. Additionally, several pervasive pest species are insects, so understanding how they will respond to climate change will have major impacts on food security. Here, I investigate thermal adaptation using *Tribolium castaneum*, which is the model beetle and a globally important pest of stored products. I use experimentally evolved populations exposed to control temperature (30°C) or stressful high temperature (38°C) for over 50 generations, to investigate how thermal adaptation occurs and the effects of population demographics and life history on this. I found that high temperature lines had lower overall reproductive output compared to control lines, even when reproduction occurred at high temperatures. I then experimentally increased population size and induced migration into the high temperature lines for 10 generations, to increase levels of genetic diversity, but found that this did not result in increased reproductive output or survival. However, when the experimental populations underwent development at high temperature, I found markedly reduced reproductive output in the control lines, but not the high temperature lines. Development at high temperature affected the fecundity of adult females and the fertility of adult males, and I identified morphological differences in the ovaries and testes of beetles following development at 38°C or 30°C. Furthermore, this adaptation appears to occur rapidly, with observable signals of change in reproductive morphology and increased reproductive output within just five generations. This thesis highlights that there is potential for rapid adaptation in insects, which suggests that there is a chance they may cope with climate change. However, it also demonstrated

the complexity of thermal adaptation, and more in-depth understanding is required to accurately predict the outcomes of changes in temperature.

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Acknowledgements

I have had a fantastic four years working on this and I have been supported by loads of amazing people. I feel incredibly lucky!

Firstly, I owe a huge thanks to my supervisor, Lewis Spurgin for giving me this opportunity. Throughout the years we have worked together, Lewis has been exactly the kind of supervisor I needed at any given time: supportive, approachable and encouraging and thanks to his fantastic supervision I have grown in confidence and competence and I'm so proud of what I've achieved here. Thank you, Lewis, for always looking out for me and for guiding me through this.

Thanks as well to Matt Gage, my secondary supervisor, who has provided great input and a sense of humour throughout, alongside DIY incubator fixing advice and screwdrivers. Thanks also to David Richardson and Tracey Chapman, who both stepped in at my peak thesis stress and agreed to do an emergency chapter read through to help me submit my thesis before my wedding, it means a lot to me.

To everybody who has been part of Team *Tribolium* during my time here, it's been awesome! The lab has been such a lovely friendly environment in which to work and I'm grateful to all of you for that: James, Kris, Ram, Andreas and Mike. An extra special thanks to the 'beetle babes', Lucy Friend and Joanne Godwin, for their endless humour, patience, wisdom and moral support (and in Lucy's case, endless tedious beetle counting!).

My PhD would not have been half as fun as it has been without all of my fantastic colleagues across CEEC, thank you all! In particular, thanks to Ryan, for being a great housemate for most of my PhD, and to Charli for always being there for a coffee or bakery run. Thanks to the Zekes: Ellen, Ents, James, Jen and Tom, for the absolute hilarity that was our office - especially to Ellen who has been such a good friend throughout. A big part of my last few years has been developing my teaching and Kelly and Bethan have made that possible, enjoyable and successful!

I am so lucky to have an absolutely amazing family who have supported me completely in everything I've ever tried to do. Mum, Dad and Amy, I can never thank you enough for all you have done for me over the past 26 years, you're the best! My incredible Mum, Alison Lewis, deserves an extra acknowledgement for measuring hundreds of photos of beetle testes for my Chapter 5.

Finally, special thanks to Joe Ribeiro, my almost husband, for his unwavering love and support. There are no words.

Chapter 1

General introduction

1.1 Climate change and thermal adaptation

The Earth undergoes natural cycles of warming and cooling, but in recent decades, the warming has taken place at an unnatural rate as a result of anthropogenic activity (Ring *et al.*, 2012). In addition to rises in global average temperatures, climate models predict an increase in unusual events, such as heatwaves, floods and heavy rainfall (Mitchell *et al.*, 2006). Furthermore, the melting of glaciers and ice sheets at the poles and the increase in temperature is causing sea levels to rise, which can result in loss of coastal habitats (Galbraith *et al.*, 2002). The effects of climate change are complex, exposing species to new and extreme climatic variables.

Climate change poses a threat to biodiversity (Botkin *et al.*, 2007), and interacts with other pressures such as overexploitation, habitat loss and pollution (Horn & Stephens, 2006; Trathan *et al.*, 2014). When faced with changes in temperature, species must respond through migration to regions with a suitable climate or through adaptation to withstand the new temperatures, otherwise they may face extinction (Aitken *et al.*, 2008; Feeley *et al.*, 2012). As warming progresses, the likelihood of species facing extinction if they fail to adapt or migrate increases. When species shift their ranges in response to climate change, it tends to be to either higher latitudes or higher elevations, where the temperature is cooler and therefore closer to the species' optimum (Chen *et al.*, 2011). The resulting addition or removal of a species from an ecosystem can have dramatic, knock-on effects for other species (Klanderud, 2004). Additionally, populations and species can undergo phenological shifts, producing new species interactions due to new temporal overlap in presence (Yang & Rudolf, 2009).

Species interactions can be complex, so it is useful to be able to predict whether a given species will migrate to find a region with a suitable temperature, or adapt to tolerate the changes in their current habitat (Franks & Hoffmann, 2012). Before we are able to make such predictions, further study into the mechanisms behind thermal adaptation are required. In addition to this, we need to understand whether it is

possible for thermal adaptation to take place at the necessary rate to keep up with global warming.

The thermal tolerance of individuals and populations can be affected by many variables, including genetic, phenotypic and age-related factors (Bowler & Terblanche, 2008; Sørensen, 2001). It has been shown that the lower limit of temperature tolerance is more variable than the upper limit, in both inter- and intraspecific insect studies (Chown, 2001; Kimura, 2004; Terblanche *et al.,* 2007). This could indicate an upper limit to physiological tolerance of temperature which restricts the ability of populations and species to adapt to higher temperatures. It is thought that tolerance to increased temperature is evolutionarily conserved, whereas tolerance of low temperatures may have evolved on multiple occasions (Araújo *et al.*, 2013). However, there is a lack of literature about the mechanistic and genetic basis of phenotypic responses to temperature changes. By increasing our mechanistic knowledge of thermal adaptation, we could gain more understanding of why thermal limits exist, as well as potentially increasing the accuracy with which we predict species responses to climate change (Porcelli *et al.*, 2015).

1.2 Population size, diversity and adaptation.

The number of individuals that make up natural populations varies enormously, both within and between species. The size of an individual population can drastically vary at different times, due to life history variation, changes in the environment, stochastic effects, or anthropogenic disturbance (Lande, 1998). This variation in population size has wide-ranging consequences for the dynamics of populations and species, over both ecological and evolutionary timescales.

A reduction in population size generally results in reduced levels of genetic diversity as a result of genetic drift and inbreeding. Genetic drift causes decreased genetic diversity in small populations because with each generation only a sample of the existing alleles are passed on from the previous generation (Ellstrand & Elam, 1993). In

small populations, this results in random fluctuations in allele frequencies, and increased probability of losing rare alleles (Allendorf, 1986; Sisterson *et al.*, 2004). Levels of inbreeding are also generally negatively correlated with population size, because the pool of potential mates will contain more related individuals in small populations. Inbreeding decreases genetic diversity within populations by increasing homozygosity and levels of relatedness (Wright, 1977). A consequence of this increased homozygosity is that it allows deleterious recessive alleles to be expressed more frequently in the population. Additionally, heterozygosity is decreased, so advantageous heterozygous phenotypes are expressed less frequently. Overall there is usually a population-wide reduction in fitness as a result of inbreeding, which is known as inbreeding depression (Wright, 1977). Inbreeding depression has been studied as far back as Darwin (1876), who observed its effects across several plant species. It has been found to have detrimental effects across plants (Richards, 2000), animals (DeRose & Roff, 1999) and humans (Ceballos & Álvarez, 2013). However, there are occasionally benefits associated with inbreeding, such as increased tolerance to high temperatures (Kristensen *et al.*, 2005).

A longstanding debate in the study of inbreeding is the extent to which it can purge deleterious mutations in small populations (Hedrick, 1994). Random genetic drift has a stronger effect in small populations, resulting in the propagation of recessive deleterious alleles, which are able to accumulate because selection can only act on them when they are expressed due to homozygosity. Inbreeding increases homozygosity, which exposes these alleles to selection, enabling them to be purged. (Keller & Waller, 2002). However, this process is dependent on various genetic factors, and therefore the efficiency and extent of purging can be very low (Bijlsma *et al.*, 2001). The optimal conditions for purging are: a large effect allele, which will be selected against rapidly once exposed; low levels of linkage between loci; gradual long-term inbreeding and population isolation to prevent reintroduction of purged alleles (Byers & Waller, 1999; Keller & Waller, 2002)

1.3 Migration, diversity and adaptation

Migration is the movement of individuals between populations or sub-populations. When a population is structured (for example, due to a fragmented habitat) the sub-populations diverge from one another due to genetic drift and selection. Migration between these sub-populations facilitates gene flow, which is the transfer of genes from the gene pool of one population to another. Gene flow between populations, or sub-populations, counteracts the effects of genetic drift and decreases overall levels of genetic structure.

Gene flow into an inbred population can increase levels of genetic variation and fitness – a process known as genetic rescue (Tallmon *et al.*, 2004). However, the relationship between the level of gene flow into a population and fitness is not necessarily linear. When genetic distance between outcrossing individuals reaches a certain point, the offspring can suffer outbreeding depression, whereby the hybridisation of individuals from different genetic backgrounds leads to reduced fitness in the resulting offspring (Fenster & Galloway, 2000). Outbreeding depression is often attributed to the separation of beneficial epistatic gene interactions which can develop within a population (Lynch, 1991; Marsden *et al.*, 2013). At present, however, we have limited understanding of when gene flow into a population will lead to genetic rescue, and when it will lead to outbreeding depression (Bell *et al.*, 2019).

Outbreeding depression can also occur when gene flow inhibits local adaptation (Garant *et al*., 2007). Adaptation to local environmental conditions results in increased individual fitness in specific environments, at the cost of decreased adaptive capability in the event of any environmental change (Aitken & Whitlock, 2013). Local adaptation is therefore beneficial if the environment is stable, because a population can become specialised and fill an adaptive niche. In these conditions, gene flow can be detrimental (Lenormand, 2002), because by introducing new alleles with each generation, gene flow can prevent fixation of beneficial alleles. However, in changing environments gene flow is expected to be beneficial, as the introduction of novel alleles can facilitate

adaptation to the new environmental conditions. Climate change can therefore be particularly detrimental for locally adapted populations when there is limited gene flow (Kremer *et al*., 2012).

Local adaptation can also directly influence migration rates and levels of gene flow. In general, individuals in locally adapted a populations are less likely to migrate (Drown *et al.*, 2013). This could be seen as an adaptation to prevent a loss of fitness due to the disruption of beneficial interactions between genes, or due to the introduction of genes less fit for the local environment (i.e. outbreeding depression; Edmands, 2007). Highly locally adapted populations are expected to suffer most from outbreeding depression, because they are likely to be more genetically differentiated from immigrating individuals. A corollary to this is that when migration does not occur, levels of inbreeding will increase, especially within small populations, and the detrimental effects of inbreeding will break down local adaptation (Falconer & Mackay, 1996; Höglund, 2009). Populations therefore exist in an equilibrium between local adaptation and inbreeding depression, maintained by migration and dependent on population size.

1.4 Life history and adaptation

The effects of demography on adaptation can vary depending on the life history of a species (Végvári *et al.*, 2009). For instance, species with short lifespans are likely to be under different selection pressures (e.g. high reproductive rates, fast rates of development) compared to long-lived species (e.g. increased parental investment, lower reproductive rates). In populations and species with long generation times, anthropogenic global warming is likely to progress further between each generation than in a species with very short generation times. However, due to various life-history differences, short-lived species are usually more vulnerable to environmental changes caused by global warming (Dalgleish *et al*., 2009), as well as to demographic fluctuations (Dunham & Overall, 1994). The increased climatic variability that is occurring due to global warming also has the strongest negative impacts on short-lived

species, in terms of both survival and reproduction (Morris *et al.*, 2008). A suggested reason for this is that short-lived reproduce regularly over short timeframes, whereas reproduction in long-lived species occurs over a longer time period, so populations are more robust to fluctuations in reproductive rate (Morris *et al.*, 2008). The reproductive strategies employed by populations and species can also be altered in response to climate change. Studies in various taxa have noted a shift towards semelparity (a single reproduction event) from iteroparity (reproduction on multiple occasions) in more challenging environments (Smith & Charnov, 2001; Nahrgang *et al.*, 2014). Evolutionary theory suggests that semelparity is advantageous in a varying environment, but iteroparity is advantageous in a stable environment (Schaffer, 1974).

In all organisms, the timing and duration of stress has implications for the outcome. In most organisms, as adults age, their fitness is reduced (Lemaître *et al.*, 2015). Stress experienced during development can have therefore have delayed consequences (Tschirren *et al.*, 2009), while stress experienced late in life can be associated with a decrease in the efficacy of stress response mechanisms and increased likelihood of fatality (Liu *et al.*, 1996). This interaction between age and stress response varies widely among species, in response to variation in life history and mode of development. For instance, in some species sex-determination is based on the environment experienced during the embryonic development (e.g. photoperiod - Naylor *et al.*, 1988; or temperature - Janzen, 1994) and therefore, sex ratios are heavily influenced by environmental changes. This is a concern for conservation, as these species (mostly reptiles and fish) may face populations declines as global warming increasingly raises average temperatures resulting in single sex progeny (Mitchell *et al.*, 2008).

In insects, holometabolism allows specialisation in different life stages, and therefore, maximal adaptability (Yang, 2001). Having such distinct life-history stages makes insects a particularly important group in which to study the effects of developmental stress. Although the effect of heat stress on adult insects has been well studied (Kingsolver *et al.*, 2013; King & MacRae, 2015), we still do not understand the effects

of stress encountered at different insect developmental stages, on both immediate survival and long-term fitness. In changing environments, generalism is often a favourable strategy (Reboud & Bell, 1997). This leads to the question of how thermal stress at the different life-history stages in holometabolous insects affects population and species responses to changing climates.

1.5 Experimental evolution

To understand how climate change may affect populations, communities and ecosystems, there are several approaches that researchers can use. Studies on wild populations can focus on specific ecosystems or habitats (e.g. the Arctic – Berteaux *et al.*, 2004; or alpine ecosystems – Fagre *et al.*, 2003), comparing populations of a species across different latitudes (e.g. Andrew & Hughes, 2004), or following changes in populations over time (Jump *et al.*, 2006). While such approaches offer 'ecological realism', it can be difficult to tease apart the complex interacting effects of biotic and abiotic variables on individuals and populations without experimental control.

There are several advantages of studying responses to environmental change in a laboratory population compared to natural populations. Firstly, using laboratory populations allows for the inclusion of replicates and controls, which is usually impossible to achieve with natural populations (Kawecki *et al*., 2012). Secondly, exact conditions can be chosen and controlled at all times. It is therefore possible to design experiments to tease apart the effects of an individual demographic factors or selection pressures, which is usually impossible to do in a natural environment. Once individual effects are well understood, we can begin to study the interactions between these processes to understand the population-level impacts of environmental change (Kawecki *et al*., 2012). The main drawback of the laboratory approach is that it is, by definition, artificial, and it is not always clear whether findings apply to real-world scenarios. There is much scope for research to bridge the gap between laboratory and natural population study, including "ecologically realistic" laboratory experiments, mesocosm experiments (Scheinin *et al.*, 2015), and combining laboratory and field

studies in the same organism (Zhan & McDonald, 2013). A further promising development in the study of wild populations is the use of museum samples to provide some insight into historical genetic variation, which may allow for temporal observations of adaptation at the genetic level (Cooper, 1994).

Experimental evolution offers a powerful tool to study the effects of environmental change at the level of genes, individuals and populations. This approach involves studying laboratory populations over multiple generations whilst subjecting them to a chosen selection pressure. Laboratory organisms usually have short generation times, allowing evolutionary adaptation to be observed within researcher lifespans, and allowing time-series data to be collected. Increasingly, therefore, experimental evolution is viewed as a powerful method for studying adaptation (Kawecki *et al*., 2012).

Experimental evolution has been widely used to study thermal adaptation across a range of organisms (e.g. Bennett & Lenski, 1999; Chakravarti & van Oppen, 2018; Mazzucco *et al.*, 2020). One common approach to studying adaptation experimentally is a technique called evolve and resequence (E & R), in which replicate populations are evolved at different temperatures and specific regions of DNA, or entire genomes, are sequenced periodically, enabling the collection of longitudinal data sets (Schlötterer *et al*., 2015; Iranmehr et al., 2017). E & R is increasingly being used in experimental studies of thermal adaptation, and studies suggest that genomic responses to novel temperatures are complex, involving many regions of the genome (e.g. Mallard *et al.*, 2018; Jakšić *et al.*, 2020). Surprisingly, there has been a limited amount of detailed phenotypic work on experimental evolution lines subjected to different temperatures.

1.6 Study system

Tribolium spp are flour beetles in the family Tenebrioinidae, the ancestors of which are thought to have originally adapted to living in rotting logs, or beneath tree bark

(Dawson, 1977). This life-history is likely to have pre-adapted them for infestation of human stored products, which has become their primary habitat and they have become a globally important pest (Sokoloff, 1974). Dispersal between grain stores is thought to have primarily been facilitated by anthropogenic movement, but some literature suggests that flight may also be a factor (Ridley *et al.*, 2011; Gurdasani *et al.*, 2019).

Tribolium provide a tractable study system: easy to rear, with a short generation time and high fecundity: features which make them an ideal laboratory model. *Tribolium* have been widely used as experimental models (Park, 1935; Desharnais & Liu, 1987; Denell, 2008) particularly in evolutionary research (Park *et al.*, 1964, Wade, 1976). The red flour beetle *T. castaneum* has become the primary model beetle, with excellent resources on phenotypes (Sokoloff, 1972), a sequenced genome (Tribolium Genome Sequencing Consortium, 2008), and other genomic resources (Posnien *et al*., 2009). Experimental populations of *T. castaneum* have been maintained at the University of East Anglia since 2004, facilitating research focusing on sexual selection (e.g. Godwin *et al.*, 2020), inbreeding (e.g. Michalczyk *et al.*, 2011) and sperm morphology and function (e.g. Vasudeva *et al.*, 2019). Of particular relevance to this thesis, is the previous research focused on the effects of high temperature on sperm (Dickinson, 2018). Recently, this group has shown that heatwaves damage sperm in *T. castaneum*, leading to reduced reproductive output and transgenerational effects (Sales *et al.*, 2018). In addition to this, thermal plasticity in gamete morphology has been described (Vasudeva *et al.*, 2019). This thesis utilises these resources and is informed by these results, with a focus on the effects of sustained high temperature, and the mechanisms of adaptation to this.

1.7 Thesis aims

In this thesis, I explore the consequences of elevated temperatures for fitness and evolutionary adaptation. In Chapter 2, I quantify fitness of adults from long-term thermal selection lines evolved at high and control temperatures. I use reproductive

output of pairs and individual survival through a heatwave as components of fitness. In Chapter 3, I investigate the effects of increased population size and assisted migration over several generations on adaptation to high temperature in the long-term high temperature selection lines. I also explore the effects of outcrossing between individuals from different populations on offspring fitness. In Chapter 4, I test for an effect of developmental environment on adult fitness at high temperature, and quantify sex-specific effects of developmental temperature on adult fertility and fecundity. I also explore how the timing of developmental stress affects adult reproduction. In Chapter 5, I test for evidence of adaptation over five generations of exposure to a novel thermal environment. I quantify how fitness changes over generations, and test for evidence of rapid phenotypic change using measurements of reproductive organs. Finally, I discuss the results from the thesis in the context of evolutionary understanding and conservation.

1.8 Chapter contributions

This project was funded by the University of East Anglia, under the supervision of Dr. Lewis Spurgin and Prof. Matthew Gage. Long-term thermal selection lines and stocks were maintained by Dr. Ramakrishnan Vasudeva, Dr. Kris Sales, Dr. Lucy Friend and the author. Dr. Lucy Friend and Dr. Lewis Spurgin contributed to line maintenance, experimental procedure and data collection for all chapters. All experiments were designed and led by the author, all analysis was carried out by the author and all chapters were written by the author. Chapter 2 was reviewed by Dr. Lewis Spurgin and Prof. Matthew Gage. Michael Pointer and Sophia Barbano assisted with data collection for Chapter 3, which was reviewed by Dr. Lewis Spurgin and Prof. Matthew Gage. Chapter 4 was reviewed by Dr. Lewis Spurgin and Prof. David Richardson. Dr. Lucy Friend performed some dissections for Chapter 5 and Alison Lewis assisted with data collection, particularly measuring testes images. Chapter 5 was reviewed by Dr. Lewis Spurgin and Prof. Tracey Chapman.

Chapter 2

Adult fitness in a long-term thermal adaptation experiment in *Tribolium castaneum*

Abstract

The pressure to adapt to higher temperatures is expected to increase for species across taxonomic groups as climate change occurs. However, we have limited understanding of how natural selection enables populations to adapt to temperature change over relatively rapid timescales, and this information will help inform areas such as conservation and pest management. The widely-distributed Coleoptera contains the highest number of known species of any animal Order, including many of conservation concern and several notable pests, making them a relevant group for the study of how populations respond to temperature change. Here, I use the red flour beetle, *Tribolium castaneum,* a globally important pest of stored products and an established research model in the life sciences, to study thermal adaptation through controlled experimental evolution in the laboratory. I use *T. castaneum* lines, established at control (30°C) and high temperatures (38°C) to investigate thermal adaptation following 54 generations of replicated selection. Specifically, I aimed to quantify the extent of adaptation to increased temperatures within two key components of fitness: i) survival through a standardised heatwave and ii) reproductive output. Following experimental evolution, I found that high temperature lines exhibited decreased reproductive output and reduced survival through a heatwave, compared to control lines. Moving high temperature lines to the 30°C control temperature prior to carrying out fitness assays significantly improved fitness, but rates of survival and reproductive output remained far below those in the control populations. Furthermore, the control lines maintained higher fitness than the high temperature lines, even after being moved to the high temperature environment prior to the fitness assays. Results therefore revealed an overall negative effect of experimental evolution at higher temperatures, and no evidence for adaptation in capacity to survive heatwaves or reproduce in 38°C temperatures. Further investigations into the effect of developmental temperature and genetic diversity in the high temperature lines may help explain these effects.

2.1 Introduction

With the increasing severity of global warming, it is important to understand how species respond to changes in their thermal environments. In particular, there is substantial interest in understanding how increased temperature affects organisms at the phenotypic and genetic levels (Sørensen, 2001; Bowler & Terblanche, 2008) and the consequences of this for population persistence. Many species exhibit phenotypic plasticity, which can enable populations to buffer small fluctuations in environmental temperature, but plasticity alone is not expected to be sufficient to tolerate long-term temperature rises (Gienapp *et al.*, 2013; Seebacher *et al.*, 2015)**.** Broadly speaking, in order to avoid extinction, species must either adapt to tolerate increases in temperature, or migrate towards the poles and follow their optimum temperature as it shifts away from the equator (Gienapp *et al.*, 2008; Porcelli *et al.*, 2015).

Rises in temperature, beyond those normally experienced, will exert environmental stress on organisms, producing selection pressure. However, phenotypic responses to temperature rise may be complex and difficult to quantify. Even when we can identify a phenotypic change that correlates with spatio-temporal variation in temperature, it can be difficult to establish whether this is adaptive (Merilä & Hendry 2014). Partly as a consequence of this, studies that have been able to predict whether species are capable of adapting to temperature change, and if so, how quickly, are uncommon. Theoretical models suggest that there are upper limits to rapid evolutionary change in response to climate, but few studies have tested this experimentally (Kopp & Matuszewski, 2013).

A commonly-used approach to studying adaptation to temperature or climate is to sample or observe natural populations from a range of environments (Porcelli *et al.*, 2016). The benefit of this approach is that it enables scientists to observe adaptation to climate in realistic ecological settings. However, the complexity of natural populations, due to the numerous confounding demographic, ecological and environmental factors, make it difficult to tease apart the causes and consequences of

temperature change (Merilä & Hendry, 2014). Experimental studies are therefore useful in this respect, as key aspects of an organism's environment can be closely controlled while specific, individual environmental variables such as temperature can be manipulated (Fry, 2003; Magalhães & Matos, 2012). Model organisms with short generation times can be maintained in novel environments over multiple generations, allowing us to observe and measure evolutionary responses to environmental change in real-time. Using experimental evolution in model organisms can therefore be a powerful tool for understanding these processes and how evolution operates in natural populations (Kawecki *et al.*, 2012). A first step towards understanding thermal adaptation is to examine fitness responses to increased temperature in relevant traits in experimentally evolved populations (Tobler *et al.*, 2015). Recent experimental evolution studies in *Drosophila* have shown that local adaptation to higher temperature can result in increased fertility after heat stress (Porcelli *et al.,* 2016), and that increased fitness can evolve in response to fluctuating thermal environments (Tobler *et al.,* 2015).

Insects are thought to outnumber all other animals and plants together in species richness (Romoser & Stoffolano, 1998). Due to their small body sizes and inability to thermoregulate, insects are particularly sensitive to changes in temperature (Overgaard *et al.*, 2014). In ectotherms, thermal tolerance breadths generally increase with latitude, as expected based on climate variability (Sunday *et al.*, 2011). However, there is relatively little variance in critical thermal maxima with latitude (Addo-Bediako *et al.*, 2000; Clusella-Trullas *et al.*, 2011; Araújo *et al.*, 2013), which suggests that tropical ectotherms may be more at risk from global warming than the more polar species (Hoffmann *et al.*, 2012). For example, the expected range shift in Australian *Drosophila* was modelled based on current critical thermal limits which predicted a southward range shift towards the more extreme latitudes (Overgaard *et al.*, 2014), and this prediction has been widely supported (Ghalambor *et al.*, 2006; Deutsch *et al.*, 2008; Kingsolver *et al.*, 2011).

The speciose Coleoptera include many economically important crop pests, invasive species and species of conservation concern (Crowson, 1981). The red flour beetle *Tribolium castaneum* is a model organism for the Coleoptera and a globally important pest of stored grains. The biology of *T. castaneum* has been well studied at a range of levels, and it has consistently shown itself to be a useful organism in the study of ecology and evolution. It has been used in studies of population demographics, dynamics and founder effects (Park, 1954; Leslie *et al.*, 1968; Szücs *et al.*, 2017), as well as sexual selection and extinction studies (e.g. Lumley *et al.*, 2015; Godwin *et al.*, 2017). Additionally, *T. castaneum* has been used extensively in research into responses to temperature (e.g. Mahroof *et al.*, 2003; Scharf *et al.*, 2016; Sales *et al.*, 2018).

In this study, I use experimental evolution to understand the adaptive responses among independently replicated lines of *T. castaneum* after 54 generations of selection at either control (30°C) or high temperatures (38°C). I assayed adaptation through two key challenges: a) the reproductive output of individual mating pairs from either selection regime under different thermal conditions, and b) the survival tolerance of individuals to heatwave conditions. I first measured the reproductive output of mating pairs from the experimental lines at both their "evolved" and "non-evolved" temperatures, to quantify the cost of high temperature, and to test whether adaptation has occurred. I then tested how individuals from the different experimental lines survived through heatwave conditions close to their thermal limit of 42°C, to test whether 54 generations of experimental evolution through increased temperature can result in an increased ability to tolerate thermal stress. The results of these fitness assays are discussed in the context of climate change.

2.2 Methods

2.2.1 Stocks and thermal selection lines

The thermal selection lines used throughout this thesis were established from an outbred stock population known as the Krakow Super Strain (KSS). This was created in 2008 by Dr. Paulina Kramarz, who encouraged genetic mixing between 11 different *T. castaneum* strains, originating from various locations worldwide (Michalczyk, 2008; Dickinson, 2018). The KSS stock populations are maintained in two replicates of 300 individuals. Each generation, approximately 150 adults from each replicate are combined in fresh fodder, in a container measuring 12 x 12 x 12 cm. In a separate container, a further 150 adults from each replicate are combined (Figure 2.1), resulting in 300 adults used to sire each replicate of the next generation, with 600 in total. This method prevents divergence between the replicates, as well as acting as a precaution, maintaining the line if one replicate is lost.

The thermal selection lines were established from the KSS stock in 2010 (Dickinson, 2018). These lines are maintained at control (30°C) and high (38°C) temperatures at 60% relative humidity (RH), with 14 independently replicated populations per treatment. The optimal temperature for population productivity under laboratory conditions in *T. castaneum* is 32°C (Howe, 1962), measured by egg hatch rate ad larval development rate. Therefore, the control temperature is 2°C below optimal, and the warm regime is 6°C above. This control temperature was selected because it is the most commonly used temperature for maintaining *T. castaneum* stocks (Beeman *et al.*, 2019). It is also the temperature that the source population had been kept at, so seemed to be the best choice of temperature for our goal of minimal selection pressures on these lines. The high temperature of 38°C was chosen to impose a strong selection pressure, whilst still being below the thermal fertility limit for *T. castaneum*. These lines are maintained in round containers (diameter 7cm, depth 7.5cm), with ~100g fodder (10% Brewer's yeast, 90% organic, strong white bread flour), topped with a thin layer of oats. Every generation, exactly 100 adults are counted and placed onto fresh fodder to mate and to lay eggs at their assigned temperature. After one

week, these adults are removed and the fodder, oats and eggs are returned to the assigned temperature regime for the eggs to develop to adulthood. After 35 days (at control temperature) or 28 days (at high temperature), 5-10 days after the adults reach sexual maturity, 100 of the adults that have developed are transferred onto new fodder to found the next generation (Figure 2.2). *T. castaneum* have a reduced development time at 38°C, presumably due to an increase in metabolic rate and, as a result, the number of generations of selection differs between the regimes. I do not anticipate this discrepancy in generations to affect the results of any experiments, as 30°C is a control treatment, not expected to impose any stress or selective pressure.

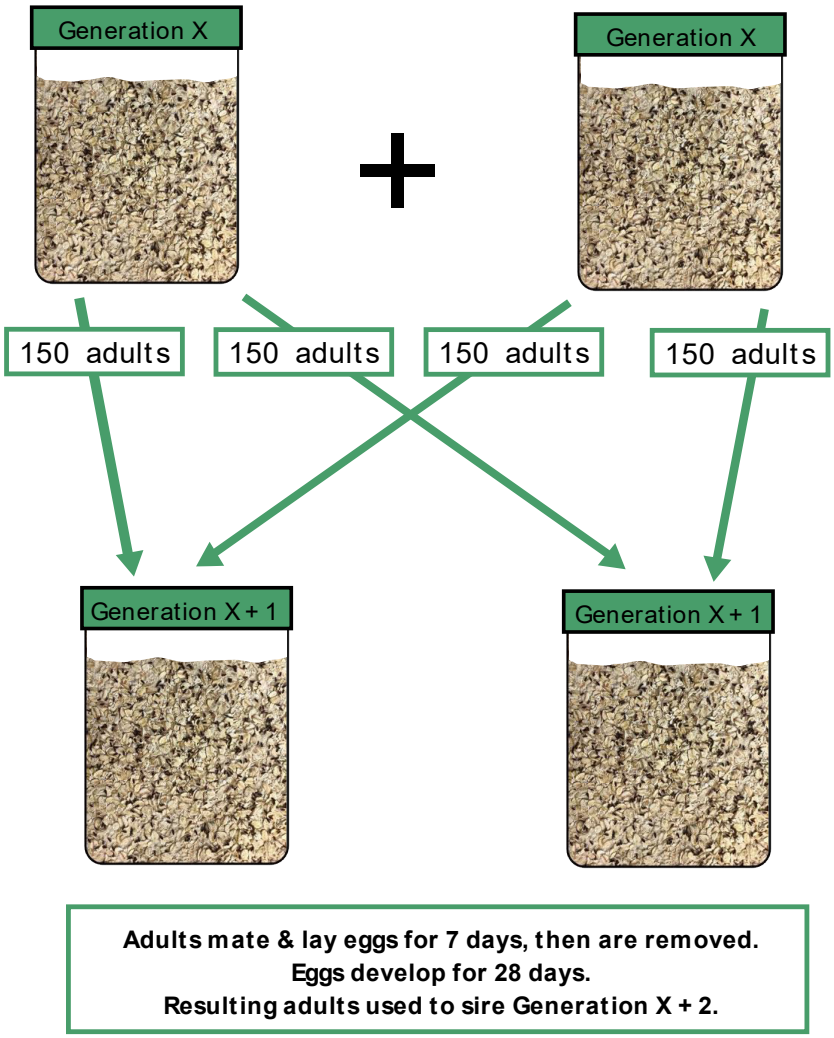

Figure 2.1. The line maintenance of the KSS stock population, repeated every 35 days. Each generation, *T. castaneum* from the tubs are mixed, as shown, to prevent genetic divergence.

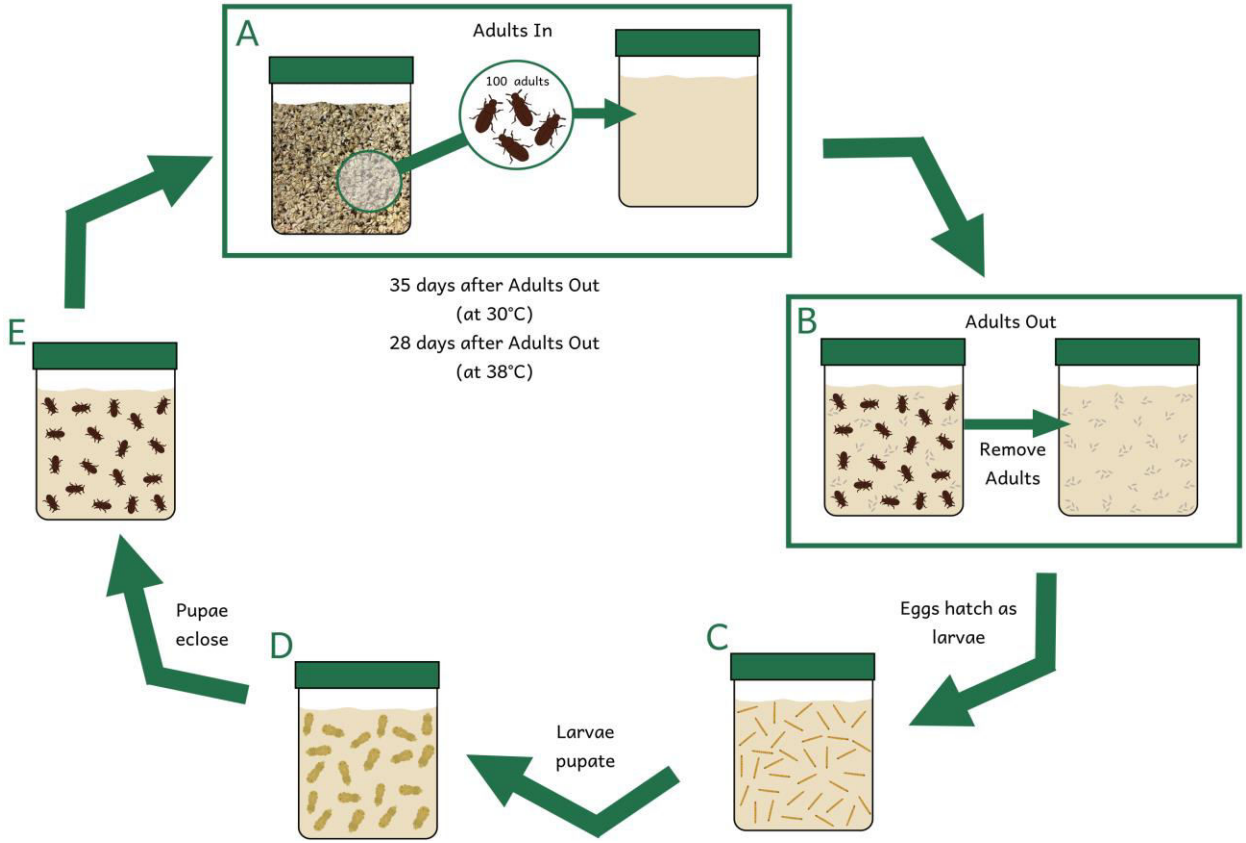

Figure 2.2. Maintenance protocol for thermal selection lines of *T. castaneum*. A) 100 random adult beetles are counted from a population and moved into clean fodder to mate and lay eggs, siring the next generation. (**B**) Those 100 adults are removed from the fodder one week later, leaving the eggs remaining. (**C**) The eggs hatch into larvae. (**D**) The larvae pupate. (**E**) The pupae eclose as adults. Ten days after eclosion, they will be reproductively mature, ready for experiments or the next generation of line maintenance (**A**).

2.2.2 Experimental procedures

The following experiments were carried out using these long-term thermal lines maintained at either 30°C or 38°C. At the time of these experiments, 54 generations had passed for the high temperature lines, while 47 generations had passed for the control lines. Three randomly-selected replicate populations from each selection regime were used for these experiments.

Reproductive output from multiple mating pairs was measured from each of the six replicate lines. At 12 days (at 38°C) or 16 days (at 30°C, to account for difference in development times) after adults were removed from their week of mating and laying in their stock populations (Figure 2.2B), 80 male and 80 female pupae were isolated from each line (sex was decided based on the appearance of genital papillae; Park, 1934) and separated by sex into groups of 20 for eclosion. These numbers allowed for excess pupae to account for any escapes, deaths or deformed adults. At 14 days after eclosion, these virgin males and females were paired for mating in 7ml vials containing approximately 1.5g fodder. Replicate pairs from each population were then mated at 30°C (n = 30 pairs) or 38°C (n = 30 pairs) for 24 hours (Figure 2.3). After the mating period, individual females of these pairs were transferred into a petri dish containing ~7g fodder and allowed seven days to oviposit at their mating temperatures (Block 1). After seven days, the females were transferred into new petri dishes with clean fodder to oviposit for a further seven days (Block 2). This was repeated up to 70 days (Block 10), at which point the females were discarded. The eggs laid in each petri dish were reared for 35 days (at their assigned rearing temperature) to develop to maturity before being frozen and adult offspring counted.

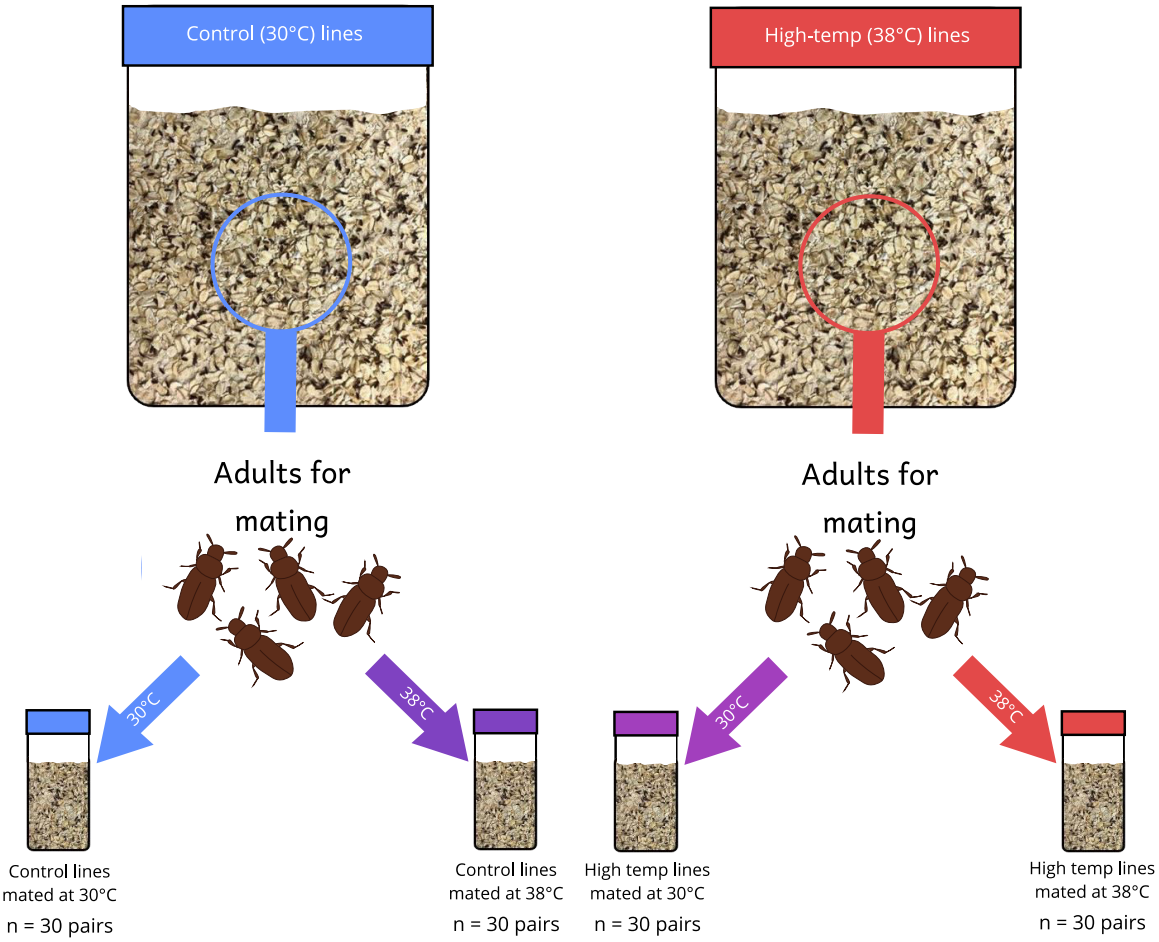

Figure 2.3. Reproductive output experimental protocol. Pairs of *T. castaneum* from three control (30°C) lines and three high temperature (38°C) lines were allowed to mate for 24 hours. From each line, 30 pairs were mated at 30°C and an additional 30 pairs were mated at 38°C to control for any plastic effects of mating temperature. The resulting eggs were reared to maturity at the allocated mating temperature and the adult offspring were then counted.

For the heatwave survival assay, approximately 600 adults were isolated from each replicate population (measured by volume, approx. 3ml) at reproductive maturity (28 days after laying for the 38°C lines, and 32 days for the 30°C lines). For one week immediately preceding the assay, half of the individuals from each line were kept at their native 'evolved' regime temperature of either 30°C or 38°C, while the other half were switched to the opposite, novel temperature of either 30°C or 38°C (Figure 2.4). After this one-week exposure period, each group of 300 individuals was put into a container with ~100g fresh fodder and oats, and exposed to a 42°C heatwave at 60% relative humidity (RH). Each day, dead beetles (identified by their complete immobility) were counted and removed. When five or fewer beetles remained alive in a population, they were transferred to smaller petri dishes containing ~7g fodder for the remainder of the experiment to ease daily counting. The heatwave assay continued for 20 days until all beetles had died.

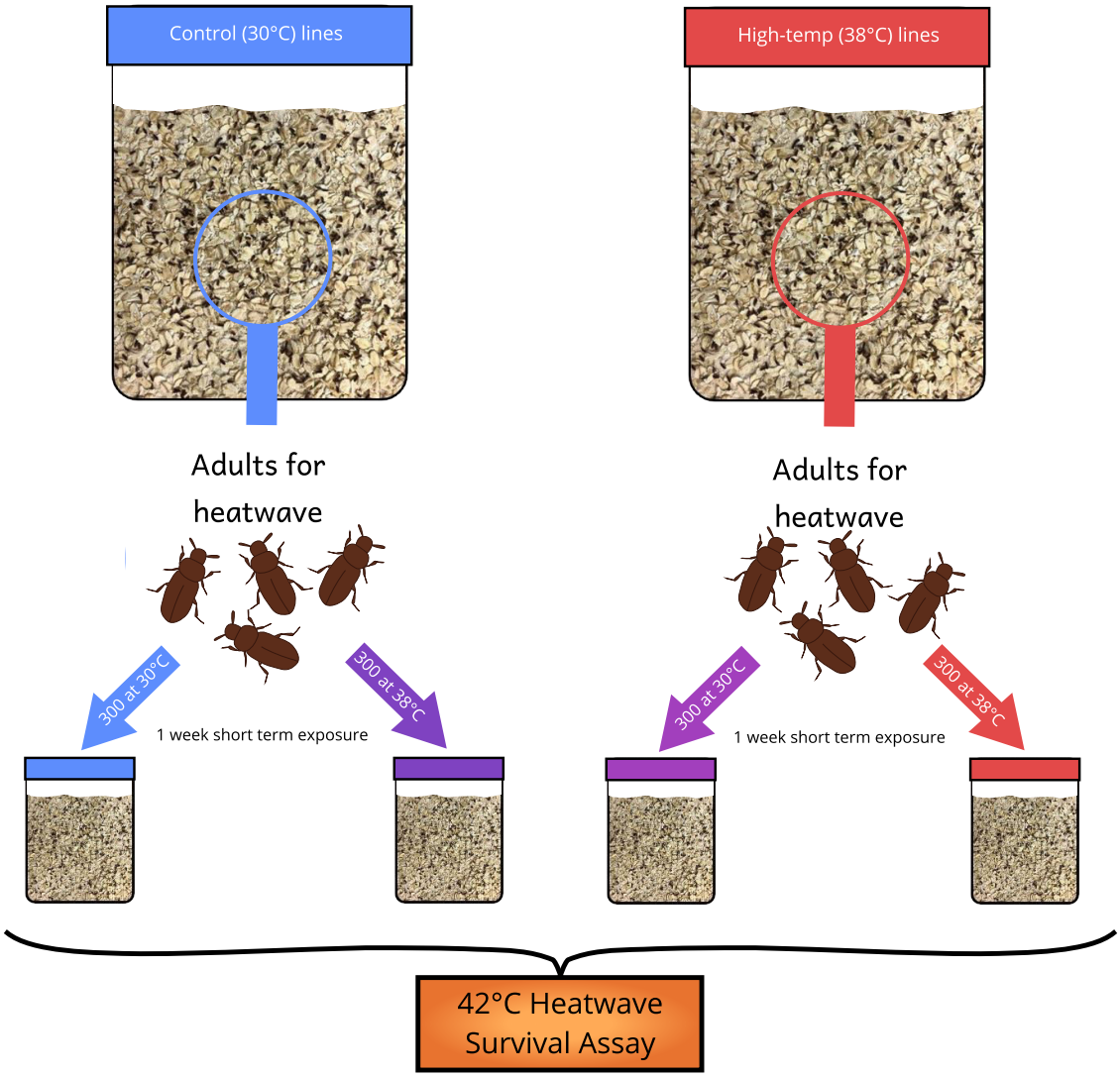

Figure 2.4. Heatwave survival assay experimental protocol. Adult *T. castaneum* were removed from long-term thermal selection lines and subject to short-term (one week) exposure to either their native temperature (n ~ 300) or a novel temperature (n ~ 300). After this exposure, the beetles were put into a 42°C heatwave and the number dead was recorded daily, until all treatments had gone extinct.

2.2.3 Statistical analyses

All analyses were carried out using R version 3.3.1 (R core team, 2016), implemented in RStudio version 1.0.136 (2009-2016). All generalised linear mixed models (GLMMs) were conducted using the 'lme4' package (Bates *et al*., 2018), while survival analyses were conducted using the 'survival' (Therneau, 2015) and 'coxme' (Therneau, 2018) packages.

To model reproductive output over time, a GLMM was fitted with Poisson error distribution, using the number of offspring produced each week as the response variable. As explanatory variables, I fitted the ancestral temperature of the line, the temperature the individuals were mated at, and number of weeks passed between mating and laying. The line ID of the mating pair was fitted as a random effect, as was the unique identity assigned to each ovipositing female. I constructed additional, separate models to identify each interaction between fixed effects, to allow for easy interpretation of the fixed effects in the main model. These models were constructed as above, but with an observation-level random effect to reduce overdispersion (Harrison, 2014). Only the effects of the interaction terms are reported from these models. To model the total reproductive output of the pairs, a GLMM was constructed as above, but with the total number of offspring per female entered as the response variable, and ancestral and mating temperature as the only two fixed effects. An observation-level random effect was included to account for overdispersion. Finally, I tested how short-term reproductive output varied among treatments using the same approach, but replacing total offspring counts with offspring counts from just the first week of laying. The total offspring count over 70 days are presented as a box and whisker plot. Here, and in all boxplots in this thesis, the midline represents the median, the two ends of the box represent the 25^{th} and 75^{th} quantiles and the whiskers extend to 1.5 x interquartile range (IQR). Data outside 1.5 x IQR are represented as points.

A mixed-effects Cox model was used to test for differences in survival between treatments in the heatwave assay experiment. Ancestral line temperature and

exposure temperature were used as fixed effects, with line as a random effect. This experiment was continued until all individuals had died, so there was no requirement for censoring. A Kaplan-Meier estimator was created using the ancestral temperature of the line and the exposure temperature as fixed effects, and this was plotted using the 'ggsurv' function, from the 'GGally' package (Schloerke *et al.*, 2017) in R.

2.3 Results

Reproductive output, measured weekly for 10 weeks, was significantly lower for pairs from the high temperature selection lines compared to control lines (Table 2.1, Figure 2.5), at both 30°C and 38°C reproductive regimes (Figure 2.7). A higher temperature during mating, oviposition and offspring development (referred to as mating temperature) resulted in fewer offspring, with no interaction between line temperature and mating temperature, suggesting that the effects of mating temperature were consistent across the high temperature and control lines (Table 2.1, Figure 2.5). Reproductive output of females decreased significantly with time since mating in all treatments, but there was a significant negative interaction with both line temperature and mating temperature (Table 2.1). This suggests that reproductive output declined more rapidly in females from high temperature lines compared to control lines, and in females mated at 38°C compared to those mated at 30°C – an effect that could be clearly observed when reproductive output was plotted as the proportion of offspring produced per week by females from different treatments (Figure 2.6).

When considering reproductive output as a whole over 70 days of oviposition, pairs from the high temperature lines produced fewer offspring than the control lines (Table 2.2, Figure 2.7). Across both control lines and high temperature lines, pairs mated at 38°C had reduced offspring production compared with those mated at 30°C, with no interaction between line temperature and mating temperature (Table 2.2, Figure 2.7). Finally, offspring counts in the first week were lower for the high temperature lines compared to the control lines, and a higher mating temperature also resulted in a decrease in short-term offspring production (Table 2.2). There was no interaction between line temperature and mating temperature in relation to initial offspring counts (Table 2.2).

Median survival time through a 42°C heatwave was six days for high temperature lines and eight days for the control lines, and this difference was highly significant (Table 2.3, Figure 2.8). Pre-heatwave exposure to high temperature also reduced survival time during a heatwave, with a median survival time of six days for individuals kept at 38°C compared to seven days for individuals kept at 30°C (Table 2.3, Figure 2.8). There was a significant interaction between line temperature and exposure temperature, suggesting that pre-heatwave exposure to 38°C resulted in more rapid survival declines in the high temperature lines compared to the control lines (Table 2.3, Figure 2.8).

Table 2.1. A GLMM modelling reproductive output of pairs of *T. castaneum* from different thermal regimes after 24 hours of mating at different thermal regimes (30°C or 38°C), with number of offspring produced as the response variable and random effects of line (Var = 1.58) and individual female (Var = 0.015). Ancestral line temperature and mating temperature were fitted as factors, with the control temperature (30°C) set as the baseline.

Fixed Effects	Estimate	Standard Error	Z	Pr(>|t|)
Ancestral Line Temperature	-1.358	0.378	-3.59	< 0.001
Mating Temperature	-1.953	0.212	-9.20	< 0.001
Weeks Since Mating	-0.938	0.017	-54.58	< 0.001
Weeks Since Mating * Mating Temperature	-0.384	0.034	-11.42	< 0.001
Weeks Since Mating * Ancestral Line Temperature	-0.090	0.031	-2.90	0.004
Ancestral Line Temperature * Mating Temperature	-0.224	0.423	-0.53	0.596

Table 2.2. GLMMs modelling initial and total reproductive output of pairs of *T. castaneum* after 24 hours of mating at different thermal regimes (30°C or 38°C). Models of **A** Initial reproductive output (first seven days) and **B** total reproductive output (70 days). Number of offspring produced was used as the response variable and both models included a random effect of the line (Var_A = 1.58, Var_B = 0) and also an observation level random effect (Var_A = 0.015, Var_B = 0.862) to account for overdispersion of the data. Ancestral line temperature and mating temperature were fitted as factors, with the control temperature (30°C) set as the baseline for each.

Fixed Effects	Estimate	Standard Error	z	Pr(>|t|)
A. Initial Reproductive Output				
Ancestral Line Temperature	-0.86	0.105	-8.18	< 0.001
Mating Temperature	-0.47	0.105	-4.43	0.023
Ancestral Line Temperature * Mating Temperature	-0.091	0.211	-0.43	0.667
B. Total Reproductive Output				
Ancestral Line Temperature	-1.06	0.173	-6.14	< 0.001
Mating Temperature	-1.07	0.143	-7.56	< 0.001
Ancestral Line Temperature * Mating Temperature	-0.059	0.283	-0.21	0.836

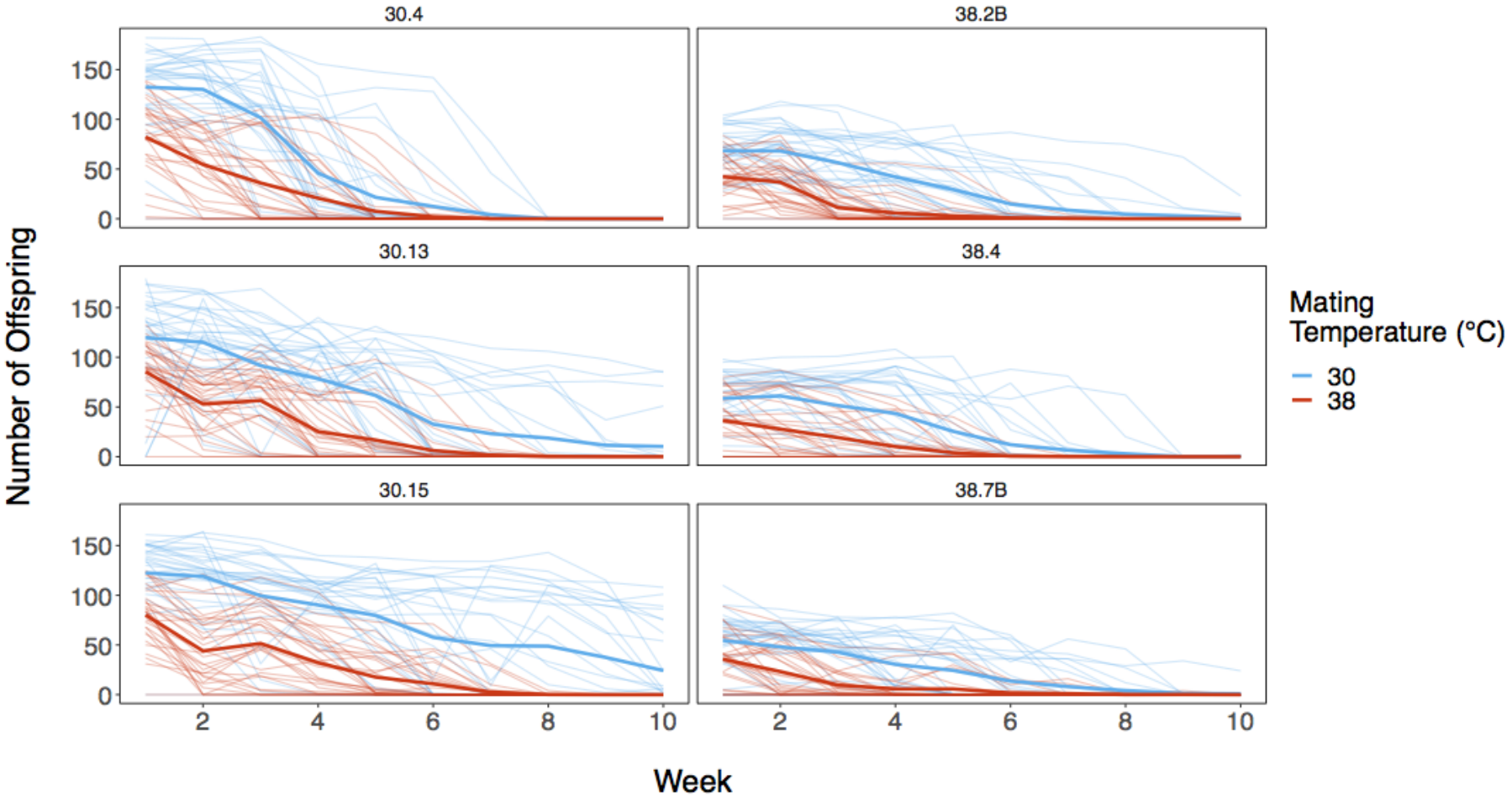

Figure 2.5. Weekly reproductive output over 10 weeks of individual females from each line of *T. castaneum* mated at 30°C (blue) and 38°C (red). Plot of raw data, with mean weekly values overlaid (dark blue/dark red). The ancestral thermal regime of the line is denoted by the first two characters of the line ID (control, 30°C lines on the left; high temperature, 38°C lines on the right). Offspring production overall was lower, and tailed off more rapidly in lines with ancestry at 38°C or pairs mated at 38°C.

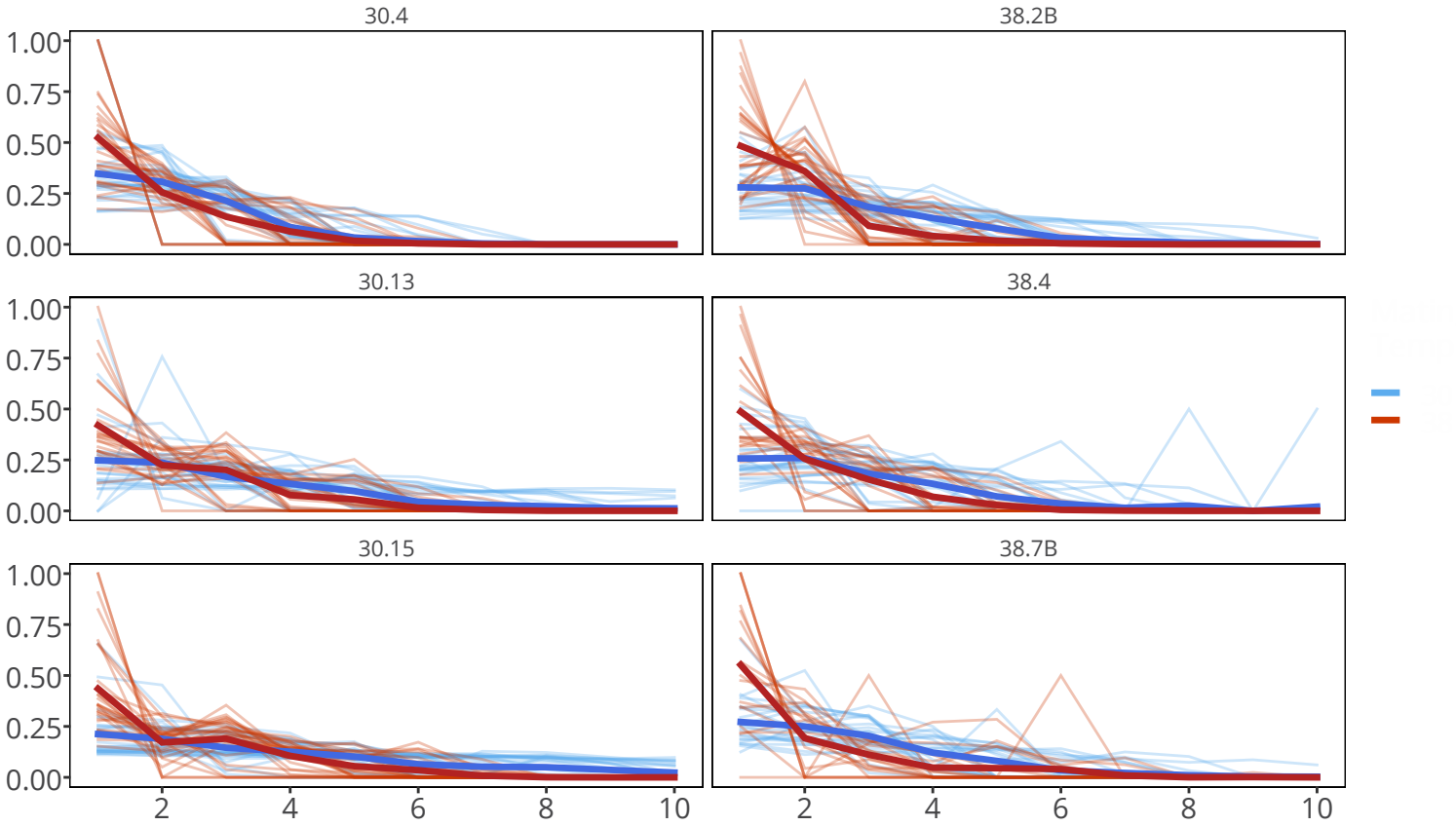

Figure 2.6. Proportion of total offspring produced per week by individual pairs of *T. castaneum* from different thermal regimes mated at control (blue) and high (red) temperatures. Plot shows offspring proportions per female in faint lines, with mean weekly proportions overlaid (dark blue/dark red). The ancestral thermal regime of the line is denoted by the first two characters of the line ID (control, 30°C lines on the left; high temperature, 38°C lines on the right).

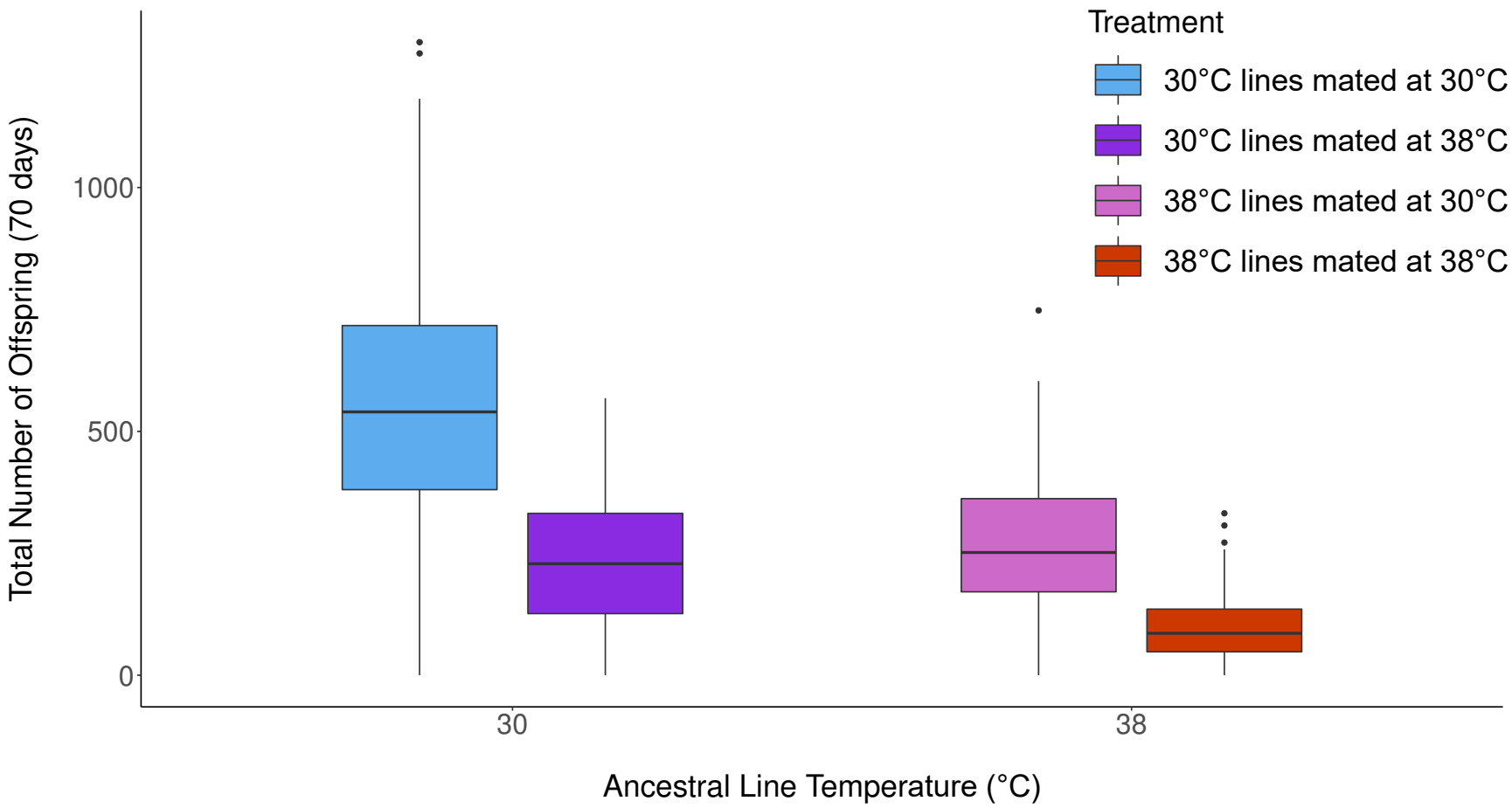

Figure 2.7. The total reproductive output from individual females of *T. castaneum* from different thermal regimes over 70 days, after 24 hours of mating with a single male. Each treatment contained 30 replicate breeding pairs from each of three thermal lines. Lines with ancestry at 30°C had significantly higher reproductive output and mating at high temperature resulted in significantly reduced reproductive output.

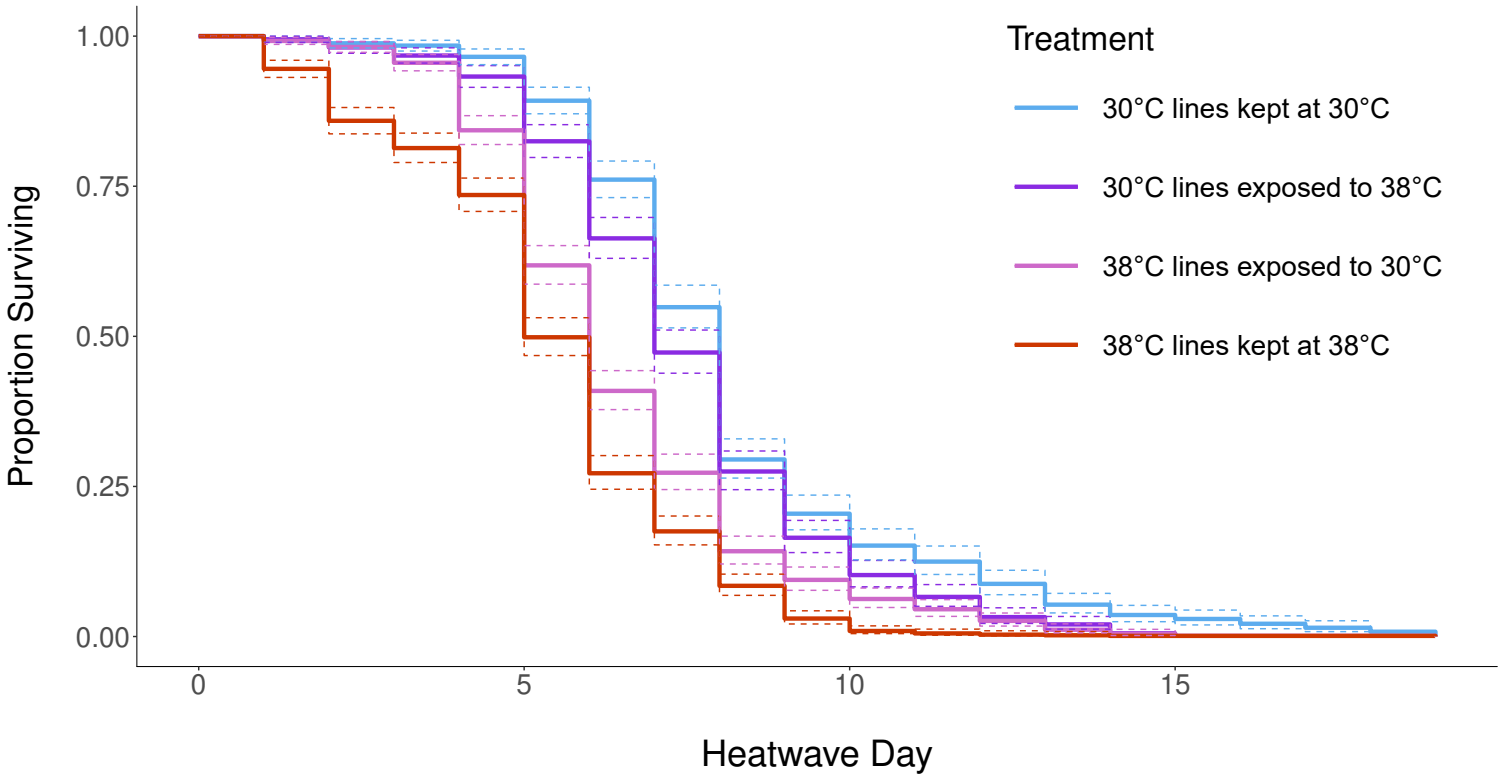

Figure 2.8. Kaplan-Meier survival curves of *T. castaneum* through a 42°C heatwave following one week of exposure to different thermal regimes (30°C and 38°C) immediately before the heatwave (42°C). Dotted lines represent 95% confidence intervals. Each treatment contained three line replicates, with ~ 300 individuals per line. Individuals from lines with control temperature ancestry (30°C) survived significantly longer than those from high-temperature (38°C) lines, and pre-heatwave exposure to high temperature had a detrimental effect on survival. Pre-heatwave exposure to high temperature was more detrimental to survival in high-temperature lines than control lines.

Table 2.3. Summary of a Cox mixed effects model used to model survival time of *T. castaneum* through a 42°C heatwave, after short-term exposure to either control (30°C) or high (38°C) temperatures (n ~ 300 individuals per line, three lines per treatment). Survival time (in days) was used as the response variable and there was a random effect of line (Var = 0.068). Ancestral line temperature and pre-heatwave exposure were fitted as factors, with the control temperature set as the baseline.

Fixed Effect	Coefficient	Standard Error	Z	p
Ancestral Line Temperature	0.092	0.027	3.42	< 0.001
Pre-heatwave Exposure	0.044	0.004	10.07	< 0.001
Ancestral Line Temperature * Pre-heatwave Exposure	0.003	0.001	2.74	0.006

2.4 Discussion

This study compared reproductive output and survival within replicate lines exposed to 54 generations of experimental evolution at 30°C control and 38°C high temperature regimes. I found no evidence for adaptation or acclimation within the high-temperature lines; on the contrary, these lines performed worse than the control lines in every fitness assay, including those carried out at high temperature. Consistent effects were observed when examining different aspects of reproductive output and survival. Here, I discuss what may explain these counterintuitive findings.

A general effect observed from this experiment was the decrease in reproductive output incurred by exposure to higher temperatures (38°C). Both long-term (multi-generation ancestral line temperature) and short-term (within-generation mating and offspring production temperature) exposure to high temperature resulted in a reduction in number of progeny produced. I predicted that short-term, within-generation exposure of the control lines to high temperature would result in a fitness decrease, due to the stress incurred by a sudden switch to the novel high temperature (as in Krebs & Loeschcke, 1994). However, after 54 generations of experimental evolution, I also expected that long-term exposure to 38°C in the high temperature lines would result in some adaptation, and improved fitness under 38°C reproductive environments, perhaps restoring reproductive output to something close to that of the control lines. By contrast, in the reproductive output assay when both the control and high temperature lines were mated at 38°C, the control lines actually outperformed the 38°C high temperature lines. This was unexpected; individuals from the control lines had never been exposed to 38°C before this experiment, yet they still outperformed those whose ancestry had been maintained constantly at 38°C for 54 generations. Additionally, although there was a partial recovery in the reproductive output of the high temperature lines when they were mated at control temperature, this increase is much smaller than might be expected if the response was purely plastic.

In addition to the aforementioned effects of temperature on offspring number, exposure to higher temperature also had an effect on the rate at which female reproductive output declined over time, with short-term exposure having a larger effect than long-term exposure. One potential explanation for this effect in my study is that *T. castaneum* may be capable of plastically responding to their environmental temperature. When exposed to more stressors, they may respond by laying eggs more quickly due to the increased mortality risk, thereby maximising reproductive output. Although overall, the beetles exposed to short-term high temperature produced fewer offspring, they did produce a higher proportion of their total offspring immediately after mating (Figure 2.6), which could indicate a difference in reproductive strategy. While this is highly speculative, there is evidence that some species can adjust their reproductive strategy according to their perceived mortality risk; for example, *Daphnia hyalia* cultured in water containing chemical cues from fish (a predator) produce more of their young in their first brood, in response to the perceived predation risk and reduced likelihood of longevity (Stibor, 1992). Previous work has established that environmental stress can cause reproduction to speed up, slow down, or stop altogether (Greenberg & Wingfield, 1987; Schrek *et al.*, 2001). An example of this is in New Zealand mussels (*Mytilus galloprovicialis* and *Perna canaliculus*), which increase spawning after translocation to a higher stress environment (Petes *et al.*, 2007); the authors suggest that this result may represent the release of existing gametes to enable resources to be allocated away from reproduction and towards survival, which could also apply here. Usually, research into this area focuses on predation risk as the cause of mortality (e.g. Candolin, 1998; Fontaine & Martin, 2006), as opposed to abiotic environmental challenges such as temperature, so responses may differ.

An alternative, more plausible, explanation for the steeper reproductive tail-off observed after within-generation short-term exposure to high temperature is that this represents another example of *T. castaneum* struggling with the exposure to higher temperature. Not only do they reproduce fewer offspring per week after a single mating, they also stop reproducing earlier. This response is comparable to that observed in *Drosophila melanogaster* (Silbermann & Tatar, 2007); adults exposed to

heat shock experienced a reduction in the hatch rate of eggs subsequently produced. My current study did not count eggs, so it is possible that a similar effect was present.

Patterns of survival through a heatwave mirror the reproductive output results, in that individuals from the high temperature lines died significantly faster at 42°C compared to individuals from the control lines. The short-term exposure treatment also had a significant effect. Individuals exposed to 38°C immediately before the heatwave died faster than those exposed to the control temperature, in both control and high temperature lines, although this exposure had a stronger effect in the high temperature lines. These results suggest that the stress of being exposed to 38°C may weaken individuals, leaving them less equipped to cope with the additional stress of further increases in temperature, and therefore providing no evidence of an ability to acclimate to an impending thermal stress. This is not always the case, some species can acclimatise to small changes in temperature. For example, a study by Scott *et al.* (2003) showed that the parasitoid wasp, *Trichogramma carverae*, survived better through a heatwave after acclimation (although this was ultimately at a cost to their longevity). There is evidence that species adapted to higher temperatures tend to have less potential to acclimatise to further temperature increases (Stillman, 2003). If the high temperature lines from this study have partially adapted, this may have affected their ability to acclimatise to the further increase in temperature.

If the short-term exposure to high temperature is detrimental to the fitness of these *T. castaneum*, then it is possible that there are also transgenerational effects involved (Boyko & Kovalchuk, 2010; Freitak *et al.*, 2009). These would only affect the high temperature lines, as the control lines have never been exposed to high temperature before. Previous research has shown that there are transgenerational effects of short-term exposure to high temperatures in male *T. castaneum* including reduced offspring fitness (Sales *et al.*, 2018), so it is possible that there are further unidentified indirect fitness costs. I test how these effects apply to this experiment in Chapter 5 of this thesis, by looking at how the high temperature lines perform over five generations of constant exposure to control temperature.

My chosen components of fitness account for the possibility of a trade-off between reproduction and survival under increased temperature. I consider survival through a heatwave to be a suitable proxy for longevity in this study because of the way the selection lines are maintained. Each generation, the adults are allowed to reproduce and mate for one week. At the end of that week, the adults are all disposed of. The reason for this is to prevent generations from overlapping, but an additional outcome is that there is no selection for long life, so long-term survival would not represent fitness in these lines, whereas short-term survival does (Parsons, 1995). There is, however, a strong selection pressure within the thermal selection lines to reproduce as much as possible within a week, as opposed to a prolonged and steady reproductive strategy. This may also contribute to explaining why a higher proportion of the total offspring produced by the high-temperature lines was in the first week. There is no fitness benefit to extended reproduction in these lines, so that ability may have been impeded to facilitate survival at high temperature. There is evidence that populations from different environments can adopt different reproductive strategies to help them best survive in their respective environments (e.g. Ellers & van Alphen, 1997).

These results, showing reduced fitness at high temperature in individuals accustomed to warmer temperatures compared with control beetles, contrast with most previous studies (Gilchrist *et al.*, 1997; Hoffmann *et al*., 2002). One recent study found strong evidence for an adaptive, genetic change in *Drosophila melanogaster* maintained at higher temperatures after over 40 generations (Tobler *et al.* 2015). It is possible that the difference between these two studies is due to differences in experimental design (e.g. Tobler *et al., 2015* using a fluctuating thermal regime, while ours was kept constant) or due to differences in how fitness was quantified. Alternatively, differences in fitness responses to temperature change could generate variation in adaptability among experimental populations and species.

It is possible that the *T. castaneum* lines using in the current study were unable to adapt to increased temperature due to low genetic diversity (Krebs & Loeschcke, 1994). The lines were established from a highly outbred stock population (Dickinson,

2018), so I am confident of the initial genetic diversity of the populations. However, due to potentially harsh selection from the novel thermal stress in the early generations of the high-temperature lines, some bottlenecking occurred which is likely to have resulted in a significant reduction in diversity of the subsequent generations and a smaller effective population size (Nei *et al.*, 1974). Low genetic diversity can result in decreased fitness via genetic drift and inbreeding depression. Inbreeding depression is a form of genetic stress, which can interact with environmental stress (e.g. increased temperature) resulting in an overall increase in the challenges faced by a population (Bijlsma *et al.*, 1997). This effect is exacerbated in small isolated populations, due to the increased impact of genetic drift resulting in a loss of allelic diversity and the lack of gene flow to alleviate this (Keller & Waller, 2002). I explore the possibility of these effects occurring in the lines used for this experiment later in the thesis (Chapter 3). There is very little literature documenting similar failures to elicit adaptation in experimental evolution studies. One of few examples is by Low-Décarie *et al.* (2013), in which seven species of phytoplankton did not demonstrate any adaptive change in response to elevated CO_2. The authors attributed this to the necessary mutations being too rare, rather than a lack of diversity.

Finally, it is possible that adaptation has occurred in the high temperature lines, but that this adaptation takes effect during the developmental stage of the beetles. I tested the immediate effects of short-term exposure to high temperature on reproductive output and survival, but I did not isolate the specific effects of development temperature on later life reproductive and survival capabilities. It is known that in insects, different life stages display different vulnerabilities and susceptibilities to stress (Klok & Chown, 2001). The environment experienced by an organism during development has been shown to have significant effects in later life (Amitin & Pitnick, 2006; Bagatto, 2005). These effects can include disease susceptibility, physiological changes and reproductive output. Insects are known to be particularly susceptible to increased temperature during developmental stages, compared with adulthood (Kingsolver *et al.*, 2011; Chiu *et al.*, 2014), an effect also observed in brine shrimp (Miller & McLennan, 1988). In the current set of experiments individuals were transferred to novel temperatures as adults. It could be that if I were

to compare individuals from high temperature lines to control beetles that have undergone development at high temperature, evidence of adaptation would be observed. This is explored in Chapter 4 of this thesis.

Chapter 3

An experimental investigation into evolutionary and genetic rescue in a maladapted population

Abstract

Small, isolated populations are often characterised by low levels of genetic diversity. This can result in inbreeding depression and reduced capacity to adapt to changes in the environment, and therefore a higher risk of extinction. However, sometimes these populations can be rescued if allowed to increase in size or if migrants enter, bringing in new alleles and thus increasing genetic diversity. This study uses experimental manipulation of population size and migration to quantify their relative effects on fitness in a challenging environment in order to better understand genetic rescue. In Chapter 2, I showed that there was reduced fitness in high temperature lines of *T. castaneum* compared with control temperature lines, at both high and control temperatures. As these populations had been size-limited for 52 generations and had been under strong selection, I suspected that a decrease in genetic diversity may have occurred in the high temperature lines. To test whether these populations could be rescued, I performed large-scale manipulations of population size and migration, and examined fitness consequences over multiple generations. I measured fitness in the long-term high temperature thermal lines maintained at their usual 'small' population size of N = 100 individuals, and with 'large' scaled up duplicates containing N ≈ 10,000 individuals. I compared these large lines with and without migration (*m* = 0.1) for 10 generations. Additionally, I assessed the effects of outcrossing at an individual level, by comparing fitness of hybrid (thermal line x stock) offspring with within-line crosses. I found that, at the population level, a rapid increase in the number of individuals in the population resulted in reduced fitness (represented by reproductive output and survival through heatwave conditions), regardless of gene flow. However, at an individual level, the hybrid offspring of migrants with native individuals generally demonstrated an increase in longevity in warm conditions compared with individuals from the lines. Overall, in these populations there was no evidence that the demographic manipulations led to genetic or evolutionary rescue. Following the effects of gene flow in individuals over several generations may be the next step in unravelling these conflicting results. I discuss these findings in the context of conservation.

3.1 Introduction

Human activity has had dramatic impacts on the environment, including raised global average temperatures and habitat destruction, which can lead to species extinction (Pimm, 2008). The outcome of these pressures is that populations are increasingly confined to small patchy habitats, resulting in population fragmentation and smaller, more isolated populations (Gibbs, 2001). In such a changing world, small populations are at risk of extinction. Smaller populations generally have lower genetic diversity due to increased impacts of genetic drift (Frankham, 1996), resulting in a reduced adaptive potential (Hoffmann *et al.*, 2017). Another feature of small populations is that, for any given individual, the pool of potential mates is smaller than that of a large population, resulting in increased levels of inbreeding and inbreeding depression (Wright, 1977). These evolutionary processes act to reduce levels of genetic diversity in small populations and can have negative consequences for individual fitness and population persistence, particularly in changing environments (Lande, 1988) and in the context of widespread anthropogenic disturbance (Young *et al*., 2016; Ceballos *et al*., 2017; Lande, 1999).

While in many cases, populations will go extinct once they drop to a certain size, there are ways in which populations can be "rescued". Evolutionary rescue is the restoration of a population through genetic changes (Bell, 2013), for example, the proliferation of adaptive genotypes following an environmental change (Gomulkiewicz & Holt, 1995; Carlson *et al*., 2014). There have been examples of this occurring both in the wild and experimentally (Bell & Gonzalez, 2009; Gomulkiewicz & Shaw, 2013). Another way of restoring populations is through the introduction of migrant individuals from conspecific populations, which is expected to increase genetic diversity, individual fitness, and population growth rates (Ingvarsson, 2001). This process is known as genetic rescue, and is supported by experimental and observational studies (e.g. Schwartz & Mills, 2005; Bouzat *et al.*, 2009; Åkesson *et al.*, 2016). One well-known example of genetic rescue occurred following a natural migration event in which a single male wolf (*Canis lupus*) joined a struggling population in Scandinavia (Vilà *et al.*, 2003), with resulting offspring exhibiting increased fitness and decreased inbreeding

coefficients (Åkesson *et al.*, 2016). As demonstrated by this population, and others (e.g. Johnson *et al.*, 2010), assisted migration can be used as a conservation tool for endangered populations, to boost genetic diversity and subsequently population size and fitness. Indeed, assisted migration has been used successfully as a conservation tool in a number of taxa (e.g. Florida Panthers, *Puma concolor coryi*, Mansfield & Land, 2002; bighorn sheep, *Ovis canadensis*, Hogg *et al.*, 2006; and adders, *Vipera berus*, Madsen *et al.*, 1999).

Despite the popularity of assisted migration as a conservation tool to bring about genetic rescue, it has been argued that migration into bottlenecked populations can also have negative effects (Tallmon *et al.*, 2004). The introduction of migrants can result in outbreeding depression, defined as a reduction in fitness as a result of outcrossing. The most commonly cited cause of outbreeding depression is that outcrossing can break down local adaptation, which can occur when there is high genetic and/or environmental divergence between outcrossing populations (Montalvo & Ellstrand, 2001; Banes *et al.*, 2016). It has also been suggested that migration of outbred individuals into a small population can result in higher genetic load being introduced into the recipient population, resulting in lower average fitness (Lynch & O'Hely, 2001). For this reason, conservation managers seek a high level of confidence that any demographic manipulations would not be to the detriment of endangered populations. Therefore, a detailed understanding of the relationship between demography and adaptation is required in order to guide conservation measures.

Experimental evolution can provide a platform to test genetic rescue in a controlled manner and to understand how it operates over multiple generations (Tallmon *et al*., 2004; Frankham, 2015). Because natural genetic rescue events are either, by their nature, one-off observations or conservation events in endangered populations, laboratory experiments on model species provide the only empirical approach for testing genetic rescue parameters in a replicated manner, without putting endangered populations at risk. *Tribolium castaneum*, the model organism for this study, is well suited for transgenerational genetic rescue research. *T. castaneum* populations can be

reared in large numbers and have short generation times, so can be monitored over several generations following a genetic rescue event, on a much shorter timescale than is possible in most natural populations. Additionally, *T. castaneum* has long been a model for population dynamics (King & Dawson, 1972), including how genetic processes affect population demographics (e.g. Desharnais & Liu, 1987; Wade & Goodnight, 1991; Pray *et al.*, 2009; Hufbauer *et al.*, 2015).

In this study, I experimentally investigate the impact of demographic manipulations in *T. castaneum* populations. Previous work has revealed no evidence of adaptation to high temperature in these beetles (see Chapter 2), which may be due to the small populations sizes at which they are maintained, and potential genetic bottlenecks in the history of these populations. To test this at a population level, I experimentally manipulated population size and migration rates, and compared how these manipulations affected fitness. One suggested way that genetic rescue might be more likely to occur is via long-term genetic restoration in place of a single rescue event (Adams *et al.*, 2011), therefore, I maintained these demographic manipulations over 10 generations. To complement these experiments, and to study the effects of outbreeding at the individual level, I quantified the fitness of crosses between individuals from small thermal populations with individuals from different genetic backgrounds. A common criticism of genetic rescue is that migrants may not be adapted to the environment of the recipient population, which could cause a loss of local adaptation (Storfer, 1999), so I also crossed individuals from different replicate thermal lines (genetically isolated for 62 generations), which removes the potential effects of maladapted migrants. The results of these assays are discussed in the context of genetic rescue and species conservation.

3.2 Methods

All experiments were carried out using the long-term thermal selection lines, and the outbred Krakow Super Strain (KSS) stocks, with line maintenance details as described in Chapter 2.

3.2.1 Population-level demographic manipulations and fitness

To simulate genetic rescue at the population level, I created sets of "large" and "migration" thermal lines, all maintained at 38°C (our high temperature, above the *T. castaneum* optimum for productivity at 32°C; Howe, 2009). These lines were established as replicates from the three high temperature thermal lines described in Chapter 2 (here referred to as the small lines) at generation 52 and scaled up to a size of 10,000 over two or three generations, depending on the number of offspring produced each generation. Once the desired population size was reached, 10,000 adults were selected each generation (by volume, ~50ml of adult beetles) to sire the following generation (Figure 3.1). These "large lines", were kept in large plastic containers measuring approximately 60 x 75 x 21cm, and containing approximately 7kg of fodder to maintain approximately equal population densities to the original, small lines. At generation 55, the large lines were then duplicated to create "migration lines", in which gene flow was introduced at a rate of $m = 0.1$ each generation. To this end, 9,000 adults from generation n of the large lines were combined with 1,000 adults from the KSS stock population to sire generation $n + 1$ (Figure 3.1). Line maintenance procedures were otherwise identical to those described in Chapter 2.

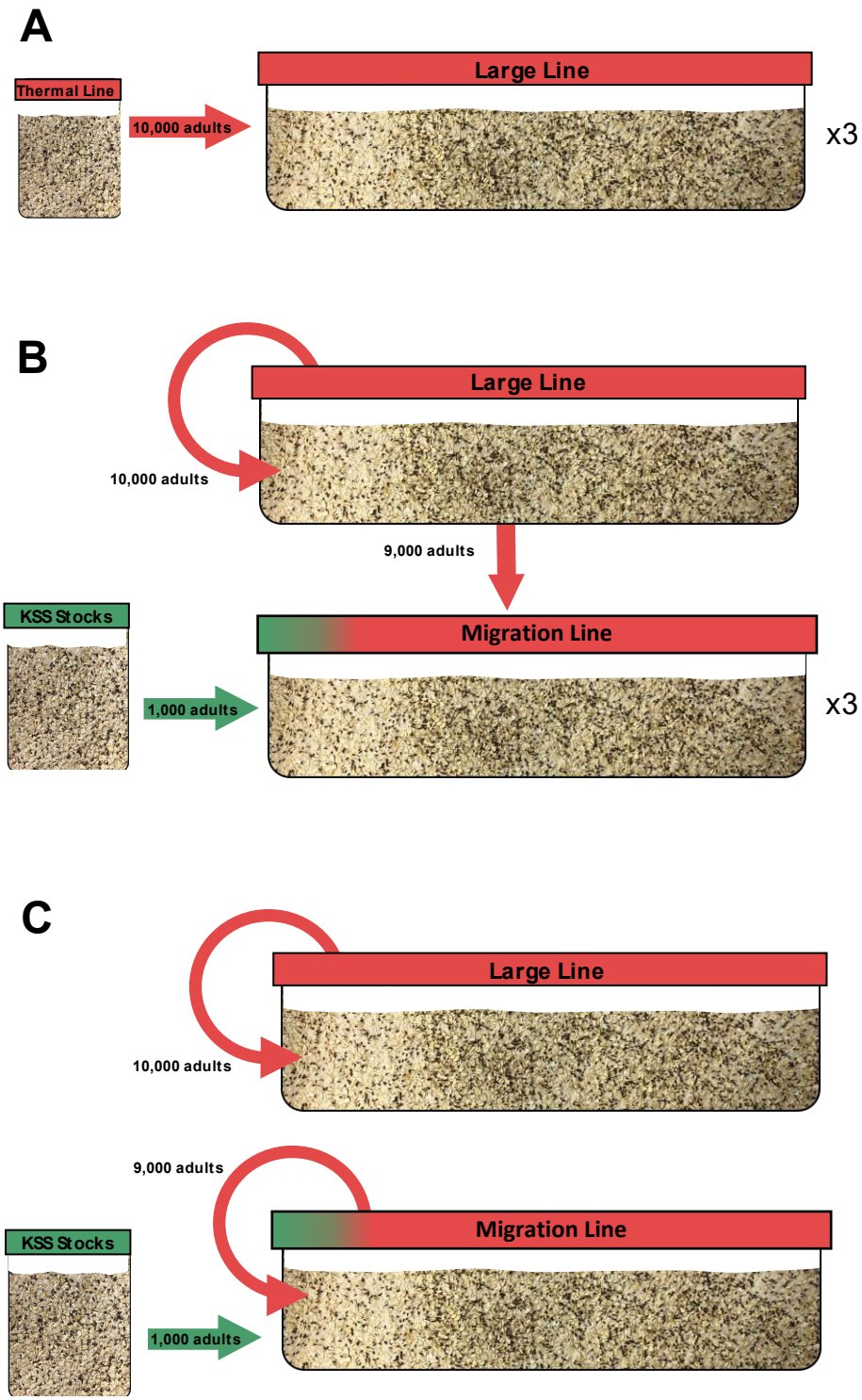

Figure 3.1. The stepwise development of *T. castaneum* large and migration lines. A. the initial generation of the large lines, starting from small, high-temperature thermal lines, **B.** subsequent generations of large lines and the start of migration lines, three generations after A. and **C.** subsequent maintenance of the large and migration lines.

I carried out reproductive fitness assays of each population of small, large and migration lines at generation 65 (after 10 generations of demographic treatment). When the adults were removed from their week of mating/laying, the eggs and fodder from each line were split into two groups. One group was put into 30°C to develop and the other into 38°C. After 12 days (at 30°C) or 16 days (at 38°C), a subset of pupae were isolated from each line at each temperature and separated by sex in groups of 20 to generate individual adults for assessment. The remaining pupae were returned to the fodder to develop. Approximately 14 days after eclosion of the isolated pupae, the resulting virgin males and females were monogamously paired for mating at their assigned development temperature in 7ml vials containing approximately 1.5g fodder for 24 hours. The females of these pairs were transferred into a petri dish containing ~7g fodder and allowed seven days to oviposit at their assigned development/mating temperatures (week one). After seven days, the females were transferred into new petri dishes with clean fodder to oviposit for a further seven days (week two), after which she was discarded. The eggs laid in each petri dish were reared for 35 days (at their assigned rearing temperature) to develop to maturity before being frozen and counted to provide a measure of the reproductive fitness of individual pairs.

I also carried out heatwave survival assays at generation 65. At reproductive maturity, approximately 300 adults from each replicate line (measured by volume, approx. 1.5ml beetles) were placed into a 200ml container (7cm diameter x 7.5cm depth) with ~100g clean fodder and oats and placed into an incubator at 42°C and 60% relative humidity (RH). These heatwave conditions are at the limits of stock *T. castaneum* survival. Each day, the beetles were sieved out of the tubs, the number of dead beetles counted and disposed of and live beetles returned to the tubs and placed back into the incubator at 42°C. When five or fewer beetles remained alive in a population, they were transferred to smaller (4.5cm diameter, 1.5cm depth) petri dishes containing ~7g fodder for the remainder of the experiment. The experiment continued until all beetles had died. This assay therefore measured survival rates through time under heatwave conditions.

Data analyses were carried out using R version 3.3.1 (R core team, 2016), implemented in RStudio version 1.0.136 (2009-2016). All generalised linear mixed models (GLMMs) were conducted using the 'lme4' package (Bates *et al*., 2018). To test for differences between demographic treatments, a GLMM was fitted with a Poisson error distribution. The number of offspring produced by individual pairs was fitted as the response variable and explanatory variables were the demographic treatment (categorical – small/large/migration) and the rearing temperature (categorical – 30°C vs 38°C). The "large" demographic treatment was fitted as the baseline in order to separately identify the effects of population size and migration. The line ID of the mating pair was fitted as a random effect, and an observation level random effect was included to account for overdispersion (Harrison, 2014). To model the interaction between the explanatory variables, a separate model was constructed, as above, but with an interaction between rearing temperature and demographic treatment and from these models only the interactions terms were reported.

All survival analyses were conducted using the 'survival' (Therneau, 2015) and 'coxme' (Therneau, 2018) packages. To test for differences in survival through a 42°C heatwave between the different demographic treatments, a Cox mixed effects model was fitted. To model the interaction between the explanatory variables, a separate model was constructed, as above, but with an interaction factor between rearing temperature and demographic treatment. This experiment was continued until all individuals had died, so there was no requirement for censoring. A Kaplan-Meier estimator was created using the demographic treatment and the rearing temperature as fixed effects, and this was plotted using the 'ggsurv' function, from the 'GGally' package (Schloerke *et al.*, 2017) in R.

3.2.2 Outcrossing and fitness

To measure the benefits of outcrossing at an individual level, I carried out crosses using individuals from the small long-term thermal lines and the outbred KSS stocks (see Chapter 2 for stock details). A total of 20 male and 20 female pupae were

collected from each thermal line (10 replicate lines at 38°C and 10 at 30°C) and 100 each of male and female pupae were collected from the outbred KSS stock. These were split into single-sex groups of 20. The pupae from the thermal lines were allowed to eclose at their native temperatures, and KSS pupae eclosed at 30°C. Ten days after eclosion, at reproductive maturity, male and female beetles were paired and put in vials to mate in three possible treatment combinations. The treatments for the pairs were: i) thermal line male x thermal line female; ii) KSS female x thermal male; and iii) thermal female x KSS male (Figure 3.2). For each type of thermal line (control: 30°C, or high: 38°C), I performed six replicates for each treatment per line, giving a total of 301 successful crosses (155 with 30°C lines and 146 with 38°C lines). After approximately 24 hours of mating, the male was discarded and the female was put into a petri dish containing ~7g fresh fodder and allowed to oviposit for 10 days. After this, the females were discarded. These offspring were allowed to mature (28 days after female was removed for 38°C lines, 35 days after female was removed for 30°C lines), at which point they were counted and the number of offspring recorded as a measure of the pair's reproductive fitness.

In conjunction with counting these offspring, the first 20 beetles counted were put into a petri dish to undergo a longevity assay at 38°C, to measure adult survival under hot conditions. This was repeated for the next 20 beetles. The third set of 20 beetles counted were put into a petri dish at 40°C. The outcome of this was that for each male x female pair described above, the longevity of their offspring was tested at 38°C (n = 40) and at 40°C (n = 20), contingent upon them producing at least 60 offspring. Any remaining offspring were counted and discarded. Each petri dish was filled with 20 beetles before the next was used. Excess beetles were discarded. At regular intervals of seven or 14 days, the number of deaths per petri dish for that time period were recorded, and the surviving beetles were put into fresh fodder and returned to the assay. The measurement intervals were adjusted during the course of the assay in response to observed death rate.

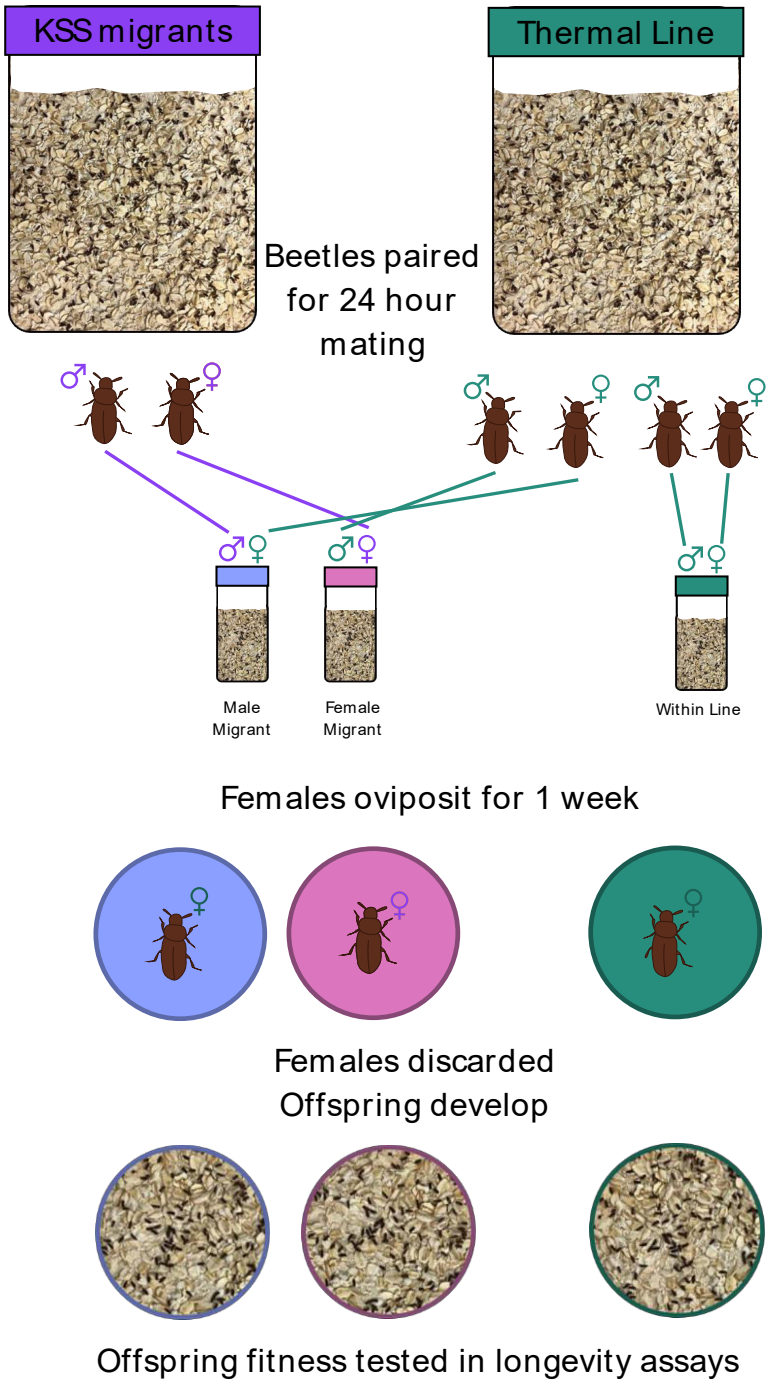

Figure 3.2. The experimental set up used to test the fitness of hybrid pairs of KSS stock and thermal line compared with isolated thermal line pairs. *T. castaneum* adults for each treatment were paired to mate for 24 hours, the females were allowed seven days to oviposit and the offspring were allowed 28 days to develop at 38°C or 35 days to develop at 30°C before the longevity assays commenced. This set up was repeated for 10 replicate control temperature thermal lines and 10 replicate high temperature thermal lines.

Differences in reproductive output between crosses within thermal lines and crosses between thermal lines (natives) and KSS (hybrid) individuals were analysed using GLMMs, fitted with a Poisson error distribution. I aimed to test for an effect of outcrossing in each of the two sets of lines, rather than for a difference between lines, so I ran two models (one for each set of thermal lines). The response variable was the number of offspring produced. The combination of parents (i.e. migrant mother, migrant father or both parents native) was fitted as a categorical explanatory variable, with the within line crosses set as the baseline. I included a random effect of the ID of the thermal line of the native parent(s), and an observation-level random effect to account for overdispersion.

To test for differences in the longevity of the offspring produced from the hybrid crosses, Cox mixed effect models were constructed, which included the treatment (male migrant, female migrant or within line cross) as a fixed effect, and the line ID of the thermal line parent as a random effect. Four models were constructed: **A** longevity of 30°C hybrids with KSS at 38°C; **B** longevity of 30°C hybrids with KSS at 40°C; **C** longevity of 38°C hybrids with KSS at 38°C; **D** longevity of 38°C hybrids with KSS at 40°C. The models were constructed with censoring, based on how many beetles were alive at the end of the experiment. For each model, a Kaplan-Meier estimator was created using the treatment as the fixed effect.

Finally, I assessed the fitness of experimental crosses between individuals from within and across the small, high temperature lines (as in the hybrid experiment, above). Ten replicate lines were used in this experiment. The lines were paired and these five pairs were labelled as groups A-E. Crosses between lines were done within these five groups. Twelve days after the removal of the adults from the lines, 20 male and 20 female pupae from each line were sexed and isolated in single sex groups. At reproductive maturity, females were paired with males from the same line (n = 4) or from the other line in the group (n = 4) (Figure 3.3). They were put into a vial containing ~ 1.5ml fodder to mate for 24 hours. The females of these pairs were then transferred into a petri dish containing ~7g fodder and allowed seven days to oviposit,

before being moved to a second petri dish for a second week of oviposition. After 35 days, the fully developed offspring were frozen and counted as a measure of reproductive fitness.

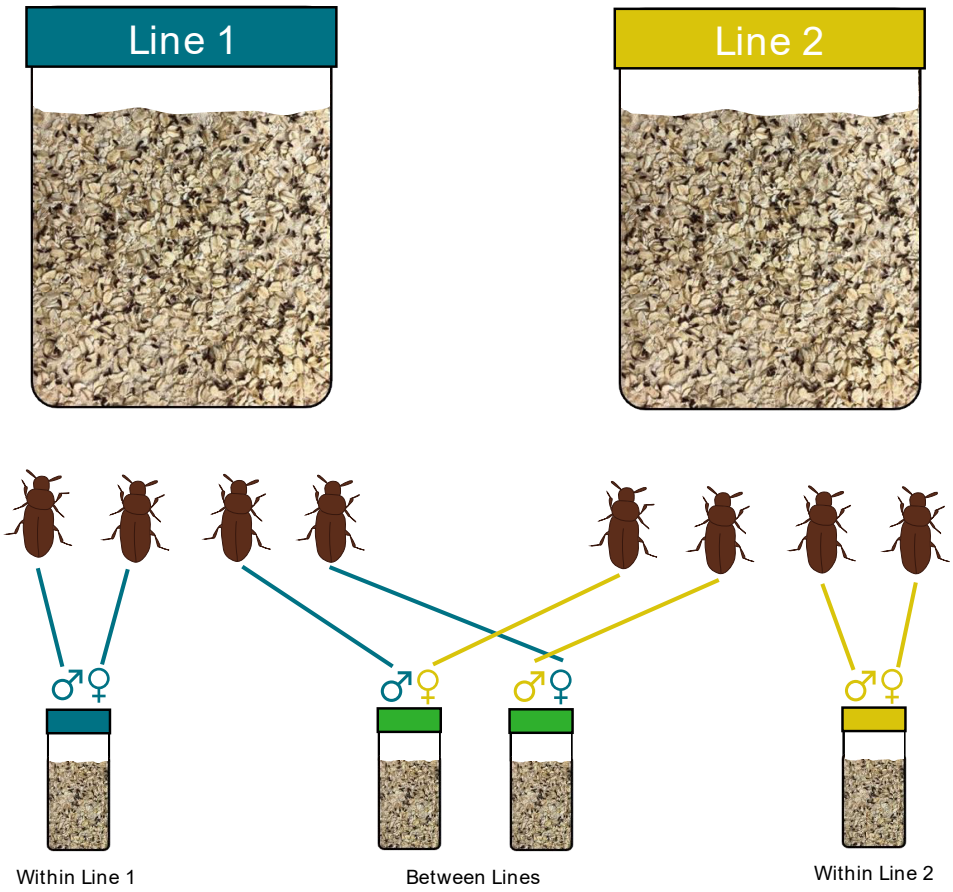

Figure 3.3. The experimental set up of within and between *T. castaneum* thermal line crosses. This figure represents just one 'group' of lines out of five (groups A-E).

A linear mixed model (LMM) was fitted to test for differences in the number of offspring produced by pairs of beetles from either the same, or different high-temperature lines. Usually, for count data, a Poisson error distribution is used, but after inspecting the residual plots and AIC values for this data set, gaussian was a better fit. The model was fitted with a response variable of number of offspring produced per pair over 14 days. The origins of the parents (same line or different lines) was fitted as an explanatory variable, with a random effect of the maternal ancestral line and the paternal ancestral line. In addition to this, differences in fitness within versus between crosses was assessed separately for each group of lines (A-E) within the experiment using a generalised linear model (GLM) per group. For each model, the number of offspring produced per cross over two weeks was fitted as the response variable. The line origin of the parents was fitted as an explanatory variable with the hybrid cross in a group always fitted as the baseline.

3.3 Results

3.3.1 Population-level demographic manipulations and fitness

Overall, reproductive pairs produced significantly fewer offspring when reared at the higher temperature (38°C) regime, indicating evidence of a fitness challenge in these conditions (Table 3.1, Figure 3.4). At 38°C, there was a significant effect of demographic treatment on reproductive output (Table 3.1), with pairs from large populations producing fewer offspring than pairs from the small populations (Figure 3.4). At both 30°C and 38°C rearing temperatures, reproductive output did not differ between pairs from the large and migration lines (Table 3.1, Figure 3.4). Across all treatments, there was a significant interaction between temperature and demographic background (Table 3.1), with the negative effect of large population size (with or without 10% migration) being more pronounced at high (38°C) compared to control temperatures (30°C) (Figure 3.4).

Survival through the 42°C heatwave condition varied significantly amongst the demographic treatments, and a significant interaction suggests that this effect depended on developmental conditions (Table 3.2, Figure 3.5). Specifically, I found no differences between demographic treatments when beetles were reared at 30°C (median survival time of nine days for all three demographic treatments; Figure 3.5A); however, I observed lower survival of individuals from the small lines (median survival time of nine days) compared to individuals from the large and 10% migration lines (median survival time of seven days) at a rearing temperature of 38°C (Figure 3.5B).

Table 3.1. A GLMM modelling reproductive output of pairs of *T. castaneum* from populations with differing demographics (size and migration) and thermal regimes (30°C and 38°C). The number of offspring produced over seven days by individual mating pairs was the response variable. Line ID was fitted as a random effect (Var = 0.009) and an observation level random effect (Var = 1.778) was added to account for overdispersion. The rearing temperature and the demographic treatment were fitted as factors with control temperature (30°C) and large population size fitted as the baselines, in order to separately identify the effects of population size and migration.

Fixed Effect	Estimate	Standard Error	Z	Pr(>|z|)
Intercept	4.503	0.067	67.57	< 0.001
Rearing Temperature	-1.149	0.069	-16.75	< 0.001
Small Treatment	0.247	0.083	2.96	0.003
Migration Treatment	0.046	0.084	0.54	0.587
Rearing Temperature * Small Treatment	0.835	0.159	5.27	0.001
Rearing Temperature * Migration Treatment	- 0.199	0.160	- 1.24	0.213

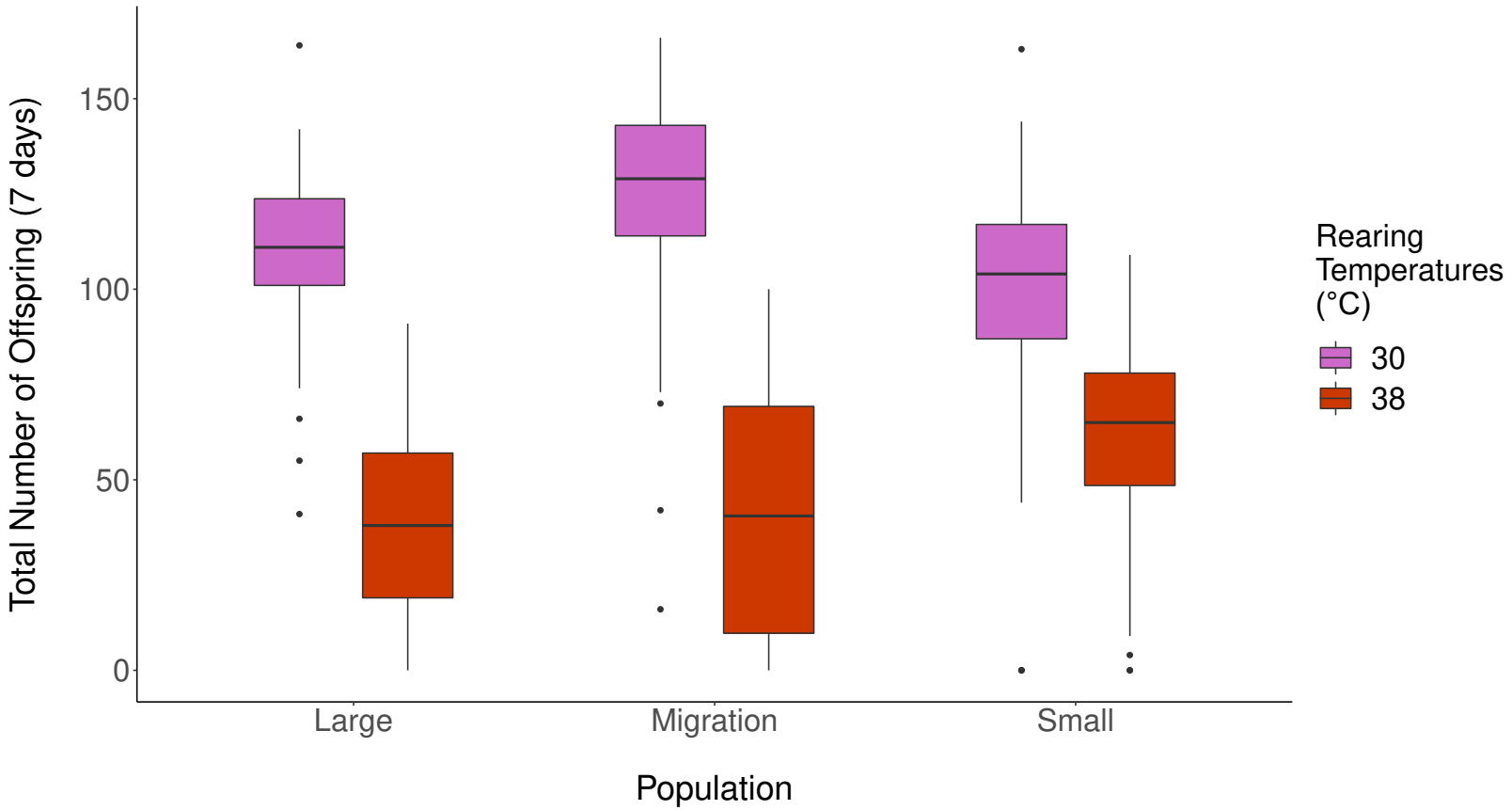

Figure 3.4. The reproductive output of individual *T. castaneum* females from different populations over seven days, immediately after 24 hours of mating. Each treatment consisted of 30 replicate pairs from each of the three demographic thermal lines, reared from eggs through to adulthood at either 30°C or 38°C. A rearing temperature of 38°C resulted in significantly fewer offspring produced than 30°C, although this effect was weaker in small populations. Overall, small populations production more offspring than large.

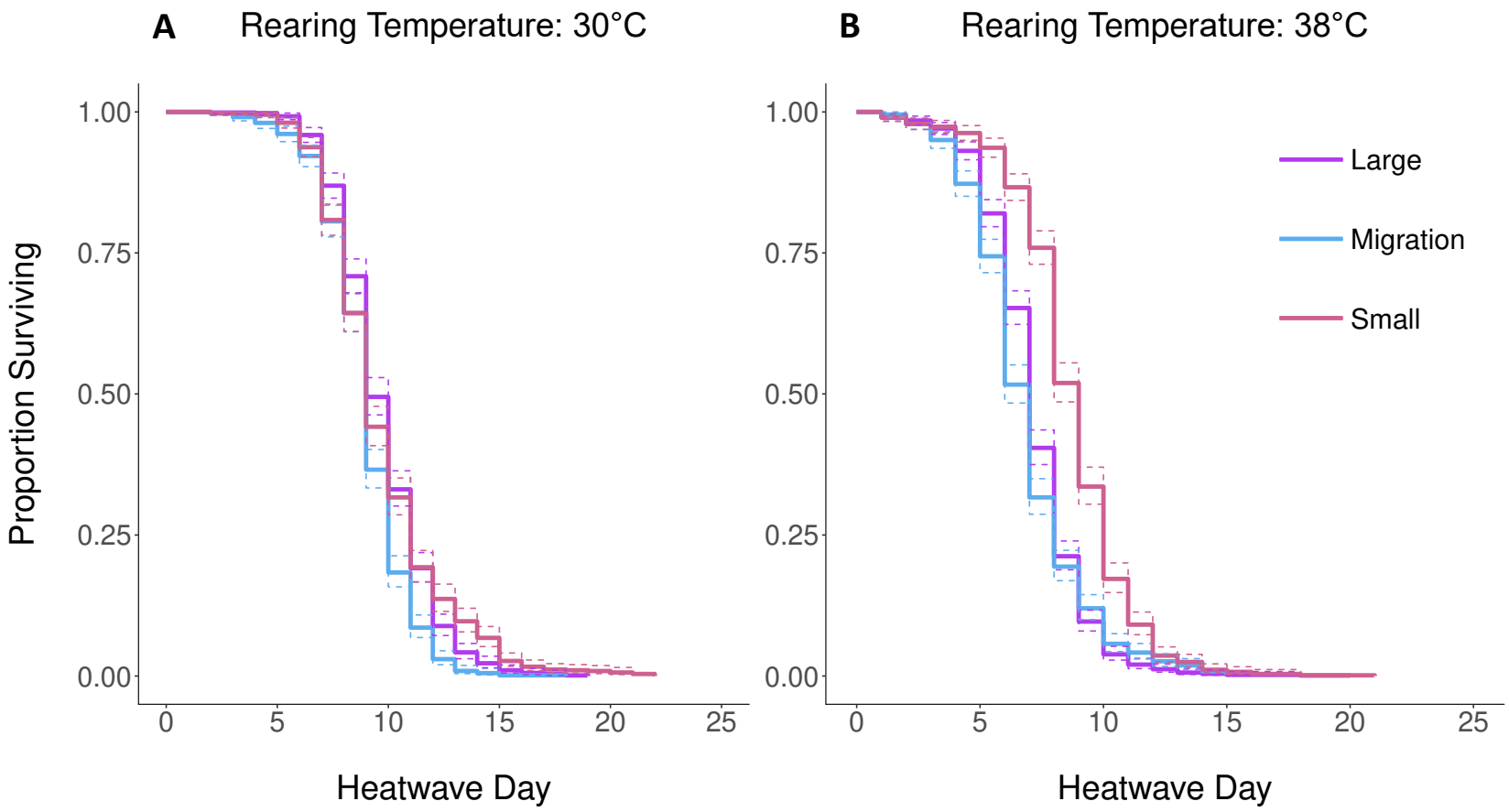

Figure 3.5. Kaplan-Meier survival curves of *T. castaneum* from each demographic treatment through a 42°C heatwave, after 10 generations of demographic control. Beetles were reared at **A.** 30°C or **B.** 38°C. Dotted lines represent 95% confidence intervals. Each treatment contains three line replicates with ~ 300 individuals per line. Individuals reared at 38°C had reduced survival time through a heatwave. Overall, the rearing temperature had less of an effect on small populations than large or migration populations, and individuals from small populations were able to survive longest in the heatwave conditions.

Table 3.2. A Cox mixed effects model of survival of *T. castaneum* through a 42°C heatwave, after ten generations of small or large population sizes and developmental exposure to either 30°C or 38°C. Each treatment was made up of three replicate lines (n ~ 300 individuals per line) and the ID of the replicate line used was fitted as a random effect (Var = 0.013). Demographic treatment and rearing temperature were fitted as factors; with large population size and control temperature (30°C) set as the baselines.

Fixed Effect	Coefficient	Exp(coef)	Standard Error	Z	P
Rearing Temperature	0.787	2.196	0.030	26.33	< 0.001
Small Treatment	-0.294	0.745	0.100	-2.96	0.003
Migration Treatment	-0.012	0.988	0.100	-0.12	0.900
Rearing Temperature * Small Treatment	-1.034	0.356	0.070	-14.71	< 0.001
Rearing Temperature * Migration Treatment	0.070	1.072	0.069	1.02	0.310

3.3.2 Outcrossing and fitness

Overall, there was no difference in the number of offspring produced by within-line pairs compared to pairs that included either a stock male or stock female in the control (30°C) thermal lines (Table 3.3, Figure 3.6). However, in the high-temperature 38°C thermal lines, outcrossing thermal line males with stock females (but not vice versa), resulted in a larger number of offspring being produced by these pairs than any other parental combination (Table 3.3, Figure 3.6).

The offspring from hybrid crosses (between 'migrant' individuals from the KSS stocks with individuals from the thermal lines) generally had greater longevity through warmer conditions than offspring from within-line crosses (Figure 3.7). This effect was observed in both the 30°C control temperature thermal lines and the 38°C high temperature thermal lines, and was evident in both 38°C and 40°C conditions. However, in the 38°C condition, there were two exceptions to this, in which the longevity of hybrid offspring was no different to that of the within-line crosses: 1) Male migrant X control female, and 2) female migrant X high-temp male. These exceptions were both for trials of longevity at 38°C, which is a less stressful temperature than the 40°C trials, and in no case was the longevity of hybrid offspring lower than the offspring from the within-line crosses (Table 3.4, Figure 3.7).

In comparisons of reproductive fitness of within-line and between-line crosses for the 38°C high temperature lines, I found no differences in reproductive output of pairs from within one line compared with pairs from two different lines. (Table 3.5, Figure 3.8). Within the groups, there was also generally no difference in the reproductive output of pairs from within-line versus between-line crosses; only one of the five comparisons showed any difference in reproductive fitness between the within-line versus between-line hybrid cross (Group D, Table 3.6, Figure 3.8). In this group, the difference was between the hybrid cross and only one of the parental lines.

Table 3.3. GLMMs modelling the number of offspring produced by *T. castaneum* KSS stock and thermal line crosses. A 'hybrid' pairs between KSS migrants and 30°C thermal beetles and **B** 'hybrid' pairs between KSS migrants and 38°C thermal beetles. The number of offspring produced, per pair, over seven days was the response variable. The origin of the parents (treatment) was fitted as a factor, with 'within line crosses' set as the baseline treatment. In both models there was a random effect of the thermal line ID used for the thermal parent beetle (Var_A = 0.09, Var_B = 0.08) and an observation level random effect was included to account for overdispersion (Var_A = 1.85, Var_B = 2.63).

Fixed Effect	Estimate	Standard Error	Z	Pr(>|z|)
A. Hybrids at 30°C				
Intercept	4.425	0.165	26.79	< 0.001
Female Migrant	- 0.253	0.191	-1.32	0.186
Male Migrant	0.184	0.192	0.96	0.339
B. Hybrids at 38°C				
Intercept	2.744	0.195	14.05	< 0.001
Female Migrant	0.538	0.245	2.20	0.028
Male Migrant	-0.363	0.245	- 1.49	0.138

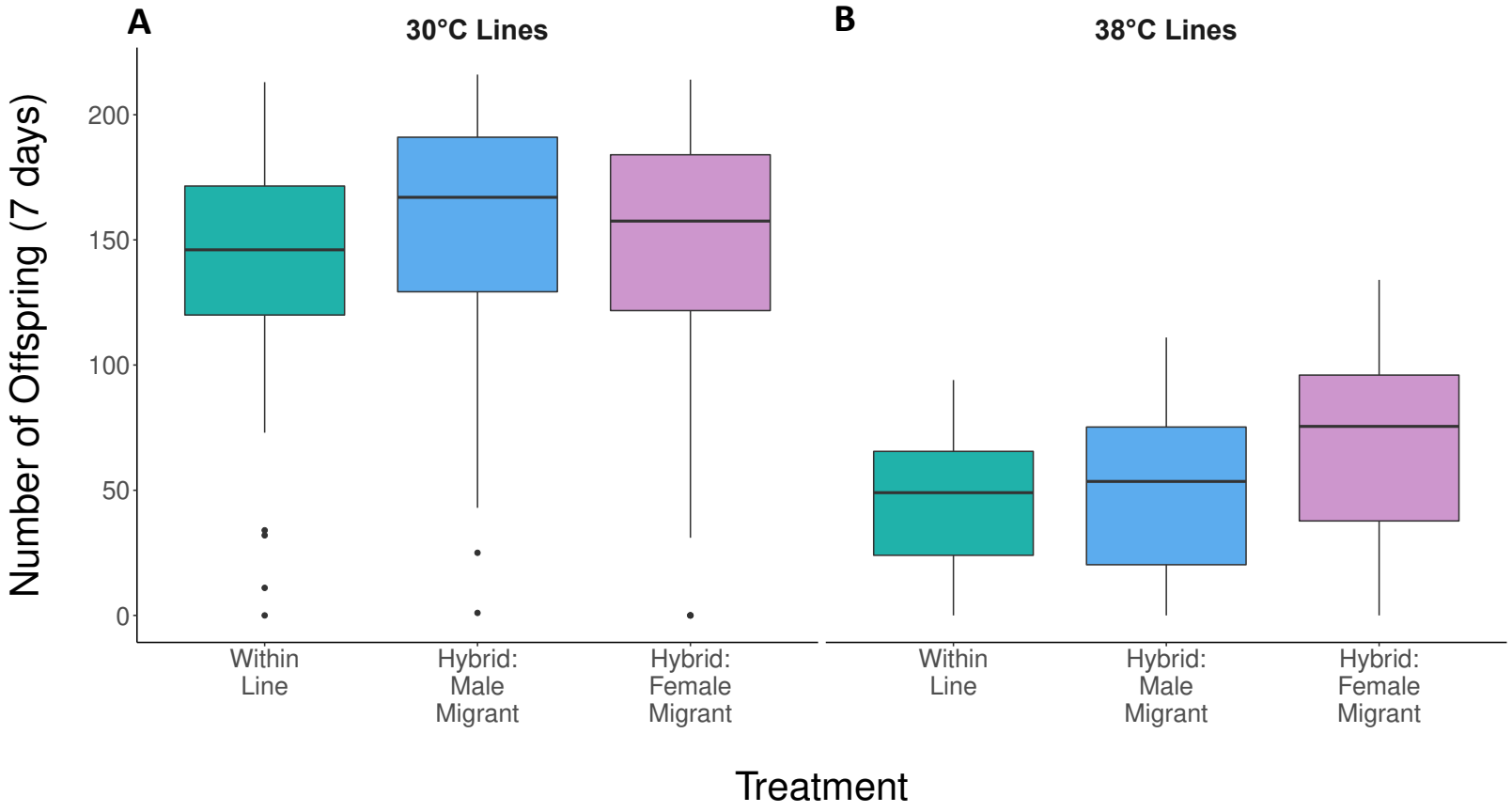

Figure 3.6. The number of offspring produced over seven days by *T. castaneum* pairs from either within one thermal line or a thermal line hybridised with a migrant from the stock population. Data are split into **A.** Control temperature lines and **B.** High temperature lines. There was no difference between the treatments in the control lines, but the high temperature lines produced significantly more offspring when crossed with a migrant female.

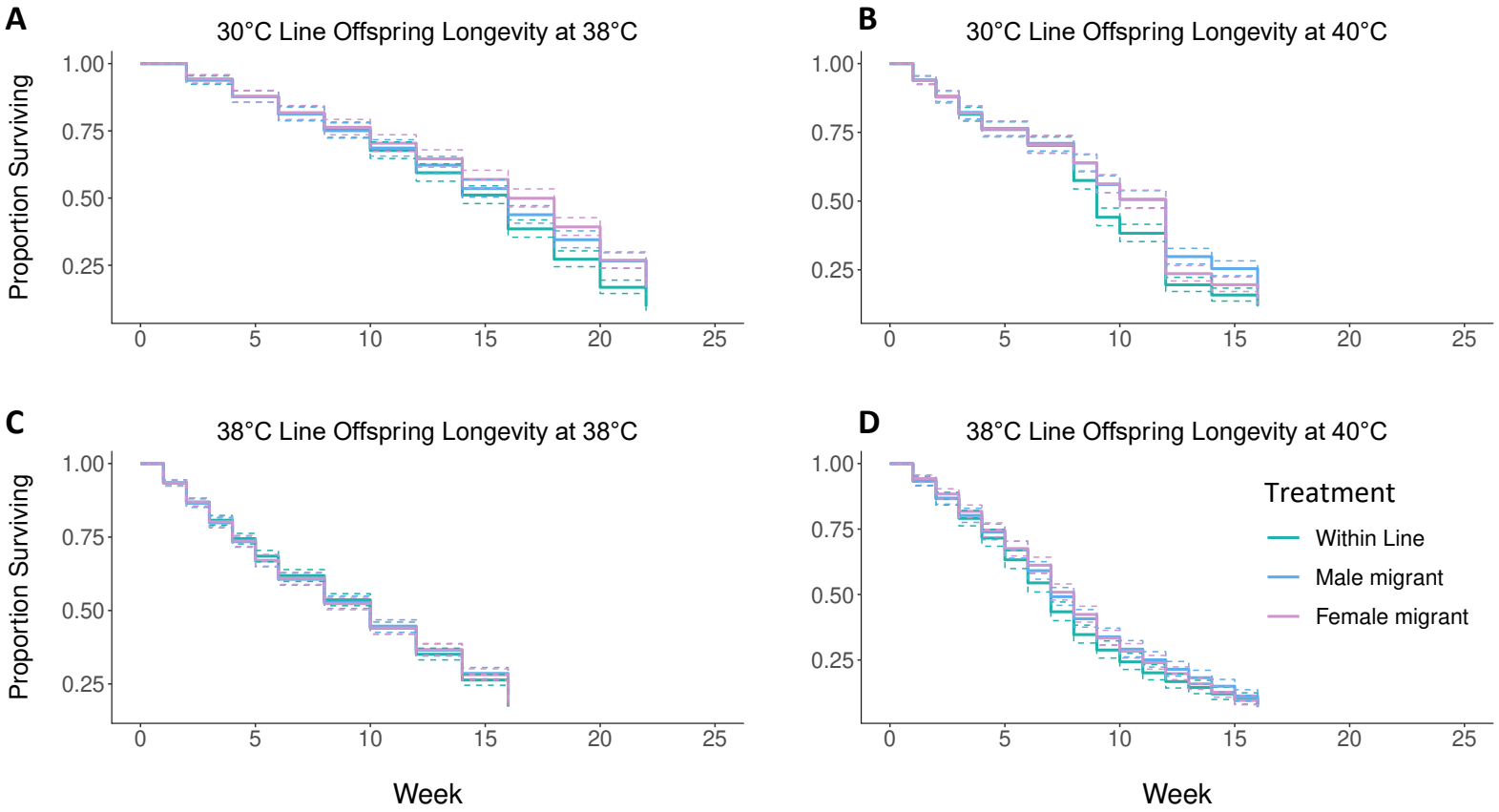

Figure 3.7. Kaplan-Meier longevity curves of *T. castaneum* from hybrid pairs of KSS with thermal lines. Dotted lines represent 95% confidence intervals. Each treatment contains six replicates of 40 individuals across 10 thermal lines. Having one migrant parent increased the longevity of individuals in all treatments except one (Table 3.4); there was no difference in longevity at 38°C between crosses within 38°C lines and those with a migrant female.

Table 3.4. A Cox mixed effects model of *T. castaneum* offspring survival of the hybrid crosses between thermal lines and KSS stock individuals (migrants). Separate models were constructed based on the thermal line ancestral temperature and the temperature of the longevity assay. In each model, survival time (in weeks) was used as the response variable and there was a random effect of Line ID (Var_A = 0.023, Var_B = 0.029, Var_C = 0.027, Var_D = 0.016). The parent combination was fitted as a factor, with crosses within thermal lines set as the baseline.

	Coefficient	Exp(coef)	Standard Error	Z	p
A. 30°C lines at 38°C					
Male Migrant	- 0.201	0.818	0.051	- 3.93	0.057
Female Migrant	- 0.267	0.765	0.052	- 5.12	0.003
B. 30°C lines at 40°C					
Male Migrant	- 0.264	0.768	0.050	- 5.30	0.001
Female Migrant	- 0.812	0.834	0.051	- 3.56	0.044
C. 38°C lines at 38°C					
Male Migrant	- 0.069	0.934	0.034	- 2.01	0.045
Female Migrant	- 0.040	0.961	0.034	- 1.18	0.240
D. 38°C lines at 40°C					
Male Migrant	- 0.129	0.879	0.055	- 2.36	0.018
Female Migrant	- 0.105	0.900	0.051	- 2.07	0.038

Table 3.5. An LMM of reproductive output of high temperature thermal lines and hybrid crosses between them. The number of offspring produced over 14 days by individual *T. castaneum* mating pairs was the response variable and the treatment (within or between line crosses) was fitted as a factor, with within-line output fitted as the baseline. Fitted as random effects were the line IDs of the mother (Var = 41.57) and the father (Var < 0.001).

Fixed Effect	Estimate	Standard Error	df	t	Pr(>|t|)
Intercept	99.18	7.82	29.56	12.68	< 0.001
Between Line Cross	-17.42	10.63	88.14	-1.64	0.11

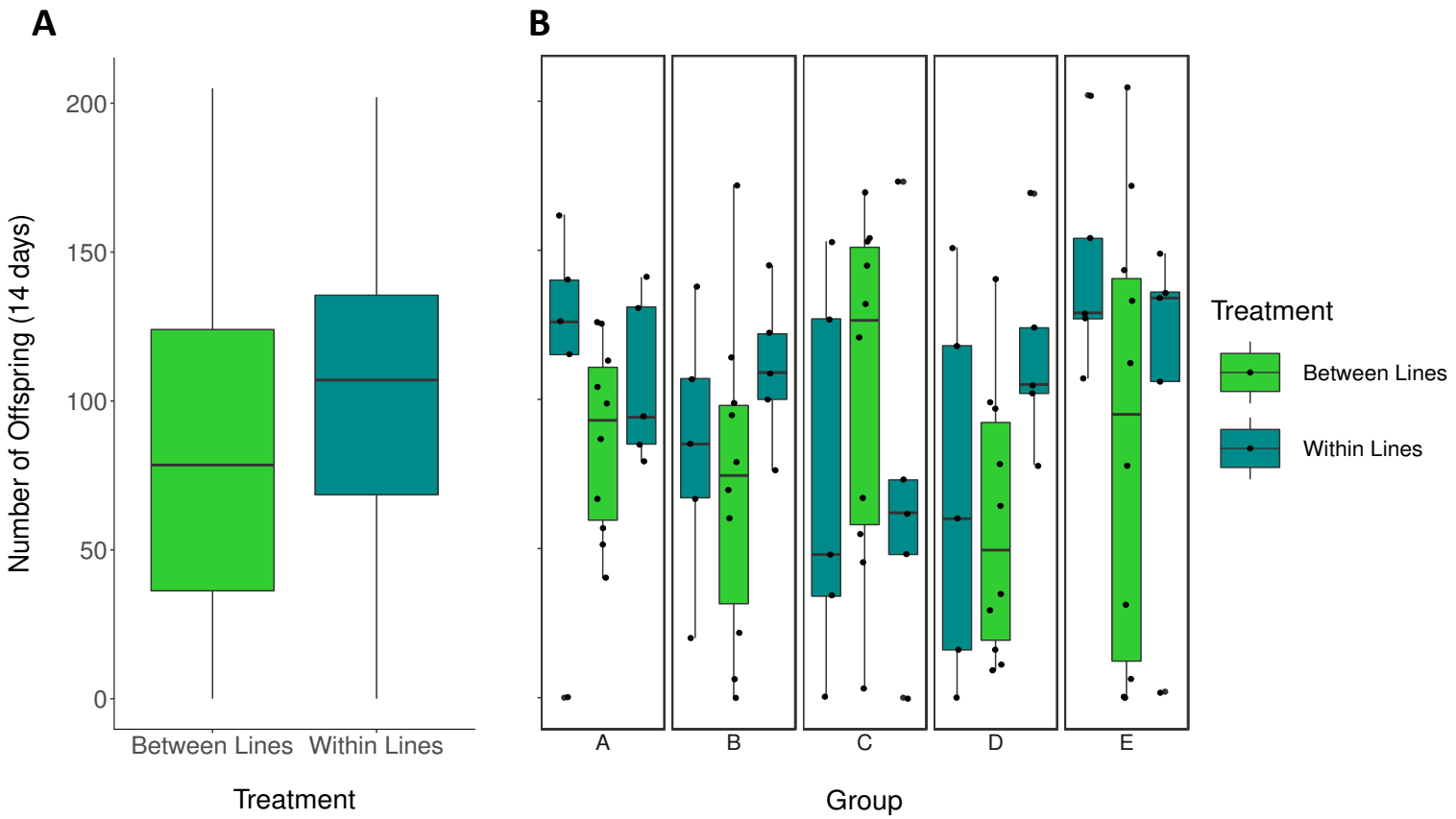

Figure 3.8. The number of offspring produced over 14 days by *T. castaneum* females paired with a mate from within their own line or from another high temperature line. Panels show **A** Summary of data across all lines and combinations. **B** A breakdown of line pairings and the crosses between them. The lines were grouped and crosses between lines occurred within groups. Relative to Table 3.6 (below), Line 1 of each group (A-E) is plotted on the left, Line 2 on the right and their cross in the middle. Overall, there was no difference in reproductive output of within- or between line crosses.

Table 3.6. Five GLMs of *T. castaneum* reproductive output of high temperature (38°C) thermal line pairs and hybrid crosses between them. Number of offspring produced over 14 days by individual mating pairs was the response variable and the line origin of the parents was fitted as a factor. The hybrid cross was always fitted as the baseline. There was no crossing between groups (e.g. Group A contained two lines and the crosses between these lines only). The replicate lines used were all maintained in the same way at 38°C for the same length of time.

	Fixed Effect	Estimate	Standard Error	t	Pr(>|t|)
Group A	Intercept	86.90	12.84	6.77	0.008
	Line 1: THL1 38.1	21.70	22.24	0.98	0.343
	Line 2: THL1 38.2	19.10	22.24	0.86	0.402
Group B	Intercept	71.70	14.87	4.82	< 0.001
	Line 1: THL1 38.3	15.80	27.82	0.57	0.578
	Line 2: THL1 38.4	38.70	25.75	1.50	0.152
Group C	Intercept	104.40	19.12	5.46	0.029
	Line 1: THL1 38.5	- 32.00	33.13	- 0.97	0.348
	Line 2: THL1 38.7	- 33.20	33.13	- 1.00	0.330
Group D	Intercept	57.80	15.25	3.79	0.002
	Line 1: THL1 38.8	11.20	26.41	0.42	0.677
	Line 2: THL2 38.3	57.80	26.41	2.19	0.043
Group E	Intercept	88.00	20.53	4.29	< 0.001
	Line 1: THL2 38.4	55.80	35.55	1.57	0.135
	Line 2: THL2 38.6	17.40	35.55	0.49	0.630

3.4 Discussion

I performed an in-depth experimental investigation into the consequences of outcrossing and genetic rescue and found that, overall, there was no evidence that increases in population size, migration or outcrossing improved fitness in *T. castaneum* populations. Despite some evidence for an increase in hybrid offspring longevity at the individual level, there was no indication that increases in population size and levels of migration could improve fitness. Further to this, crossing individuals between independent replicates of the high temperature lines did not result in increases in reproductive output or survival. These results were unexpected, as the thermal lines had been maintained for over 50 generations at small population sizes, so were likely to be suffering from some level of inbreeding depression. However, in these conditions, it seems that fitness was not improved, but was in fact impaired, by the attempts at rescue.

The small thermal line populations could be considered a bottlenecked version of the large stock populations (the source of the migrants for this study), where the number of adults founding each generation was reduced from 600 (in the KSS stocks) to 100. Additionally, due to strong selection pressures, especially at the start of experimental evolution, it is likely that the 38°C high temperature lines underwent more significant bottlenecks than the control lines. Previous studies have shown that warm temperature conditions can be stressful for *T. castaneum* and reduce their reproductive fitness (Sales, 2018; Sales *et al.*, 2018). Therefore, it is likely that effective population size was reduced before adaptation could take place. Moreover, the early 38°C lines produced approximately half the number of adults compared to their 30°C equivalents, and some replicates did not produce enough adults to maintain them (Dickinson 2018). Levels of inbreeding and genetic drift are therefore expected to have increased within these populations which, in turn, could result in reduced fitness or adaptive capacity (Lande & Barrowclough, 1987).

At the whole-population level, I found unexpected evidence of decreased fitness after the populations had been free to rapidly expand to 100 times their original size, contained within a much larger, but otherwise identical, habitat. Much of the research into population expansion focuses on spatial expansion and colonisation of new territories. Individuals at the edge of the expansion tend to be less fit than those remaining in the ancestral range (Peischl *et al.*, 2013). However, this logic applies to populations undergoing range expansion into heterogenous habitats, and numerous factors, including spatial sorting and gene surfing, can create changes in fitness. The format of my study did not necessarily enable colonisation of novel areas, but allowed expansion into the same habitat but on a much larger scale. Theoretical work has shown that a rapid increase in population number can result in some interesting genetic effects, including an excess of rare mutations (Slatkin & Hudson, 1991; Zeng & Charlesworth, 2010), and a decrease in linkage disequilibrium (Slatkin, 1994). This effect has been observed in humans (Keinan & Clark, 2012), but models predict that although there are a larger number of deleterious variants present, the more detrimental ones are more effectively purged in a large population, so the resulting individual fitness should be similar (Gazave *et al.*, 2013). There is currently a lack of experimental research to explore these issues in more detail and across different conditions. An increase in population size could also result in a change in density, however, in this experiment I was careful to scale up the size of the population enclosure and resources available proportionally to the number of adults founding each generation, so density remained constant.

It is generally accepted that gene flow into a small population can increase genetic diversity and levels of fitness (Frankham, 2016), and high-profile success stories of genetic rescue exist (Madsen *et al*., 1999; Ingvarsson, 2002). However, I found no evidence that 10 generations of migration into previously bottlenecked populations resulted in increased fitness. Gene flow as a result of migration is not guaranteed, as migrant individuals may fail to reproduce viably (Turček & Hickey, 1951), but I am confident that at least some gene flow occurred in these lines, as genome sequences of 12 individuals from each migration and large line show marked differences in

heterozygosity and evidence of introgression (Lewis & Spurgin, unpublished data). However, gene flow may not always result in increased fitness, as high levels of migration into a population can disrupt local adaptation, possibly resulting in offspring with reduced fitness (Storfer, 1999). In these cases, migration will oppose naturally selected optima, resulting in suboptimal fitness, a phenomenon known as 'migration load' (Lenormand, 2002). It is possible that the more diverse KSS migrants in this study were also less fit than the thermal lines adapted to higher temperatures, and this gene flow would therefore not confer any fitness benefits to the population. However, if this were the case, I would expect to see some fitness cost of such crosses at the individual level (Edmands, 2007). Instead, I found that crossing males from high temperature thermal lines with migrant females resulted in increased fitness at high temperature, but there was no such effect in any of the other crosses. Furthermore, in almost all of the treatments, the offspring resulting from these crosses had increased longevity when surviving through trials at high temperatures, compared with the 30°C controls.

It is important to consider that the KSS stock may not, in fact, be as diverse as assumed. It was constructed from 11 separate lineages of *T. castaneum* (Michalczyk, 2008), but over the years, may have lost some of this initial diversity. As the thermal selection lines descend from this population, this would suggest that the assisted migration between them may not have led to any genetic changes. I believe this to be highly unlikely, for a number of reasons. Firstly, as explained above, I was able to identify evidence of introgression and increased heterozygosity from the genomes of individuals from the migration lines, which would not have been possible had the migrants been genetically very similar to the thermal lines. Secondly, genetic drift alone is unlikely to cause homogenisation, either within the stocks, or between lines, as loss of genetic diversity is stochastic. Thirdly, due to the much smaller population sizes of the thermal lines, and therefore the stronger effects of drift in these lines, basic simulations suggest that 52 generations of isolation from the stocks would lead to some genetic differentiation between these populations, solely due to drift (data not shown). As these high temperature lines were under strong selection, I would expect that this would further enhance their genetic divergence from the KSS stock.

Finally, if the stock and thermal lines were essentially the same, I would not expect to see fitness benefits when I studied outcrossing at the individual level.

Migration into a population can also lead to 'swamping' of the local gene pool if a migrant has particularly high short-term productivity, resulting in a breakdown of local adaptation (Hedrick & Fredrickson, 2010), or an introduction of deleterious alleles (Hedrick *et al.*, 2014). My study maintained a 10% migration rate into the population for 10 generations, which is arguably unrealistically high. In conservation, genetic rescue attempts are usually a single gene flow event at a higher frequency (e.g. Florida Panther, ~30% (Johnson *et al.*, 2010); Mexican wolves, three lineages of three, two and two individuals merged (Hedrick *et al.*, 1997). However, there are arguments that longer-term genetic restoration (low-level gene flow over several generations) is the more effective approach to salvaging vulnerable populations (Adams *et al.*, 2011). Yet, such sustained gene flow may be more likely to result in genetic swamping (Haygood *et al*., 2003). I found that gene flow at the population-level did not affect fitness, despite evidence that individual outcrossing showed some limited positive effects. When I investigated the individual-level effects of outcrossing, I found that female migrants mating with native males resulted in more progeny than any other pairing. However, I found no fitness benefits when high temperature females were mated with either a high temperature male from an independent line, or a migrant male. These findings are consistent with a scenario in which initially increased fitness of migrant individuals can be lost in future generations, either as a result of the breakdown of local adaptation, or as a result of the introduction of deleterious alleles, which then spread. Further research that directly monitors fitness of individual lineages over multiple generations would help confirm this possibility.

One suggestion for the sex-specific difference in fitness of outcrossed pairs may be that sex-specific adaptation has occurred in the lines. If male-specific function is thermally-sensitive, and males from the 38°C high temperature lines have adapted to function at higher temperatures, the migrant (unadapted) males will introduce poorly-adapted male traits for high temperature, leading to outbreeding depression in such crosses. This theory is supported by recent work on *T. castaneum* showing that male

reproduction is especially sensitive to temperature (Sales *et al.*, 2018). However, if this were the case, I would also expect to see an increase in fitness due to inbreeding avoidance when crossing between 'adapted' thermal lines compared to within-line crosses. The lines had been separated for approximately 62 generations of experimental evolution, so I expected genetic drift in these smaller populations to have caused them to differentiate enough that back-crossing between them would have shown some genetic rescue from inbreeding. However, I found no difference in reproductive fitness between parents crossed from the same thermal line compared with parents crossed from different thermal lines (intra-line and inter-line crosses, respectively). An alternative explanation is that the high-temperature females were maladapted, reducing their fecundity. This idea is supported by the lack of improvement in the thermal lines when the reproductive pairs were of mixed thermal line origin. The data also suggest that this potential female maladaptation is not an artefact of drift, in which case it would not be evident across so many lines, but instead is likely to be the result of a trade off or incomplete adaptation. Although there is some evidence that female reproduction can be affected by temperature (Irwin & Lee, 2000; Berger *et al.*, 2008), there is little evidence in the literature that female fertility is more vulnerable than male fertility with regards to high temperature (Sales *et al.*, 2018; Iossa, 2019), so further work in this area is needed.

There is some debate amongst population geneticists with regards to the ideal population structure for conservation management (Simberloff & Abele, 1976). The argument here is whether it is better to have a single large mixing population, or several small populations between which migration can occur (Diamond, 1975; Miller-Rushing *et al.*, 2019). My work suggests that neither is necessarily better. Although I have found evidence that large populations were less fit than small ones, I did not find evidence that mixing small populations improves fitness, although there was no cost to this mixing. I suggest that the genetic backgrounds of small populations to be mixed may play an important role in the success of genetic rescue attempts. Populations that are genetically very similar are likely to experience little to no effect of mixing. However, if the differences between the populations are too great, outbreeding

depression may occur (Lynch, 1991). These effects have been observed in both animals (e.g. the Arabian oryx, *Oryx leucoryx*; Marshall & Spalton, 2000) and plants (e.g. the common primrose, *Primula vulgaris*; Barmentlo *et al.*, 2018).

In this study, I aimed to explore the role of habitat size (and therefore effective population size) on adaptive potential and fitness. While size is clearly an important factor for population success (Hoffmann *et al.*, 2017), I find here that issues arising from past bottlenecks can persist, or be exacerbated, even in a large population. I find no evidence that evolutionary rescue can address this within relatively short time scales. To facilitate population rescue, assisted migration can be implemented in the hope that genetic rescue may follow, however, I also found no evidence of this. My study highlights the need for more empirical research into what factors might enable the success of population interventions.

Chapter 4

The effects of heat stress during development

Abstract

As the global climate warms, incidences of extreme weather events, such as heatwaves, are expected to increase in frequency. Heatwaves exert stress on individuals, which can have important consequences for populations, species and ecological communities. It is likely that heat stress during development can have detrimental fitness effects later in life, but we have little understanding of the mechanisms and impact of such developmental effects. Here, I use *Tribolium castaneum*, an established insect model and worldwide pest of stored grain, to explore the effects of exposure to high temperature during development on subsequent adult fitness. In female*s*, the pupal stage – during which the ovaries and spermatheca mature – was particularly sensitive to high temperatures, with reduced later life reproduction in individuals exposed to 38°C as pupae. Further experimentation enabled separation of how developmental temperature affects fecundity and fertility. In females, development temperature impacted fecundity (egg laying rates) while in males it impacted the proportion of eggs laid by female mates which hatched and survived to adulthood, which may represent fertility. Intriguingly, these effects were not observed in long-term selection lines (see Chapter 2), which maintain fecundity and fertility despite development at high temperature, indicating evolutionary adaptation to such heat stress. Overall, the results of this study suggest that in *T. castaneum,* there are considerable fitness costs associated with developmental temperature stress, but these may be overcome via rapid evolution. Further investigations into the reproductive biology and morphology of *T. castaneum* following development at high temperature could help us to understand the mechanisms for the observed evolution.

4.1 Introduction

When organisms are exposed to stressors, there can be immediate, short-term consequences, such as changes in hormone levels (Demers & Bayne, 1997) and/or behavioural modifications to try to mitigate the stress (e.g. to dissipate excess heat, Bryant, 1983; or avoid predators, Adamo *et al.*, 2013). However, there can also be longer-term consequences, which can persist in the individual after the stressor has been removed (Kaufer *et al.*, 1998; Adamo *et al.*, 2013), or even be transmitted to subsequent generations (Sales *et al.*, 2018). Stress during development appears to be particularly important and long lasting, with negative effects on later-life fitness identified across multiple taxa (Spencer *et al*., 2003; Roseboom *et al*., 2006; Eyck *et al.*, 2019). A recent meta-analysis on the effects of developmental stress across animal taxa concluded that the effects of developmental stress depend on the latency and duration of exposure (Eyck *et al.*, 2019), with older juveniles exposed for longer experiencing the greatest reductions in later-life fitness. However, we know little about how responses to developmental stress vary between taxa, nor how developmental stress leads to evolutionary adaptation.

Insects represent over half of all described species (Mayhew, 2007) and provide a number of important ecosystem services. Their life-history offers an excellent opportunity to study the later life effects of developmental stress. Most insects are holometabolous, undergoing metamorphosis during development, which has likely contributed to diversification in this clade (Mayhew, 2007). However, the separate life stages often differ markedly in their morphology and ecology, and may have different habitat requirements (Kingsolver *et al.*, 2011). For example, many aquatic insects spend their juvenile stages in water but their adulthood in the air, so environmental changes to waterways would only affect developing individuals (Derka *et al.*, 2019). In other insect taxa there is spatiotemporal overlap in the presence of the different developmental stages, with populations containing individuals at different life stages (Kiritani & Nakasuji, 1967). In such instances, all individuals may be exposed to the same environmental stressors, but may exhibit varying responses due to differences in physiology, morphology or microhabitat associated with their life stage (Lamb &

Gerber, 1985; Bauerfeind & Fischer, 2005). Understanding how stress affects different insect life-history stages is therefore important for understanding broader ecological and evolutionary processes.

Changes in temperature are a major source of stress for natural populations (Gates *et al.*, 1992; Sage *et al*., 2015). As global warming progresses, extreme climatic events, such as heatwaves, are predicted to increase in frequency (Meehl & Tebaldi, 2004; Christidis *et al.*, 2014). As yet, there is limited understanding of the effects that these events can have on ecological systems, partly due to the complexity of such systems (Ummenhofer & Meehl). Insects, one of the most abundant taxa, are thought to be under particular pressure from climate change due to their inability to thermoregulate (Paaijmans *et al.*, 2013), and it is predicted that we will see drastic changes in insect communities due to temperature rises (Nooten *et al.*, 2014). It is also becoming increasingly clear that understanding responses to climate change requires multidisciplinary study, from the level of the gene to the ecosystem (Scheffers *et al.* 2016). An important part of this is developing a detailed understanding of how variations in temperature across life-history stages impact individual fitness. In insects, there is limited understanding of how thermal stress during development affects later life fitness. This is likely to have important consequences for understanding, and ultimately predicting, population and species-level responses to climatic change.

Many studies into thermal tolerance focus on survival or loss of motility, without taking into consideration other crucial life-history effects such as reproduction (Hoffmann, 2010). In insects, due to their short lifespans, even reversible reproductive damage can be particularly important due to the high proportion of total lifetime spent recovering after a heat stress (Jørgensen *et al.*, 2006). As the frequency of heatwaves is predicted to increase, it is important to understand how these events will affect the fertility and reproduction of the individuals that survive them, to better understand their ecological impact (Sultan, 2007). Several studies provide evidence that exposure to high temperatures causes a decline in reproductive success in insects, for both males (Zizzari & Ellers, 2011; Sales *et al.*, 2018) and females (Krebs &

Loeschcke, 1994). Experimental studies using insect populations have shown that developmental thermal stress can result in reduced sperm number and egg viability (Green *et al.*, 2019), reduced fecundity (Wang *et al.*, 2014), increased egg development time (Piyaphongkul *et al.*, 2012) and changes in oviposition patterns (Zhang *et al.*, 2015). However, we still do not understand how general these effects are across taxa, nor to what extent exposure to thermal stress across generations can result in evolutionary adaptation to these developmental effects.

Tribolium castaneum is an ideal species in which to study the effect of temperature on development due to its popularity in developmental biology studies (Brown *et al.* 2009). They differ from *Drosophila*, the other common model insect, in both juvenile and adult morphology and so can provide different insights into development (Brown *et al.* 2009*;* Angelini & Jockusch, 2008). The ancestral developmental mechanism of insects is more closely represented by *Tribolium spp* than by *Drosophila* (Lynch *et al.*, 2011). They are holometabolous, along with 80% of all insect species and 50% of all animal species (Kristensen, 1999; Whiting, 2002). *Tribolium* species have been used extensively in studies of responses to temperature change (e.g. Mahroof *et al.*, 2003; Scharf *et al.*, 2016; Sales *et al.*, 2018). Surprisingly, however, there is limited research on the interaction between temperature and development in this model species.

Here, I use the *T. castaneum* model to investigate the effects of exposure to increased temperature during development on later-life fitness. Existing literature suggests that developmental stress will have negative effects later in life, with longer lasting stress having more pronounced effects (Eyck *et al.*, 2019). I first quantify overall effects of developmental thermal stress on adult reproductive output and survival. Second, I test how the duration and timing of this stress affects adult reproductive output. Finally, I separately test how developmental thermal stress affects fecundity (the number of eggs laid) and fertility (the proportion of these eggs that hatch and survive to adulthood) and how these differ in stock populations and long-term thermal selection lines. This study will inform our understanding of developmental stress in holometabolous insects, and its fitness consequences.

4.2 Methods

Unless stated otherwise, all experiments here were carried out using Krakow Super Strain (KSS) stocks maintained at 30°C, with ~600 adults producing each subsequent generation (as described in Chapter 2). Count data are generally presented as boxplots, as described in Chapter 2.

4.2.1 Effects of developmental heat stress on fitness

Two subsets of the KSS stock population were created by taking 150ml (~100g) of fodder (9:1 mix of organic strong white bread flour and Brewer's yeast) from the original population after females had oviposited, thereby maintaining consistent egg density with the source population. One subset was immediately moved to 38°C, to experience increased temperature throughout development and the other was kept at 30°C until sexual maturity. At pupation, 100 male and 100 female pupae were sexed from each treatment and isolated by sex in groups of 20. At reproductive maturity (10 days after eclosion), individual males and females were paired, both within and between treatments in a full factorial design (n = 45), and allowed 24 hours to mate at 38°C. After this, the female from each pair was moved onto fresh fodder to oviposit for a week, at which point she was discarded and the eggs produced were allowed 35 days to reach adulthood before being frozen and counted.

To test whether survival through heat stress differed between beetle populations subjected to different developmental temperatures, adults from both treatments (described above) were kept at 38°C for a week before ~300 individuals (1.5ml) from each treatment were put into 42°C. This was to ensure that any effects observed were not due to thermal acclimation. Each day, the number of deaths per treatment were counted and recorded. The dead adults were removed and the remaining live beetles were returned to the 42°C environment. The dead adults were then sexed (by identifying the male-specific sex patches on the ventral side of the first femur (Hinton, 1942; Faustini *et al.*, 1981).

To test for differences in reproduction of beetles reared at 30°C and 38°C, a generalised linear mixed model (GLMM) was fitted, with Poisson error distribution. The number of offspring produced was fitted as the response variable and the developmental temperature of the male and of the female parents were fitted as explanatory variables. An observation level random effect was added to account for overdispersion (Harrison, 2014). A separate model was constructed to test for an interaction between the paternal developmental temperature and the maternal developmental temperature. Only the effect of the interaction term was reported from this second model.

A Cox proportional hazards model was used to test for differences in survival between developmental treatments and sexes. The survival time (in days) was fitted as the response variable, with the developmental temperature and the sex of the beetle fitted as factors. This experiment was continued until all individuals had died so there was no requirement for censoring. A Kaplan-Meier estimator was created using the developmental temperature and the sex as the fixed effects and this was plotted using the 'ggsurv' function from the 'GGally' package (Schloerke *et al*., 2017) in R.

4.2.2 Relative vulnerability of reproductive output following thermal stress at different times during development

To test the importance of the timing of developmental stress, 300 adults from KSS stocks were added to each of eight containers (7cm diameter, 7.5cm depth), containing 100g fodder) at 30°C to mate and oviposit for two days to create replicate populations. After this, the adults were removed from all replicates (this point subsequently referred to as day 0). Six replicates were exposed to 38°C for different five-day blocks, creating six treatment groups spanning the entirety of development from egg to adulthood. Two additional replicates were used as controls: one was exposed to high temperature (38°C) for 30 days, covering the entirety of development, while the other was kept at a control temperature (30°C) throughout. On day 20, 40

male and 40 female pupae were sexed from the 38°C control and from each treatment group (here named treatments 1-6, Table 4.1), while 200 pupae of each sex were collected from the 30°C control. These pupae were separated in groups of 20, by sex and treatment and allowed to develop at their assigned temperature regime. On day 30, all treatment groups were put into control temperature to acclimatise. On day 35, pairs were mated in cross-treatment combinations.

Males from each five-day heat treatment (i.e. treatments 1-6) were paired with females from the 30°C control line, to isolate the effect of high temperature on different male developmental stages. There was also a control treatment in which males and females from the 30°C control lines were paired. Conversely, females from each five-day heat treatment were paired with males from the 30°C control line to identify developmental stages in females that may be more vulnerable to high temperatures. There was an additional treatment in which males and females from the 38°C control lines were paired. This treatment was excluded from the analysis and was used as a visual comparison to demonstrate the effect of high temperature exposure throughout development.

Table 4.1. The temperature regimes for each of the heat treatments that *T. castaneum* were exposed to test the impact of heat stress on later adult reproduction. Red indicates the treatment was kept at 38°C for the time period and blue indicates 30°C.

Time	Days 0 - 5	Days 5 - 10	Days 10 - 15	Days 15 - 20	Days 20 - 25	Days 25 - 30	Days 30 - 35
Treatment 1	X	X	X	X	X	X	X
Treatment 2	X	X	X	X	X	X	X
Treatment 3	X	X	X	X	X	X	X
Treatment 4	X	X	X	X	X	X	X
Treatment 5	X	X	X	X	X	X	X
Treatment 6	X	X	X	X	X	X	X
38°C control	X	X	X	X	X	X	X
30°C control	X	X	X	X	X	X	X

I tested for female and male developmental effects using separate models. This was preferred over a single model with an interaction term, as i) I wished to test for separate male and female effects, rather than for a difference in effects, and ii) the treatment variable had a large number of categories. To test whether short-term exposure to high temperature had a significant effect at any particular developmental stage in males, a GLMM was constructed with Poisson error distribution. The number offspring was fitted as the response variable, while treatment of the parent (i.e., the developmental stage that the focal parent was exposed to high temperature, with 30°C control set as the baseline) was used as an explanatory variable. These models both required an observation level random effect to account for overdispersion.

4.2.3 Isolating the effects of developmental heat stress on fecundity and fertility

Subsets of the stock KSS population were created by taking 150ml (~100g) of fodder from the original population after females had oviposited. One subset was moved to 38°C and the other was kept at 30°C until sexual maturity. At pupation, 180 pupae were sexed from each treatment and isolated by sex in groups of 20. At reproductive maturity, individual males and females were paired, both within and between treatments (n = 20), and allowed 24 hours to mate at 38°C. In addition, 20 pairs from each of three replicate high temperature lines (developed at high temperature) were included as a positive control (see Chapter 2 for maintenance details).

After mating, the females were each put into a petri dish containing fresh fodder and allowed to oviposit for seven days at 38°C. After this, females were moved into a vial containing fresh fodder, made from finely sieved flour and yeast (to maximise egg visibility), and allowed two days to oviposit. The eggs were then removed, counted and put into a clean petri dish with ~7g fresh fodder to develop. After 35 days, the number of adults in each petri dish were counted to identify the proportion of eggs that survived through to adulthood. This experiment was replicated three times. Pooling

the data from these replicates had no effect on the model outcomes (data not shown), experiment number was included as a random effect in the models to account for this.

To test for differences in the number of eggs produced by KSS parents reared at high and low temperatures, a GLMM was constructed, with Poisson error distribution. The number of eggs produced after two days of oviposition was entered as the response variable and maternal and paternal developmental temperature were both explanatory variables and the experiment was included as a random effect. The model residuals were overdispersed so an observation level random effect was included. Additionally, a GLM was fitted to compare how the fecundity of the long-term high temperature selection lines compared with the KSS when both parents had developed at 38°C. As before, the egg count was the response variable and there was a Poisson error structure. The explanatory variable was the line origin (KSS stock or thermal selection line) of the parents.

A separate model was constructed to test for differences in viability of eggs laid by KSS parents reared at control or high temperatures. The number of offspring that survived until adulthood was counted and divided by the total egg count to calculate the proportion of viable offspring (i.e. those that developed to adulthood). This was modelled as a binomial response in a GLMM. The explanatory variables were maternal and paternal developmental temperature and, as above, experiment was fitted as a random effect and an observation level random effect was included to account for overdispersion. As described previously, the interaction term was reported from a separate model. A GLM was constructed with binomial error structure, to test for differences in the thermal selection lines and the KSS stocks. The proportion of viable offspring produced between KSS parents when both had developed at high temperature was compared with that of the high-temperature thermal lines. The proportion of viable offspring was fitted as the response variable, coded as above. Line origin (KSS stock or thermal selection line) was fitted as an explanatory variable.

4.3 Results

4.3.1 Effects of developmental heat stress on fitness

Development at high temperature (38°C) was associated with a reduction in the reproductive output of both males and females (Table 4.2, Figure 4.1). There was a reduction in offspring produced by pairs in which the mother or father (or both) were reared at high temperature, and median reproductive output was lowest when both parents were developed at high temperature (Figure 4.1), although there was no interaction between the maternal and paternal developmental temperatures (Table 4.2). As well as a reduced number of offspring, beetles that developed at high temperature also had reduced median survival (four days) in heatwave conditions (42°C), compared with those reared at control temperature (seven days; Table 4.3, Figure 4.2). There was no difference in survival between male and female beetles within each developmental regime (Table 4.3, Figure 4.2).

Table 4.2. A GLMM testing the reproductive output of pairs of *T. castaneum* from different thermal regimes (30°C or 38°C). All pairs mated for 24 hours at high temperature. The number of offspring produced was fitted as the response variable and an observation level random effect (Var = 1.62) was included to account for overdispersion. The developmental temperatures of the mother and the father were fitted as factors, with control temperature (30°C) set as the baseline in each case.

Fixed Effect	Estimate	Standard Error	z	Pr(>|z|)
Intercept	4.434	0.170	26.073	< 0.001
Male Developmental Temperature	- 0.937	0.206	-4.558	< 0.001
Female Developmental Temperature	- 1.085	0.205	-5.282	< 0.001
Male Development Temperature * Female Development	- 0.121	0.411	0.295	0.768

Table 4.3. A Cox proportional hazards model of the survival of adult *T. castaneum* through heatwave conditions (42°C) following development at different temperatures (30°C or 38°C). Survival time (in days) was set as the response variable and the developmental temperature and sex of the beetle were fitted as factors, with baselines set as control temperature (30°C) and male, respectively.

	Coefficient	**Standard Error**	**Z**	**p**
Development Temperature	2.273	0.118	19.340	< 0.001
Sex	- 0.093	0.084	- 1.106	0.269
Development Temperature * Sex	-0.168	0.169	- 0.996	0.319

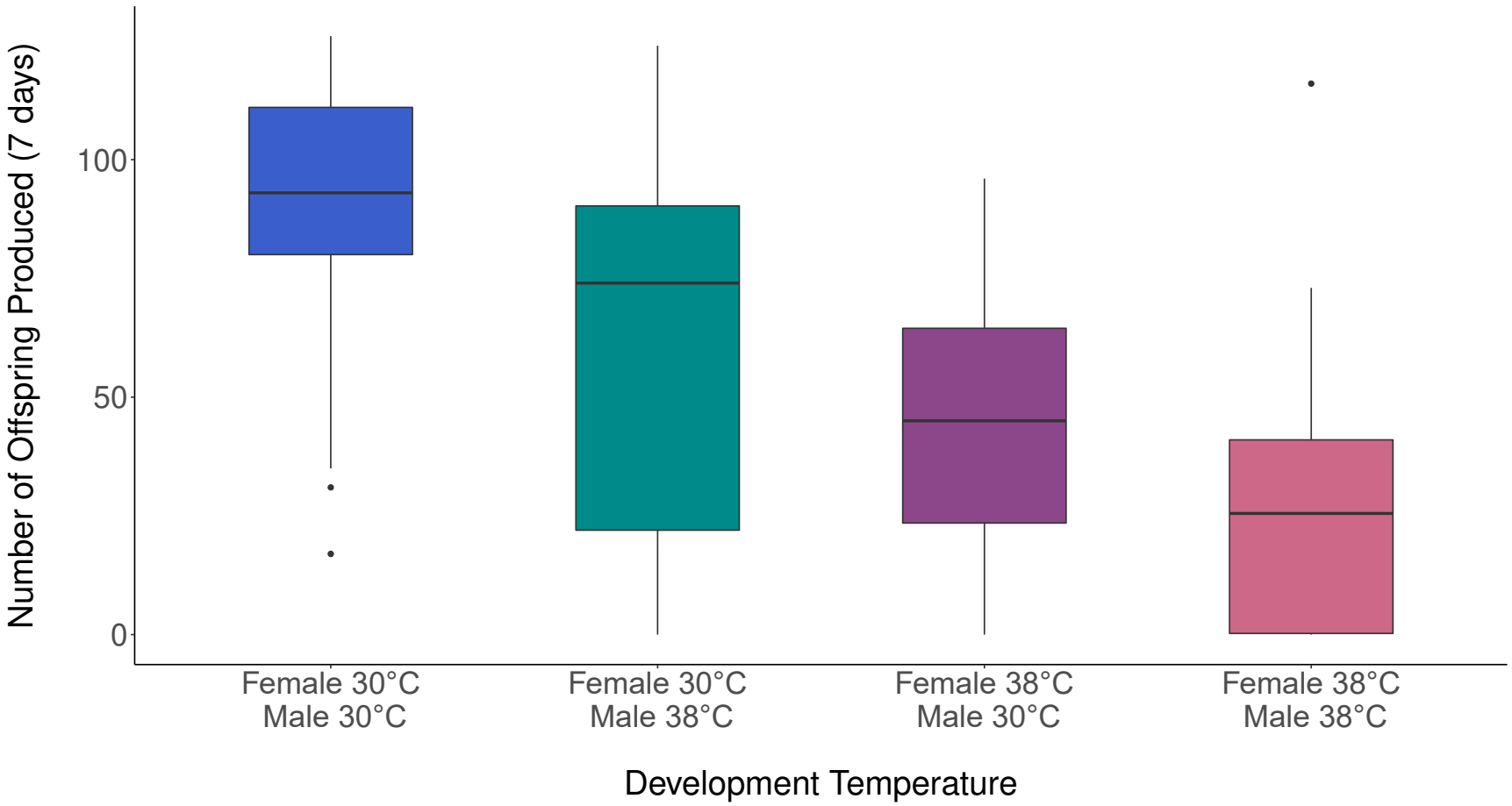

Figure 4.1. The number of offspring produced over by *T. castaneum* parents reared in different thermal regimes. Females (reared at either 30°C or 38°C) were mated with males (reared at either 30°C or 38°C) and allowed seven days of oviposition. Both male and female development at high temperature had a detrimental effect on their later life reproductive output.

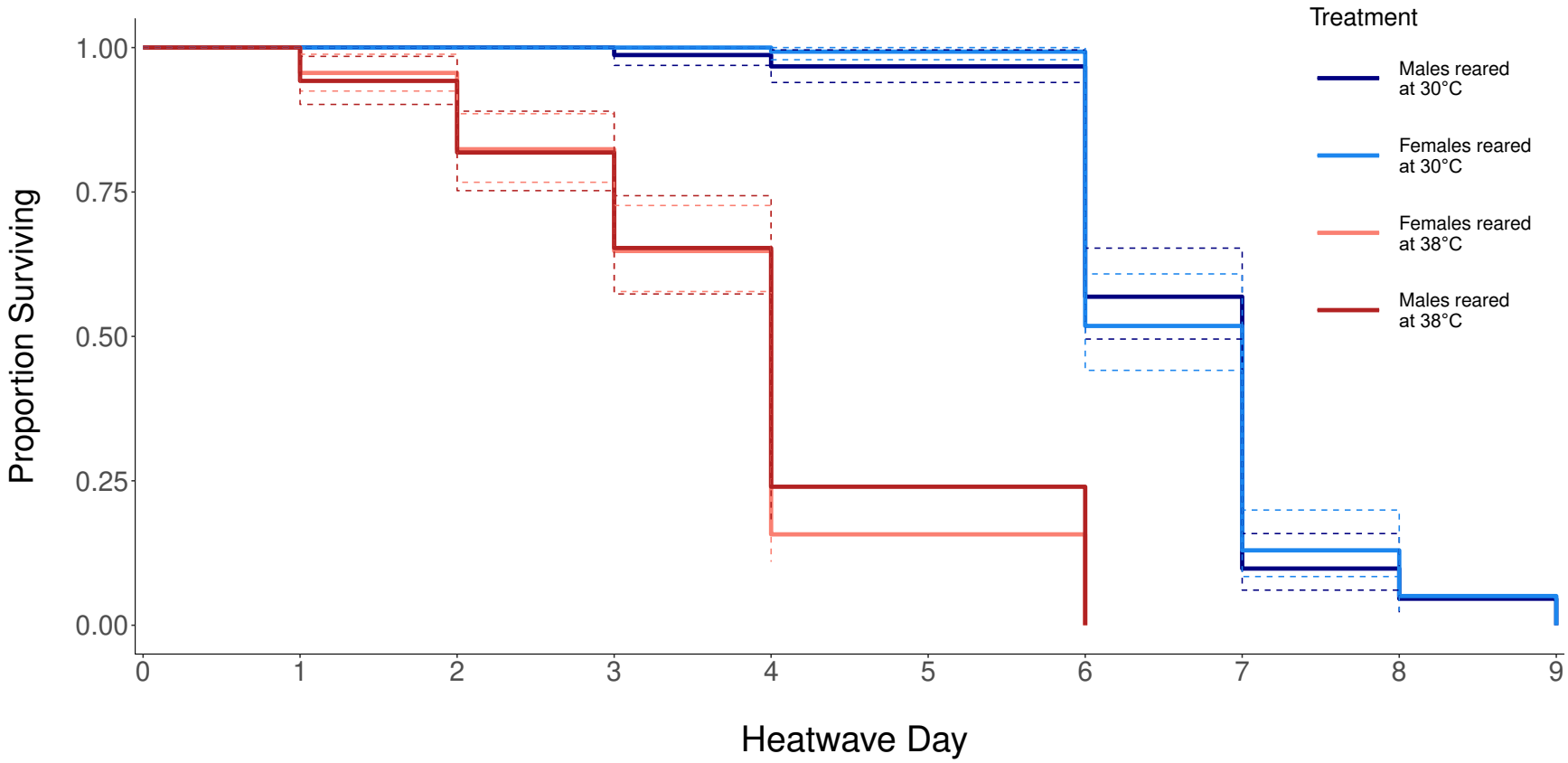

Figure 4.2. Kaplan Meier survival curves for *T. castaneum* through 42°C heatwaves following development at different thermal regimes (30°C or 38°C). Blue lines represent beetles reared at control temperatures (30°C), red lines represent beetles reared at high temperatures (38°C). Dark lines show male survival, paler coloured lines show female survival. Dotted lines represent 95% confidence intervals. Individuals developed at high temperature (38°C) did not survive as long though a heatwave (42°C) as those that developed at control temperature. There was no effect of sex.

4.3.2 Relative vulnerability of reproductive output following thermal stress at different times during development

In males, none of the five-day high temperature exposure treatments resulted in significant reductions in adult reproductive output relative to controls (Table 4.4A, Figure 4.3A). In females, exposure during days 15-20 or days 20-25 of development resulted in a reduction in reproductive output relative to controls (Table 4.4B, Figure 4.3B). Subjecting *T. castaneum* to five-day periods of high temperature exposure at different points throughout development showed that in no cases were reductions in fitness as severe as when individuals were exposed to high temperature for their entire development (Figure 4.3).

Table 4.4. GLMMs comparing the number of offspring produced by pairs of *T. castaneum* after one parent had been exposed to high-temperature for five days during (different periods of) development. Focal parent (**A.** fathers or **B.** mothers) was exposed to 38°C for five days. The 30°C control treatment was set as the baseline. Number of offspring produced was used as the response variable and the treatment of the focal parent was used at the explanatory variable (with no exposure to high temperature set as the baseline). Both models included an observation level random effect (Var_A = 0.305, Var_B = 0.319) to account for overdispersion.

	Fixed Effect	Estimate	Standard Error	Z	Pr(>|z|)
A: Male Effects	Intercept	4.953	0.102	48.60	< 0.001
	Days 0-5	- 0.065	0.147	- 0.44	0.660
	Days 5-10	- 0.219	0.147	- 1.49	0.136
	Days 10-15	- 0.166	0.149	- 1.11	0.265
	Days 15-20	- 0.086	0.147	- 0.58	0.559
	Days 20-25	- 0.159	0.146	- 1.09	0.275
	Days 25-30	- 0.224	0.146	- 1.53	0.125
B: Female Effects	Intercept	4.953	0.104	47.54	< 0.001
	Days 0-5	- 0.143	0.152	- 0.94	0.347
	Days 5-10	- 0.180	0.152	- 1.19	0.236
	Days 10-15	- 0.122	0.152	- 0.80	0.423
	Days 15-20	- 0.324	0.152	- 2.13	0.033
	Days 20-25	- 0.405	0.151	- 2.68	0.007
	Days 25-30	- 0.055	0.152	- 0.36	0.719

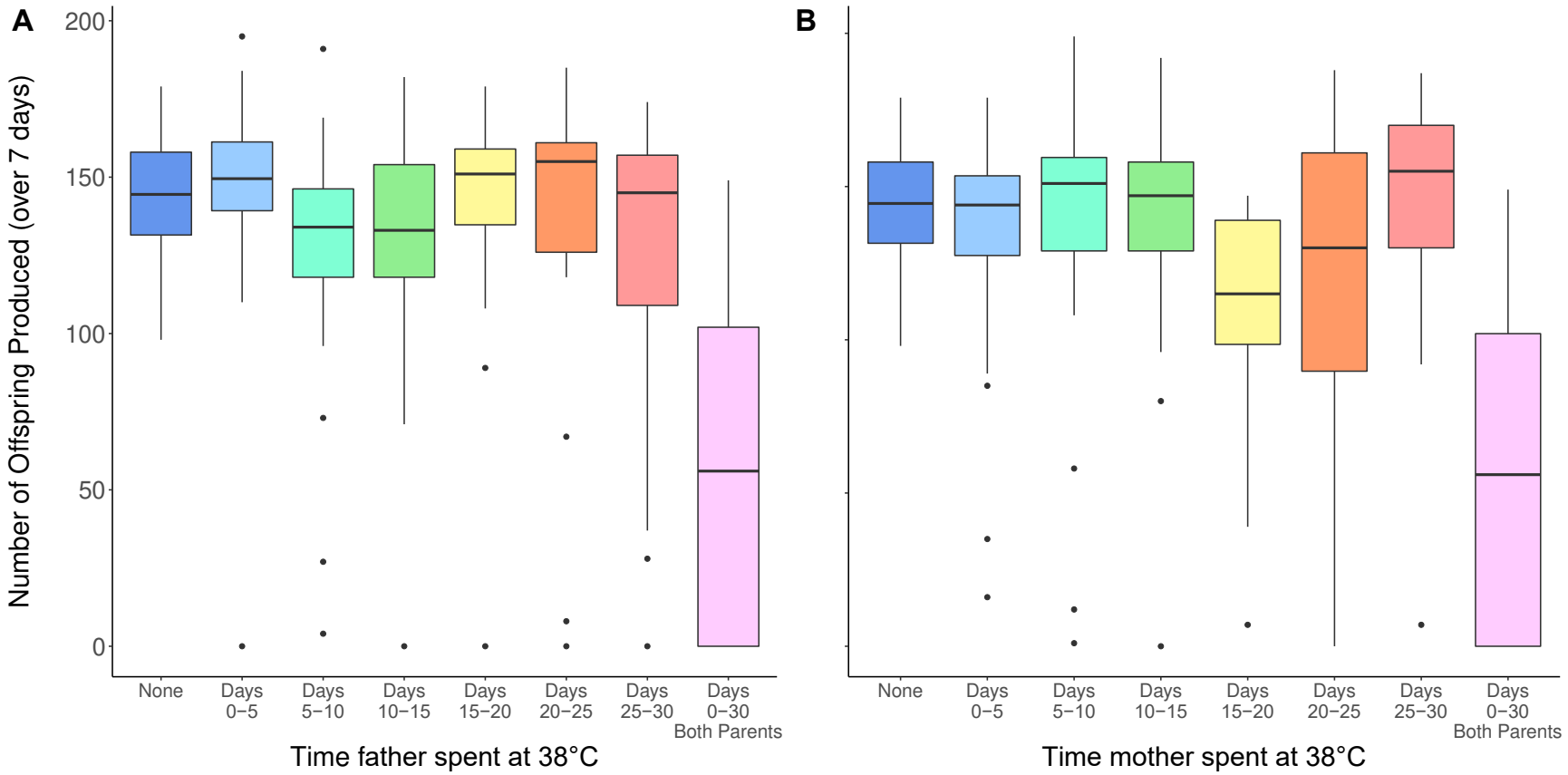

Figure 4.3. The number of offspring produced by pairs of *T. castaneum* after the focal parent (A. fathers and B. mothers) was exposed to five days of high temperature (38°C) during different stages of development. Offspring counts represent seven days of oviposition and pupae from these treatments were collected on Day 20. Female exposure to high temperature during days 15-20 or 20-25 resulted in reduced offspring production, but no other treatment was significant. Exposure of both parents to 38°C throughout development is included for visual reference only.

4.3.3 Isolating the effects of developmental stress on fecundity and fertility

Females developed at high temperature laid fewer eggs in a two-day period than those developed at control temperatures (Table 4.5, Figure 4.4). There was no effect of paternal development temperature on the number of eggs laid by the female (Table 4.5A, Figure 4.4). Females from long-term high temperature selection lines laid more eggs than KSS females from the corresponding treatment (i.e. where both parents developed at high temperature; Table 4.5B, Figure 4.4). There was no interaction between the development temperatures of the male and female on the number of eggs laid (Table 4.5).

Eggs produced by pairs in which fathers were developed at high temperature were less likely to survive to adulthood than those fertilised by fathers that developed at control temperature (Table 4.6A, Figure 4.5). There was no effect of maternal developmental temperature and no interaction between maternal and paternal developmental temperature (Table 4.6A). Eggs from KSS pairs in which fathers were developed at high temperature had a lower overall survival to adulthood than those from the high-temperature lines (Table 4.6B, Figure 4.5).

Table 4.5. Parameters from models for the fecundity (two-day egg count) of *T. castaneum* following development at different thermal regimes (30°C or 38°C). A. A GLMM to test for differences between different parental development temperatures. Fitted as factors were maternal development temperature and paternal development temperature (baselines set to 30°C). The experiment number was fitted as a random effect (Var = 1.02) and an observation level random effect was included due to overdispersion in the data (Var = 1.43). **B.** A GLM to test for differences in the effects of development at high temperature in the KSS and long-term high temperature selection lines. Line origin was the fitted factor, with selection lines were set as the baseline.

	Fixed Effect	Estimate	Standard Error	z	Pr(>|z|)
A.	Intercept	4.194	0.658	6.372	< 0.001
	Maternal Development Temperature	- 2.805	0.355	- 7.902	< 0.001
	Paternal Development Temperature	0.053	0.197	0.271	0.786
	Maternal Temperature * Paternal Temperature	0.482	0.452	1.065	0.287
B.	Intercept	2.853	0.031	91.250	< 0.001
	Line Origin	- 0.402	0.047	- 8.552	< 0.001

Table 4.6. Parameters from models of the fertility (proportion of the eggs laid that grew to adulthood) of *T. castaneum* raised in different thermal regimes (30°C or 38°C) during development. A. A GLMM including maternal and paternal development temperatures (baselines set to 30°C). Experiment was coded as a random effect (Var < 0.001) and there was an observation level random effect (Var = 9.61) to account for overdispersion. **B.** A GLM to compare the fertility of eggs laid at high temperature between KSS and long-term high temperature selection lines (selection lines set as baseline).

	Fixed Effect	Estimate	Standard Error	Z	Pr(>|z|)
A.	Intercept	- 1.440	0.663	- 2.172	0.030
	Maternal Development Temperature	- 0.572	0.701	- 0.816	0.415
	Paternal Development Temperature	- 4.122	0.713	- 5.784	< 0.001
	Maternal Temperature * Paternal Temperature	0.647	1.426	0.454	0.650
B.	Intercept	- 0.094	0.063	- 1.502	0.133
	Line Origin	- 2.470	0.150	- 16.463	< 0.001

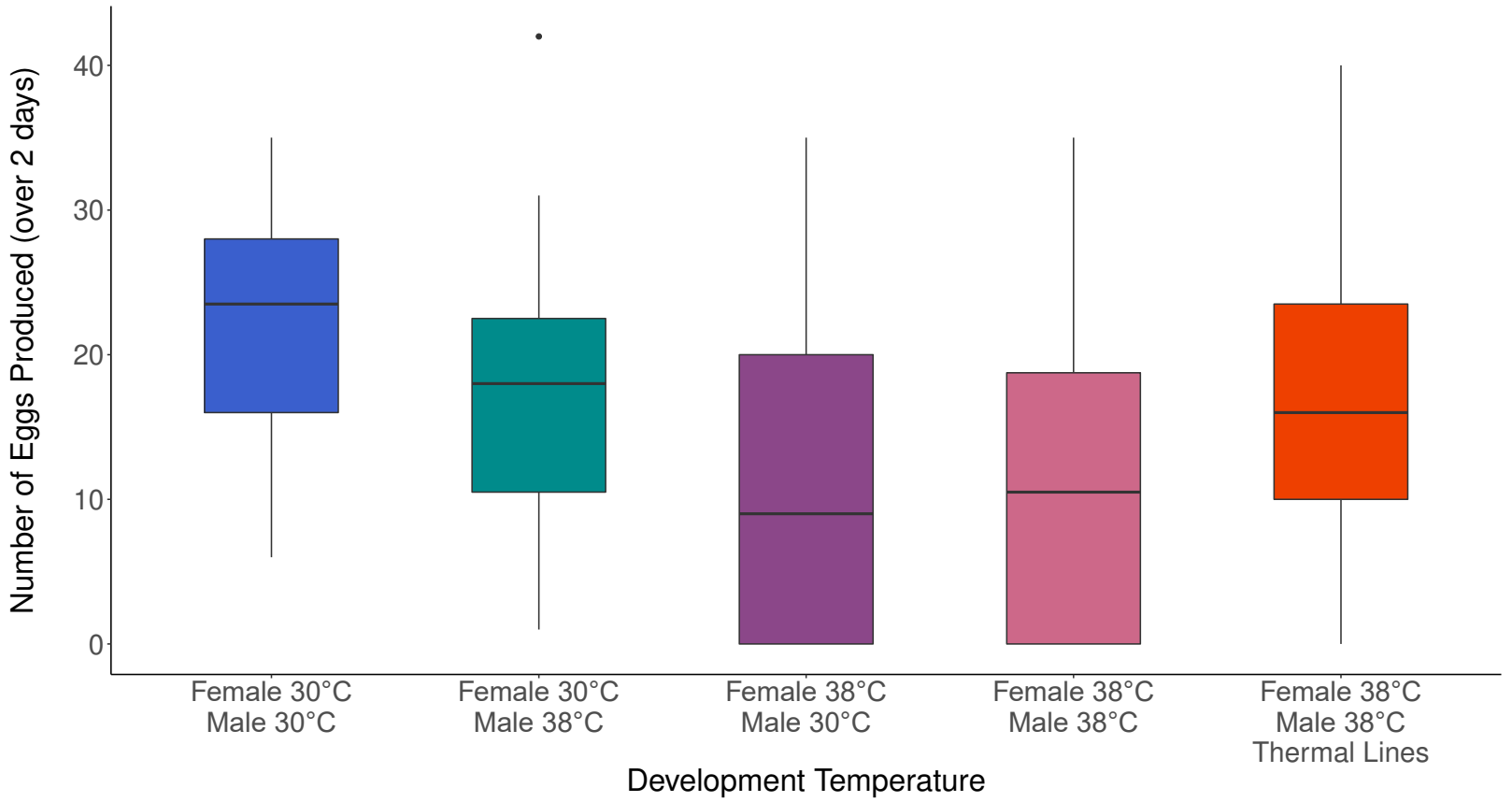

Figure 4.4. The number of eggs produced over two days by female *T. castaneum* that were raised in different thermal regimes (either 30°C or 38°C) mated with males (reared at either 30°C or 38°C). Female development at high temperature was associated with a significant reduction in the number of eggs produced. There was no effect of male development temperature. The long-term selection lines produced significantly more eggs than the KSS stocks following the same treatment.

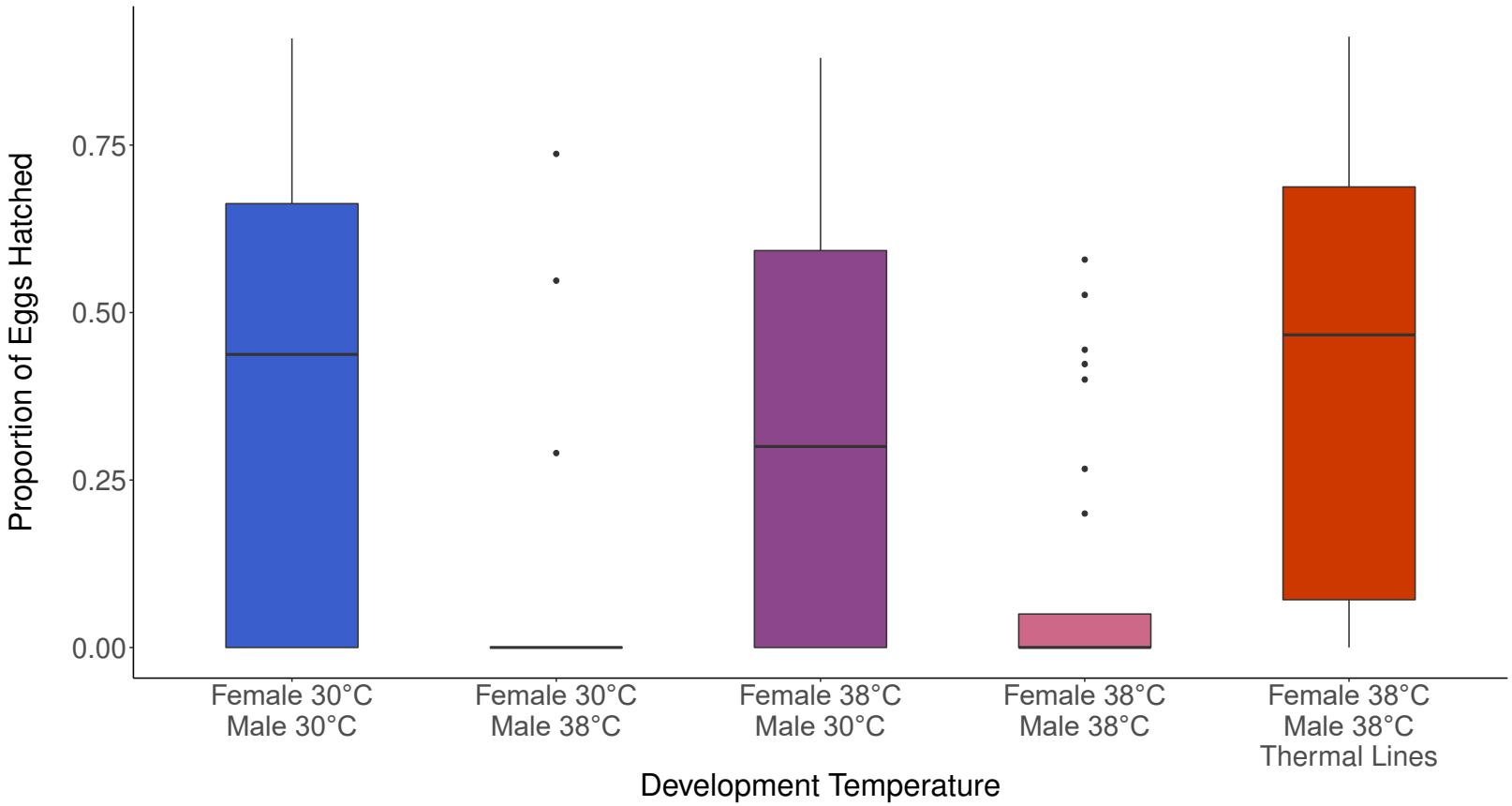

Figure 4.5. The proportion of eggs surviving to adulthood, laid over 2 days by female *T. castaneum* raised in different thermal regimes (either 30°C or 38°C) mated with males (reared at either 30°C or 38°C). There was no effect of female development temperature, but males development at high temperature led to a decrease in eggs hatching and surviving to adulthood. The long-term selection lines had higher hatch and survival success than the KSS stocks from the corresponding treatment (Female 38°C, male 38°C).

4.4 Discussion

The aim of this study was to improve our understanding of the effects of exposure to high temperature during development on later life fitness. In *T. castaneum*, I found a negative effect of temperature during development on later life reproductive output in both males and females. This effect was much stronger in individuals (both males and females) exposed to high temperature for the entirety of their development, than those exposed for short periods. Further experimentation showed that in females, the pupal stage is most vulnerable to a short period of temperature increase, in terms of later-life reproductive output, and that effects on female productivity were explained by reduced fecundity (number of eggs laid). In males, I found no single developmental stage was particularly sensitive, but prolonged exposure to high temperature during development resulted in a reduction in egg viability. This latter effect encompasses the fertilisation of the eggs as well as their survival to adulthood – for convenience, I refer to this overall effect as 'fertility', here.

I found that exposing *T. castaneum* to high temperatures for their entire development had a negative effect on both later life survival and reproduction, in both males and females. I performed an experiment designed to test the how effect of timing of exposure affected adult reproduction, and found that a five-day exposure time to elevated temperate was not enough to elicit a response in males at any stage of development. However, in females, two time period treatments showed a modest, but significant, decline in reproductive output after five days exposure during development. Over these two treatment times, I expect a high proportion of the individuals would pass through the pupal stage, as the treatments fell either side of the pupae collection for this experiment (Day 20). It is possible that, if I were to re-run this experiment with a treatment encapsulating the entire pupal stage, I would see a more dramatic response. To simulate a more natural scenario, I would also include treatments combining variations in the timing, duration and even the number of exposures to high temperature, to see which of these combinations of factors had an effect. Previous work in *Tribolium spp* has shown that pupae are particularly sensitive, in terms of future reproductive ability, to temperature increases (Mahroof *et al.*,

2005), likely because most ovary and spermatheca maturation occurs at this stage (Sokoloff, 1974). Interestingly, this does not correspond with immediate survival through a heat shock. In this regard, the *T. castaneum* pupal stage has been found to be the most robust (Mahroof *et al.*, 2003). In *Drosophila*, pupae are also relatively robust to heat stress in terms of survival (Krebs & Loeschcke, 2008), potentially due to their immotility, and thus inability to avoid heat stress, resulting in the evolution of mechanisms to avoid heat shock damage. One theory is that different insect stages have different metabolic rates (Emecki *et al.*, 2002) and that these differences in metabolite production could affect survival and stress tolerance (Mahroof *et al.*, 2003)

This study further investigated how fecundity and fertility, two separate aspects of reproductive success, are affected by developmental stress. Exposure to high temperature during development affected fecundity in females in these experiments, which concurs with anecdotal reports from the 1930s (Park, 1935) following an incubator malfunction in a laboratory keeping *T. castaneum*. My results suggest that either females are less able to produce eggs, perhaps because eggs are unable to mature fully at high temperature (Berger *et al.*, 2008), or that eggs are being produced but not released. Evidence from Coleoptera indicates that oviposition can be delayed in an unfavourable environment to preserve resources for the future (Danho, 2002). However, as virgin *T. castaneum* lay unfertilised eggs before mating, it seems unlikely that the reduced oviposition after developmental heat shock is due to a strategic delay in egg laying. Alternatively, exposure to high temperature during development may affect ovary development - as shown in thrips (*Frankliniella occidentalis*) which developed ovaries with fewer ovarioles under such conditions (Sun *et al.,* 2019). The possibility that the ovaries of *T. castaneum* females exposed to heat shock during development may not have fully developed is a theory I will explore in Chapter 5 of this thesis.

We did not see any effect of the male developmental treatment on the fecundity of their female mates. In some insect species, males transfer a variety of seminal fluid proteins (SFPs) to females during copulation, (Chapman, 2011). These proteins have a

range of effects in females, including increasing post-copulatory egg laying (Herndon & Wolfner, 1995) and decreasing female receptivity to further matings (Baumann, 1974). In *T. castaneum*, SFPs have been identified, but less is known about their functions (South *et al.*, 2011). Xu *et al.* (2013) suggested that they may work to protect and nourish sperm and to increase egg production. In the current study, the number of eggs laid by female was not affected by the heat treatment of the male, which suggests that if SFPs do influence post-copulatory oviposition in *T. castaneum* females, this is not affected by male developmental temperature.

Male exposure to high temperature during development had a strong negative effect on the survival to adulthood of eggs laid by female mates. It is known that exposure to high temperature can cause immediate sperm damage in *T. castaneum* (Sales *et al.*, 2018), but it is not clear whether this damage would persist to subsequently affect adult reproductive fitness. We do not know whether development at high temperature has any morphological effects in males, but later life reductions in fertility and even complete sterility, following developmental thermal stress have been observed in a range of organisms. For instance, research in the parasitic wasp (*Anisopteromalus calandre*) has shown a decrease in sperm number following a heat treatment during the pupal stage (Nguyen *et al.*, 2013), and several studies have reported male sterility in plants following developmental thermal stress (e.g. Sage *et al.*, 2015; Begcy *et al*., 2019). Previous work in this laboratory has shown that after exposure to high temperature, *T. castaneum* can suffer deformation of the testes (Sales, 2018a), which could cause reduced fertility, and is something I will explore in Chapter 5 of this thesis. I found no effect of the female mates' developmental regime on the hatch rate of her eggs, suggesting that the reduced viability of those eggs is driven largely by male effects.

In a pest-control study, Mahroof *et al.* (2005) found that the pupal stage is more sensitive to high temperatures in terms of adult reproduction than the adult stage in *T. castaneum*, which supports my findings here. Mahroof *et al.* (2005) showed that heat treatment during the development of either parent had a negative effect on the egg to

adult survival, which differs from my results, indicating a male specific effect. This difference may be due to the nature of the heat treatment; Mahroof *et al.* (2005) heated pupae to 50°C for one hour, whereas my heat treatments were to 38°C for the entire development period. Despite these differences in approach, the findings are remarkably similar, indicating that this effect is robust to different heat intensities and durations, though whether variation in these parameters affect the severity of the effects is largely unknown. Extreme high temperatures can cause rapid death, but longer exposure to moderately high temperatures can cause "thermal wounding" (Denlinger & Yocum, 1998). For example, adolescent phases may survive the duration of a heat stress, but later fail to reach adulthood (Cupp & Horspall, 1970). I saw clear decreases in reproductive output following developmental exposure to elevated temperature, which suggests that *T. castaneum* can suffer thermal wounding at 38°C.

A novel finding from my current study was that the high temperature selection lines (described in Chapter 2) maintained their ability to lay and fertilise eggs, despite developing at high temperature, whereas control lines did not. In previous work (Chapters 2 & 3), I found no evidence of adaptation in the adults from these selection lines. It is only now, when investigating the effects of temperature on developmental stages, that I have evidence of adaptation. Thermal adaptations with effects during development, such as an adjusted development rate in frogs (Laugen *et al.*, 2003) and butterflies (Karl *et al*., 2008), have been found previously across a variety of taxa (Keller & Seehausen, 2011).

In conclusion, my results concur with previous findings from this field, that high temperature during development has negative effects on adult fitness (Mahroof *et al.*, 2005; Porcelli *et al.*, 2017; Eyck *et al.*, 2019), but also identify important sex-specific effects on fecundity and fertility. The results of this current study are likely to be applicable across many organisms, as loss or reduction of reproductive success is a common effect of sub-lethal thermal stress (Jørgensen *et al.*, 2006). Additionally, gamete production in invertebrates is usually initiated during the immature developmental stages (Porcelli *et al.*, 2017) so reproductive traits are likely to be

affected by stress encountered at this point. Thermal fluctuations can influence developmental time in insects, increasing the amount of time they exist in these more vulnerable stages (Ratte, 1984). Such results highlight the importance of considering life history when investigating thermal stress and adaptation. Moving forward, a fuller understanding of the effects of elevated temperatures at different life stages can lead to a more thorough understanding of how heat waves resulting from climate change may affect the dynamics of insect populations.

Chapter 5

Thermal adaptation and reproductive morphology

Abstract

Changes in temperature due to global warming are likely to impose strong selection pressures on individuals from different populations and species worldwide. Due to their inability to thermoregulate, ectotherms are expected to be particularly vulnerable to temperature changes, and there is considerable interest in understanding whether and how ectotherms may be able to adapt. Previously, I showed that *Tribolium castaneum* exhibit markedly lower reproductive outputs after development at high temperature (see Chapter 4). Evidence from long-term selection lines suggests that this effect can be alleviated within ~60 generations. Here, I study how reproductive morphology and fitness change within the first five generations of exposure to a new thermal environment. I found that reproductive fitness increased over five generations of exposure to high temperature, but also that fitness fluctuated over generations. In males, I saw an increase in the number of testis follicles present over generations of exposure to high temperature, but in females I saw no change in ovary "blockage" over the generations, despite linking this trait to fecundity. Further work in this area is needed in order to identify and explain the physiological changes associated with elevated temperature in this species. A deeper understanding of these changes and the mechanisms of their spread through populations will help us to understand the effects of global warming on insect reproduction and population viability.

5.1 Introduction

Evolution is classically thought of as a process spanning millennia. However, with anthropogenic factors accelerating the warming of the world at an unprecedented rate, environments are changing rapidly, with profound consequences for natural populations. To avoid thermal stressors, organisms may respond plastically to environmental changes, or they may modify their behaviour, range or distribution, risking contact with new diseases and predators (Holt, 1990). Alternatively, populations may be able to evolve rapidly to adjust their average phenotype to be one that is fitter at higher temperatures. Failure to modify either a phenotype or range can result in extinction, and there is increasingly a consensus that we are currently in the midst of the sixth mass extinction due to anthropogenic climate change (Ceballos & Ehrlich, 2018). Projections indicate that global average temperatures will continue to rise for many years (Brown & Caldeira, 2017) so extant species will have further challenges to face.

Observational studies in natural populations and modelled projections are conflicting in the conclusions drawn regarding the ability of populations to rapidly adapt to novel thermal environments. Quintero & Wiens (2013) studied representative species from major tetrapod clades and concluded that the rate of adaptation required to keep up with the expected changes in climate is orders of magnitude (>10,000 x) faster than the typically observed rates. However, other studies of individual populations suggest that the rapid change in temperature does not always result in an increase in the strength of selection (Cresswell & McCleery, 2003), which may be indicative of evolutionary change at a rate that is sufficient to maintain fitness despite temperature change (Visser & Both, 2005). Rapid adaptation can occur in nature, such as in the case of invasive species and pests, in which rapid adaptation facilitates colonisation (Prentis *et al.*, 2008). There have been several suggested mechanisms that can facilitate rapid adaptation, including bottlenecks, hybridisation and stress inducing genomic modification, through either epigenetics or transposable elements (Molinier *et al.*, 2006). Rapid adaptation in natural populations can lead to speciation occurring on

ecological timescales, which can be driven by divergent habitat preferences, sexual selection and natural selection (Hendry *et al.*, 2007).

The Insecta are the largest Class within the largest animal Phylum (Wigglesworth, 2020), and offer useful models for studying rapid adaptation to environmental change. Due to their typically short generation times, they have the facility to adapt quickly to changes, and this adaptation can be observed over human lifetimes (Kawecki *et al*., 2012; Mukherjee *et al.*, 2017). However, as ectotherms, unable to thermoregulate, insects can be also particularly susceptible to changes in environmental temperature, not just in terms of survival but of subsequent reproductive viability (Sales *et al*., 2018). Given that insects perform a variety of ecosystem services, and can be pollinators, food sources, detritivores and predators (Schowalter, 2000), their thermal sensitivity is an issue of key importance for global sustainability

Holometabolous insects undergo metamorphosis during development - each developmental stage is distinct, with potentially different susceptibilities to changes in temperature (Zhang *et al.* 2015). In the short-term, thermal stress can have different effects on the development rate of different life stages (Folguera *et al*., 2010), but on a longer-term basis, thermal stress encountered at immature stages can have life-long impacts on fitness (Klockmann *et al.*, 2017). Research on insects has shown that the timing and duration of early-life stress is important for predicting later-life effects (Folguera *et al*., 2010; Zhang *et al*., 2015). However, there is very little research on the mechanisms by which early-life stress in insects results in reduced later-life fitness, and less still on whether (and if so, how quickly) insect populations can adapt to these effects of early-life stress.

This thesis has shown that experimental *Tribolium castaneum* populations are highly susceptible to thermal stress (see Chapters 2 and 4) and produce fewer offspring when exposed to thermal stress during development. In addition to this, the previous work in this thesis using long-term selection lines has shown that this species can apparently

adapt to increased temperatures (Chapter 4). The specific form of adaptation observed here has enabled the long-term high temperature selection lines to reproduce following development at high temperature with a significantly higher reproductive output than non-adapted stocks. This adaptation was observed following >70 generations of constant exposure to high temperature in a very strong selective environment. However, we do not yet understand the mechanistic basis of this adaptation, or how rapidly this adaptation has arisen and spread through the experimental populations. Adaptations that take place during development, such as this, have been suggested as a route for rapid adaptation, due to the potential knock-on effects throughout the life of the organism (Lin *et al.*, 2017).

Here, I investigated the real-time evolutionary dynamics of populations in new thermal environments. Specifically, I tested how stock *T. castaneum* populations of different genetic backgrounds adapt to high temperature over five generations, to gain insights into their capacity to respond rapidly and uncover the mechanisms allowing them to do so. I performed reproductive fitness assays over five generations, to track the number of offspring that pairs from the different lines/genetic backgrounds could produce. Additionally, I quantified variation in ovary and testis morphology of all populations, to determine i) whether this provides an explanation for the reduction in fecundity and fertility observed after development at high temperature (See Chapter 4), and ii) to determine whether (and how quickly) these traits change over time as a result of natural selection. I discuss these results in the context of how natural populations may respond to changing climates.

5.2 Methods

5.2.1 Experimental procedures

Two experimental replicates were created for each of three evolutionary replicates of the long-term high temperature selection lines and of three standard control temperature lines (See Chapter 2 for details of long-term thermal selection lines). This was done by allowing 100 adults one week to mate and oviposit into fodder, removing the adults, and splitting the fodder between two containers (7cm diameter x 7.5cm depth), assigned to different thermal regimes (30°C ad 38°C). In addition to this, six identical replicate lines were created from the diverse KSS stock population, which was maintained at a control temperature (30°C) and at higher population numbers than the thermal lines (600 adults per generation and 100 adults per generation, respectively, see Chapter 2 for details of stocks). One replicate of each of the thermal lines and three replicate lines from the stock were put into each experimental test temperature (control: 30°C and high temperature: 38°C) and maintained according to the usual thermal line maintenance routine (described in Chapter 2). The outcome of this was that at each temperature, there were three replicate control temperature thermal lines, three replicate high temperature thermal selection lines and three replicates of the stock population.

Every generation, 60 male and 60 female pupae were sexed from each line, at each temperature. These pupae were stored in single sex groups of 20. Approximately 10 days later, after eclosion, 30 males and 30 females were paired within lines and allowed 24 hours to mate. The females were then moved to a petri dish with clean fodder to oviposit for seven days and the males were stored together in one petri dish for this time. At the end of this seven-day period, these mated males and females were used to seed the next generation, supplemented up to 100 individuals with randomly selected (unsexed) beetles from the line. The eggs laid in the petri dish were allowed 35 days to develop to adulthood before being counted.

To identify differences in testis and ovary morphology following development at different temperatures, excess beetles collected for the reproductive output assay in generation 1 were frozen and their testes or ovaries were dissected and photographed (up to 10 individuals per sex, per line). To test for changes in ovary and testis morphology over generations of exposure to high temperature, this protocol was repeated in generations 3 and 5 of the experiment, with the adjustment that only testes and ovaries from the stocks and the 30°C thermal lines at high temperature were dissected and photographed. The photographs of the reproductive organs were measured using Image J (Schneider *et al.*, 2012). The length and width of each egg in the ovaries was measured (Figure 5.1A) and recorded, along with the position of the egg within the ovary. The length and width of each follicle of the testes was measured (Figure 5.1B) where possible, and the total number of follicles visible on the testis was recorded. The elytra length of each individual was also measured and used as a proxy for body size (as in Conner & Via, 1992; Figure 5.1C).

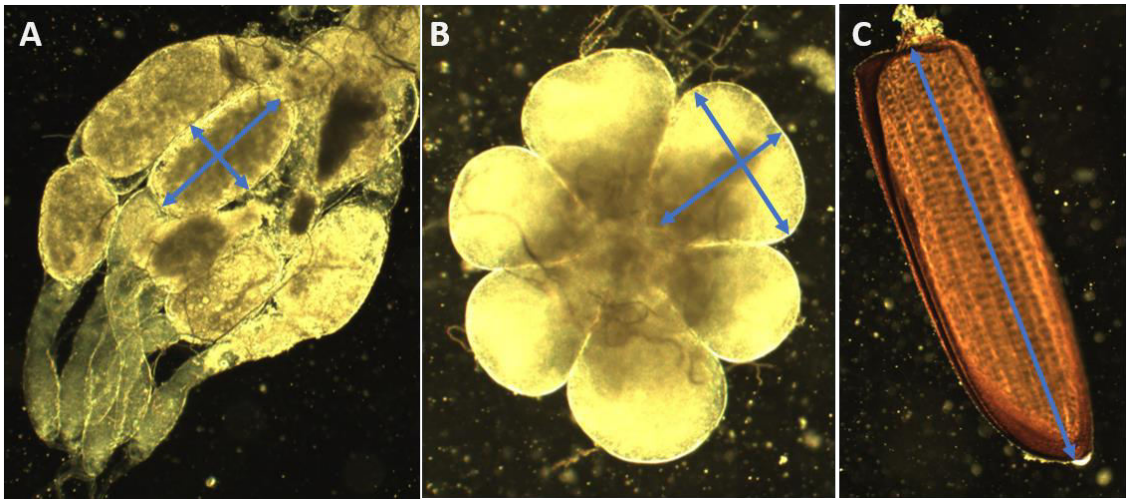

Figure 5.1. Phenotypic measurements used in *T. castaneum* evolution experiments. Images detailing the metrics used to measure **A.** Eggs in an ovary and **B.** Testis follicles. Length at longest point was measured, followed by width, at widest point, at a 90° angle to the length vector. **C.** Elytra length. The length at longest point was measured to provide a proxy for body size. Images not to scale – for indicative purposes only.

Because I observed considerable variation in ovary morphology within and between lines (see results), I tested how ovary morphology was related to fecundity in an additional experiment using mated females. Two replicate KSS populations were created using the eggs laid in ~50ml fodder following seven days of oviposition. This was supplemented with 50ml of clean fodder, to prevent overcrowding and the resulting density-dependent effects on fitness. One replicate was kept at control temperature (30°C) and the other at high temperature (38°C) through development. At pupation, 40 male pupae were collected from each population and 80 female pupae were collected from the high temperature population. These pupae were stored in single sex groups of 20 individuals for 10 days, until approximately five days after eclosion. At this point, the high-temperature females were paired with either a high temperature or a control temperature male and allowed 24 hours to mate. At the end of this period, the males were discarded and the females allowed to oviposit in a petri dish of clean fodder for seven days. Previous work in this laboratory has shown that peak oviposition begins seven days after mating (unpublished), so after this they were transferred to a vial with clean fodder for a further two days of oviposition. At the point of removal from the vial, the female was frozen at -20°C for dissection and her ovaries and elytra were photographed and measured, as described above. The eggs from the vial were counted as used as the measure of fecundity.

5.2.2 Statistical analyses

To test for differences in offspring counts between lines and over generations, linear mixed models (LMMs) were fitted. The data were split by developmental temperature and modelled separately. For each developmental temperature, the number of offspring produced was fitted as the response variable, with the type of line (KSS stock/high temperature thermal lines/control thermal lines) and the generation as explanatory variables, fitted as a categorical factor and linear covariate, respectively. I also fitted the replicate line ID as a random effect. I ran a similar model, with generation fitted as a categorical factor instead of a linear covariate, which returned qualitatively identical results.

I predicted that testes from beetles developed at high temperature would be smaller, or have greater variation in follicle size, than those developed at control temperatures. To test this, I compared the mean testis follicle size per individual and within-individual variance in follicle size across different treatments in generation 1, using LMMs. Follicle size was calculated by multiplying the two perpendicular measurements taken from each follicle (Figure 5.1B). Mean follicle size per individual and within-individual follicle size variance were entered as response variables in separate LMMs, and line type, thermal regime and elytra length were fitted as explanatory variables. Line ID was included as a random effect. During the dissection and measurement of testes, it was apparent that some testes were more fragile than others, and therefore more difficult to remove without damage or loss of follicles. To test whether this differed between treatments, an LMM was performed on data from the first generation of the experiment, with the number of follicles as the response variable, and explanatory variables and random effects entered as above. To test for changes in testes morphology over five generations of exposure to high temperature, three LMMs were constructed, with mean follicle size, variance in follicle size within an individual and number of follicles entered as response variables. Line type, generation and elytra length were entered as the explanatory variables, and line ID was entered as a random effect. Interaction models were also built in the same way, but only the interaction term was reported from these models. I also performed separate analyses of differences in elytra length between lines and over generations (Appendix B).

A principal components analysis (PCA) was conducted on the ovary measurements, using the number of eggs in the ovaries of an individual, the mean egg length and width per individual, the number of eggs found in the calyces (plural of calyx, i.e. where the bases of the ovarioles meet, Figure 5.2) and the proportion of eggs found in the calyces as input variables. The first two principal components (PC1 and PC2) were then used as response variables in two LMMs constructed to test for difference in ovary morphology across the different treatments in generation one. Each model used either PC1 or PC2 as the response variable, with line type, thermal regime and elytra length as explanatory variables and a random effect of line ID. To test for changes in ovary morphology over the five generations of exposure to high temperature, another

two LMMs were constructed, using PC1 and PC2 as response variables. Both models had the line type, the generation and elytra length fitted as the explanatory variables and a random effect of line ID.

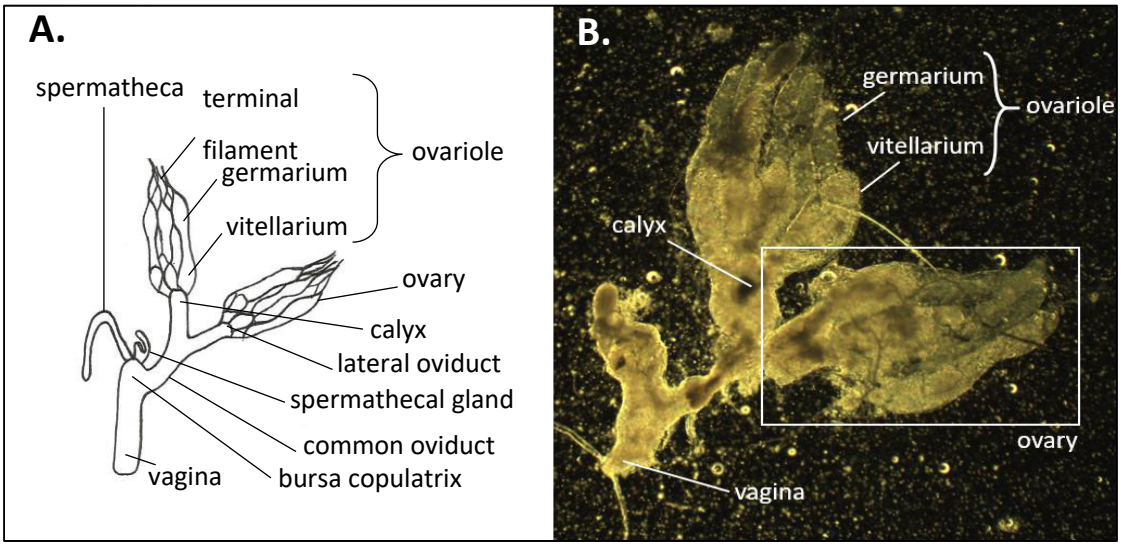

Figure 5.2. *T. castaneum* ovaries. **A.** An ovary diagram, adapted from Sokoloff (1972). **B.** labels from the ovary diagram applied to a photograph of a dissected ovary from a wild-type sexually mature female.

As outlined above, I carried out a second, separate experiment to directly test how ovary morphology was related to fecundity within individuals, using mated females. A separate PCA was conducted for these females' ovary measurements, as above. A linear model was constructed to test whether values of PC1 and PC2 were significantly related to the number of eggs laid by individual females, with number of eggs as the response variable and PC1, PC2 and elytra length coded as linear covariates. Because the males mated with the dissected females came from two thermal environments (30°C or 38°C), I also tested for differences in PC1, PC2 and the number of eggs laid between male treatments, using independent samples t-tests.

In boxplots throughout this chapter, the midline represents the median, the two ends of the box represent the 25th and 75th quantiles and the whiskers extend to 1.5 x IQR. Data outside 1.5 x IQR are represented as points. Asterisks (*) represent the mean.

5.3 Results

We first studied how reproductive output changed over generations among long-term selection lines and control lines, at control and high temperatures. At 30°C, I found a marginally non-significant increase in reproductive output over the five generations, and no evidence of an interaction between line type and generation (Table 5.1A). However, the 38°C lines had consistently and significantly lower reproductive output compared to the stock populations (Table 5.1A, Figure 5.3A). At 38°C, there was a significant increase in reproductive output over the five generations (Table 5.1B, Figure 5.3B), although there was substantial fluctuation between generations. Reproductive output in the 38°C lines did not differ significantly from that in the stock lines, but the 30°C lines had lower reproductive output (Table 5.1B). There was a significant interaction between the line type and the generation, with the reproductive output of the 38°C lines increasing to a lesser degree than the stock populations over the five generations. Conversely, the 30°C lines showed a greater increase in reproductive output than the stock populations (Table 5.1B). These interactions were largely driven by differences between the lines in the first generation, which were no longer apparent in generation five (Figure 5.3).

Table 5.1. LMMs testing for differences between line types in the number of *T. castaneum* offspring produced over five generations at **A.** 30°C or **B.** 38°C. The number of offspring was modelled as the response variable and the generation (which was modelled as a linear covariate) and line type (with the baseline set to the stock populations) were fitted as explanatory variables. There was a random effect of Line ID (Var_A = 1.619, Var_B = 4.274).

	Fixed Effect	Estimate	Standard Error	T value	Pr(>|t|)
A. 30°C	Intercept	102.196	2.812	36.341	< 0.001
	Line Type (30°C Lines)	- 3.755	2.658	- 1.413	0.215
	Line Type (38°C Lines)	- 20.334	2.664	- 7.631	< 0.001
	Generation	1.371	0.701	1.956	0.051
	Generation* Line Type (30°C Lines)	1.096	1.705	0.643	0.520
	Generation * Line Type (38°C Lines)	- 3.156	1.721	- 1.834	0.067
B. 38°C	Intercept	13.840	2.805	4.935	< 0.001
	Line Type (30°C Lines)	- 9.395	2.831	- 3.319	0.016
	Line Type (38°C Lines)	3.533	2.810	1.258	0.257
	Generation	6.997	0.653	10.726	< 0.001
	Generation * Line Type (30°C Lines)	3.987	1.596	2.498	0.013
	Generation * Line Type (38°C Lines)	- 4.081	1.578	- 2.586	0.010

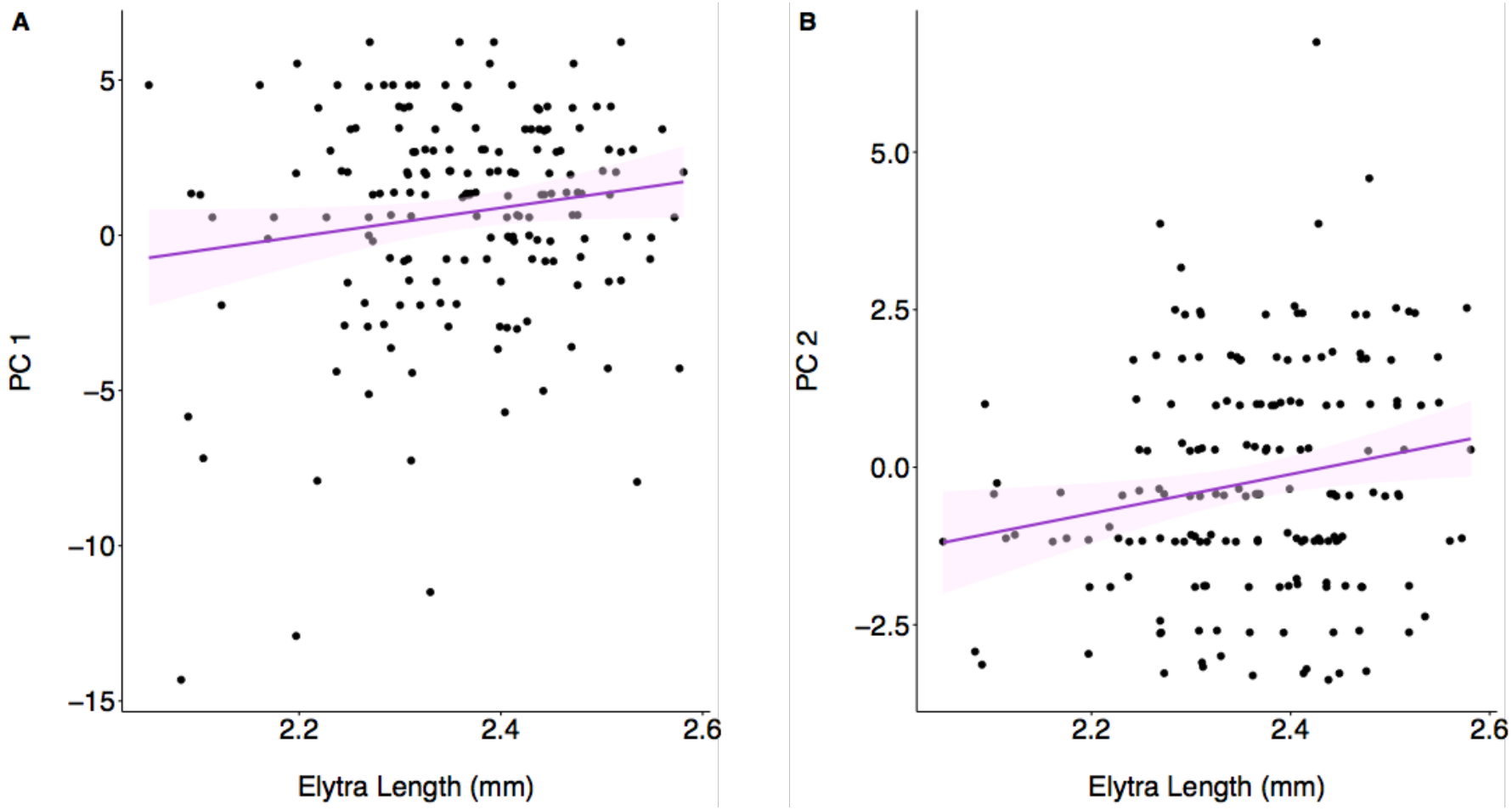

Figure B.3. The correlations between elytra length and ovary morphology in virgin *T. castaneum*. A. Elytra length with PC1 (R = 0.15, p = 0.054) and **B.** Elytra length with PC2 (R = 0.19, p = 0.013) in females from various lines (stocks, 30°C thermal lines and 38°C thermal lines) and reared at different temperatures (30°C and 38°C).

In generation one, there was no difference in mean testis follicle size across any of the line types at either temperature. There was no effect of body size and there were no significant interactions between fixed effects (Table 5.2, Figure 5.4A). Over the five generations of this experiment, there was an increase in mean testis follicle size (Table 5.3, Figure 5.4B), and the stock populations had larger mean follicle size over this time than the 30°C lines (Table 5.3). There was no difference in the variance in follicle size between the different line types in generation one, but development at high temperature resulted in more variance in follicle size within individuals. There was no effect of elytra length on the variance in follicle size and there were no significant interactions between these variables (Table 5.4, Figure 5.5A). Over the five generations of exposure to high temperature, there was no difference in variance in follicle size between the stocks and 30°C lines and no change over the generations. There was no effect of elytra length (used as a proxy for body size) and no interactions between any of these variables (Table 5.5, Figure 5.5B). There was no difference in the number of follicles on testes from individuals from different lines, or reared at different temperatures (Table 5.6, Figure 5.6A). There was no effect of elytra length on number of follicles. However, over the five generations of exposure to high temperature, both the stocks and the control temperature thermal lines showed in increase in the number of follicles present on testes (Table 5.7, Figure 5.6B). There was no difference between the lines and no interaction between line type and generation.

To summarise the results from male morphological measurements, I found very little difference in testis morphology between lines, but over the five generation of exposure to high temperature, the average testis follicle size increase and the number of follicles per testis increased.

Table 5.2. An LMM testing whether the mean testis follicle size (mm) per *T. castaneum* male varies across different line types and different rearing temperatures. The mean follicle size was the response variable, with a random effect of Line ID (Var < 0.001). There were fixed effects of line type and temperature, with baselines set to stocks and 30°C, respectively.

Fixed Effect	Estimate	Standard Error	T value	Pr(>|t|)
Intercept	0.108	0.041	2.612	0.010
Line Type (30°C Lines)	- 0.006	0.009	- 0.754	0.479
Line Type (38°C Line)	- 0.012	0.009	- 1.439	0.200
Temperature (38°C)	- 0.005	0.004	- 1.352	0.178
Elytra Length	0.005	0.017	0.319	0.750
Line Type * Temperature: (30°C Lines at 38°C)	- 0.005	0.009	- 0.617	0.538
Line Type * Temperature: (38°C Lines at 38°C)	0.001	0.009	0.117	0.907

Table 5.3. An LMM testing whether the mean follicle size (mm) per individual *T. castaneum* changes over generations in lines exposed to 38°C. The response variable was mean follicle size and the explanatory variables were line type (with the stock set as the baseline) and generation (modelled as a linear covariate). There was a random effect of Line ID (Var < 0.001).

Fixed Effect	Estimate	Standard Error	T value	Pr(>|t|)
Intercept	0.057	0.032	1.770	0.079
Line Type	-0.006	0.003	- 2.063	0.041
Generation	0.002	0.001	2.005	0.047
Elytra Length	0.023	0.014	1.642	0.103
Line Type * Generation	0.002	0.002	0.969	0.334

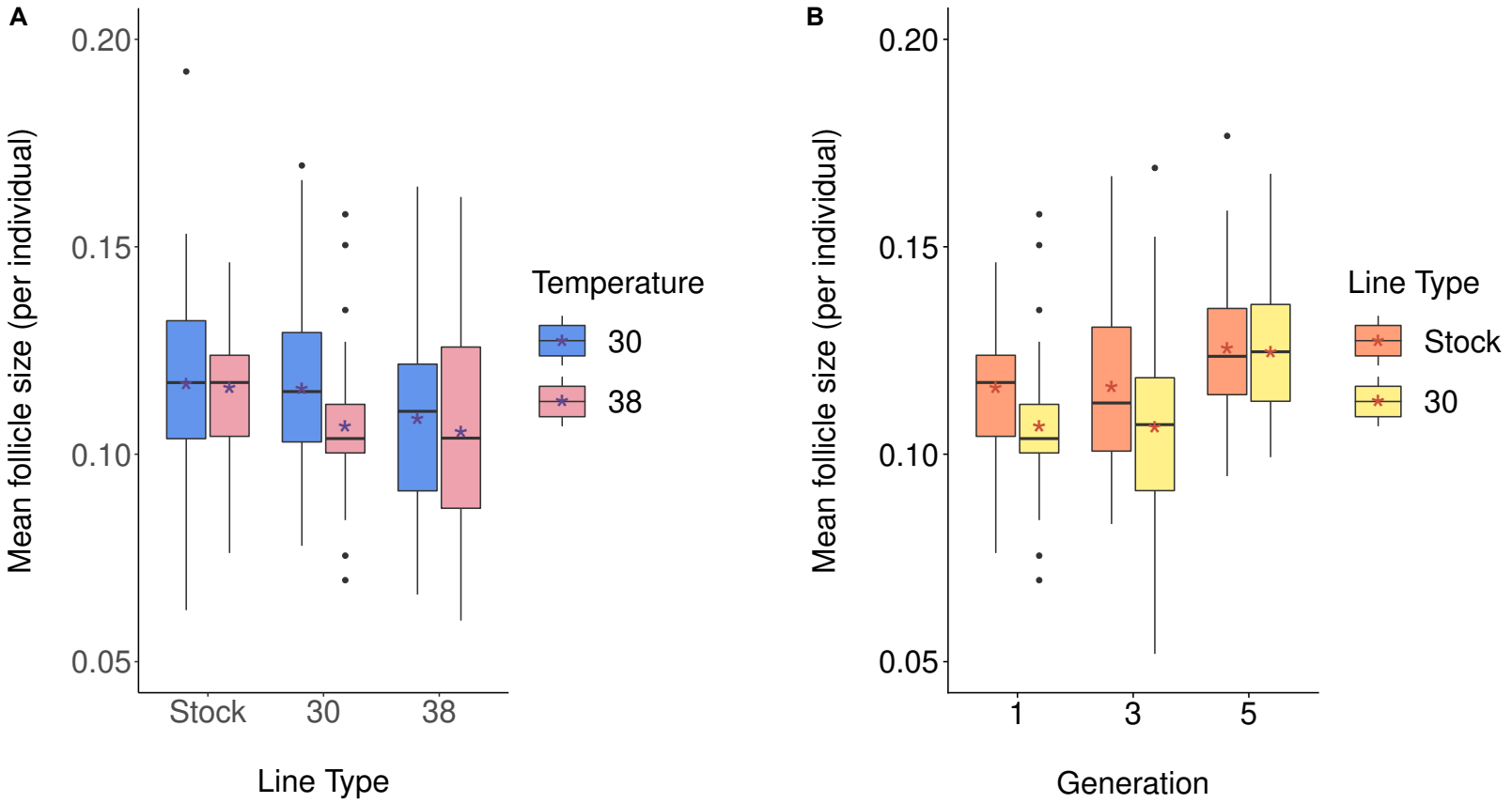

Figure 5.4. Mean follicle size (mm) of testes dissected from *T. castaneum* males A. between treatments in generation 1 and **B.** over five generations of exposure to high temperature in stocks and 30°C thermal lines. Mean values are represented by *. There were no differences between lines in generation 1, but there was a marginally significant increase in follicle size over the five generations and the follicles of the 30°C lines were smaller than those of the stocks.

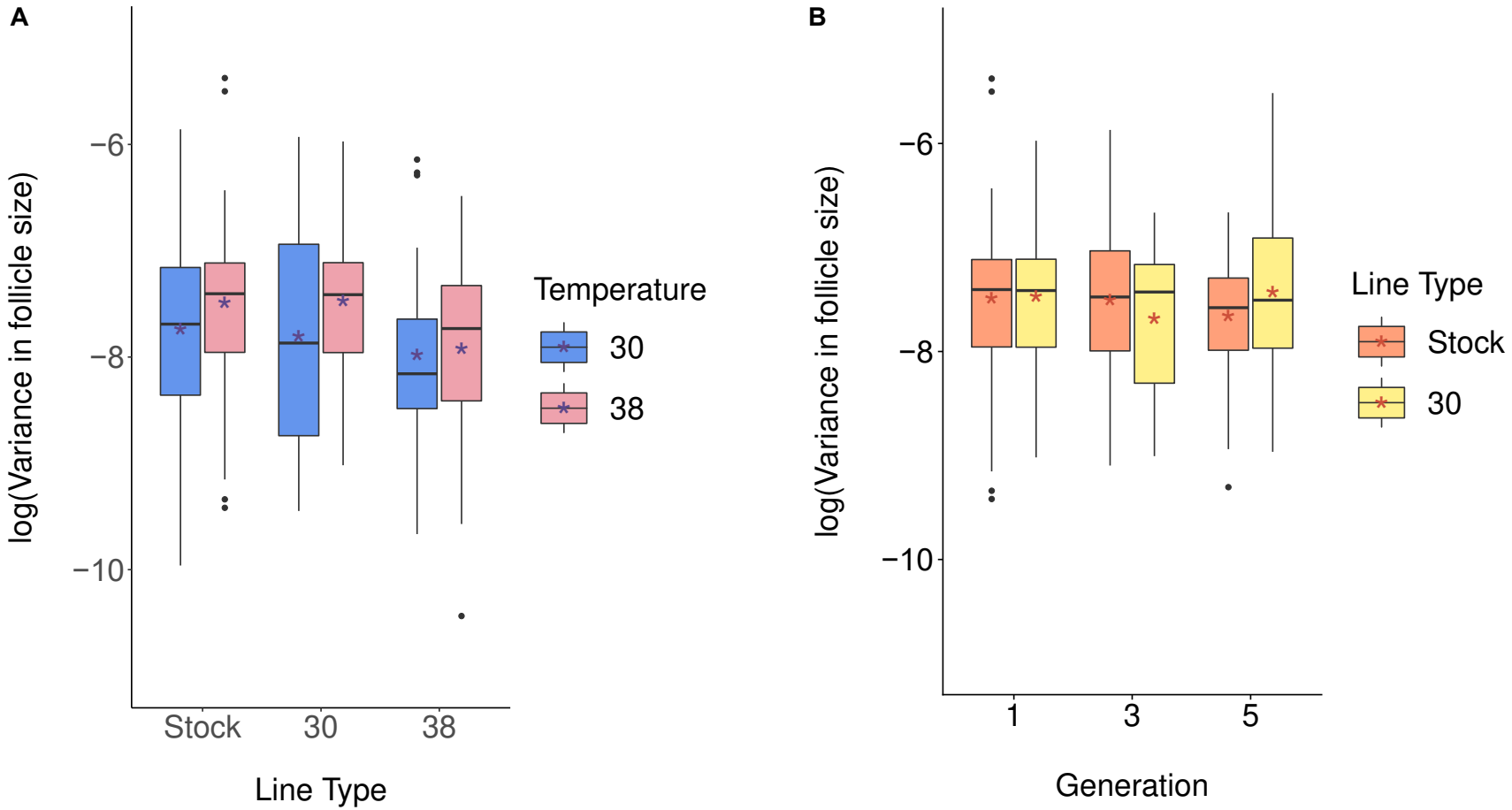

Figure 5.5. Log of the variance in testis follicle size within individuals, from testes dissected from *T. castaneum* males **A.** between treatments in generation 1 and **B.** over five generations of exposure to high temperature in stocks and 30°C thermal lines. Mean values are represented by *. There were no differences between treatment in generation 1 or over the five generations of exposure to high temperature.

Table 5.4. An LMM testing for differences in the log of within-individual variance in testis follicle size across *T. castaneum* testes from different types of populations and different rearing temperatures. The log of the within-individual variance in testis follicle size was the response variable, with a random effect of Line ID (Var = 0.009). There were fixed effects of line type and temperature, with baselines set to stocks and 30°C, respectively.

	Estimate	Standard Error	T value	Pr(>|t|)
Intercept	-9.640	1.586	- 6.079	< 0.001
Line Type (30°C Lines)	0.013	0.186	0.068	0.948
Line Type (38°C Lines)	-0.363	0.185	- 1.967	0.100
Temperature (38°C)	0.292	0.148	1.972	0.050
Elytra Length	0.806	0.664	1.212	0.227
Line Type * Temperature (30°C Lines at 38°C)	0.106	0.339	0.312	0.756
Line Type * Temperature (38°C Lines at 38°C)	- 0.084	0.346	- 0.242	0.809

Table 5.5. An LMM to test whether log within individual variance in testis follicle size varies over generations in lines of *T. castaneum* exposed to 38°C. Response variable was variance in follicle size, explanatory variables were line type (with the stock set as the baseline) and generation (modelled as a linear covariate). There was a random effect of Line ID (Var = 0.026).

Fixed Effect	Estimate	Standard Error	T value	Pr(>|t|)
Intercept	- 7.971	1.289	- 6.182	< 0.001
Line Type	0.011	0.176	0.065	0.951
Generation	- 0.028	0.045	- 0.627	0.532
Elytra Length	0.211	0.565	0.374	0.709
Line Type * Generation	0.046	0.072	0.631	0.529

Table 5.6. An LMM modelling changes in number of testis follicles across *T. castaneum* treatments. Treatments represent different line types and thermal regimes. The number of follicles was set as the response variable, with explanatory variables of temperature and line type (baselines set as 30°C and stock populations, respectively). There was a random effect of line ID (Var < 0.001).

Fixed Effect	Estimate	Standard Error	T value	Pr(>|t|)
Intercept	4.547	3.281	1.386	0.168
Temperature	- 0.308	0.307	- 1.002	0.318
Line Type (30°C Lines)	0.012	0.354	0.034	0.973
Line Type (38°C Lines)	0.652	0.307	1.864	0.064
Elytra Length	2.208	1.375	1.606	0.110
30°C Lines at 38°C	- 0.750	0.701	- 1.069	0.287
38°C Lines at 38°C	- 0.959	0.715	- 1.340	0.182

Table 5.7. An LMM modelling changes in the number of follicles on testes dissected from male *T. castaneum* over generations of exposure to high temperature. The total number of follicles across both testes per individual was the response variable, with fixed effects of line type (baseline set as stock populations) and generation. There was a random effect of line ID (Var = 0.134).

Fixed Effect	Estimate	Standard Error	T value	Pr(>|t|)
Intercept	8.751	3.024	2.894	0.004
Line Type	- 0.163	0.410	- 0.397	0.712
Generation	0.404	0.105	3.863	< 0.001
Elytra Length	0.076	1.326	0.057	0.954
Line Type * Generation	0.118	0.167	0.695	0.488

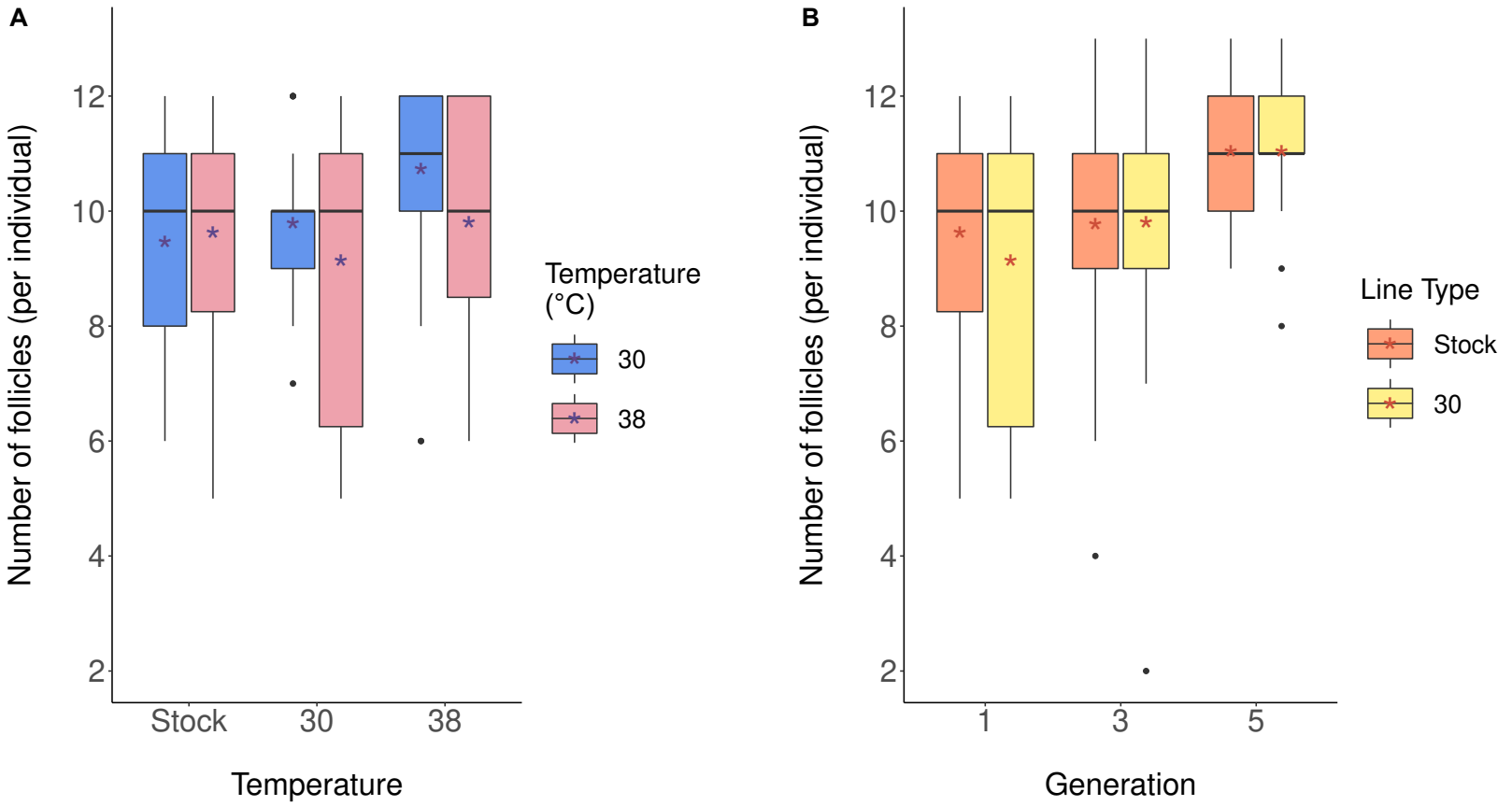

Figure 5.6. The number of follicles across both testes of dissected *T. castaneum* males from **A.** different populations (stocks, 30°C thermal lines and 38°C thermal lines), reared at different temperatures (30°C and 38°C) in generation 1 and **B.** stocks and 30°C thermal lines exposed to high temperature for five generations. Mean values are represented by *. There was no difference between treatments in generation 1, but over five generations of exposure, the number of follicles increased in the stocks and 30°C lines.

The first two principal components from the PCA of ovary morphological measurements and egg distribution explained 83.3% and 16.6% of the total variation, respectively. PC1 was strongly negatively correlated to the number of eggs in calyces and moderately negatively correlated to the total number of eggs in the ovaries (Table 5.8). A low value of PC1 represents an individual with ovaries that appear blocked due to a mass of eggs in the calyces. Ovaries with a high value for PC1 contain very few eggs. PC2 was strongly positively correlated to the total number of eggs present in the ovaries and was negatively correlated to the number of eggs in the calyces (Table 5.8). Therefore, a high value of PC2 represents an individual with lots of eggs present in the ovarioles, but none/very few present in the calyces. A low value for PC2 would represent an individual with very few eggs present, but those that are present would be in the calyces, as opposed to the ovarioles.

There was no difference in PC1 between the different lines in generation one, but individuals developed at high temperature had lower values of PC1. This effect was strongest in the 30°C thermal lines (Table 5.9, Figure 5.7A). Over the five generations of exposure to high temperature in this experiment, there was no change in PC1 in either the stocks or the 30°C lines and there was no difference between these two line types (Table 5.10, Figure 5.7B). There was no difference in PC2 between the three line types (stocks, 30°C thermal line and 38°C thermal lines) in generation one. Development at high temperature resulted in a decrease in PC2, consistent across all line types. There was no effect of body size on PC2 in generation one, and no significant interactions between any of the fixed effects (Table 5.11, Figure 5.8A). There was no change in PC2 over the generations and no difference between the line types (Table 5.12, Figure 5.8B), but across the lines and generations, larger beetles tended to have higher values of PC2.

Finally, I used measurements of non-virgin ovaries to test how ovary morphology was related to fecundity. A PCA on non-virgin ovaries showed very similar loadings to the virgin ovary data (Table 5.13). There was no effect of the developmental environment (30°C or 38°C) of the male mated with the dissected female on PC1 ($t_{30.3} = 0.57$, p =

0.57), PC2 ($t_{28.1}$ = 1.49, p = 0.15) or the number of eggs laid by the female ($t_{32.9}$ = 0.26, p = 0.80) (Figure 5.9). There was no effect of body size (measured by elytra length) on the number of eggs laid (Table 5.14). Higher values of both PC1 and PC2 increased the number of eggs laid by an individual (Table 5.14, Figure 5.9).

To summarise the results from female morphological measurements, I found that the morphology of ovaries developed at high temperature differed from those developed at control temperature. This effect manifested as a decrease in a principal component which represented a multivariate measure of ovary morphology, and which was associated with reduced fecundity. There was little change in ovary morphology over five generations of exposure to elevated temperature.

Table 5.8. A summary of the loadings of a PCA of ovary morphology from *T. castaneum* virgin females from different line types (stocks, 30°C thermal lines and 38°C thermal lines) developed at different temperatures (30°C and 38°C), over five generations. Five variables were input into the analysis.

	PC 1	**PC 2**	**PC 3**	**PC 4**	**PC 5**
Proportion of Variance	0.833	0.166	< 0.001	< 0.001	< 0.001
Standard Deviation	4.117	1.840	0.141	0.057	0.020
Correlation to number of eggs in ovaries per individual	- 0.692	0.721	- 0.041	- 0.006	< 0.001
Correlation to mean egg length per individual	- 0.004	- 0.009	- 0.199	0.890	0.411
Correlation to mean egg width per individual	- 0.002	- 0.005	- 0.125	0.393	- 0.911
Correlation to number of eggs found in calyces per individual	- 0.721	- 0.686	0.097	0.013	- 0.002
Correlation to proportion of eggs found in calyces per individual	- 0.042	- 0.097	- 0.966	- 0.232	0.033

Table 5.9. An LMM of how PC1 varied in virgin *T. castaneum* ovaries across different types of populations and different rearing temperatures. PC1 was the response variable, with fixed effects of line type and temperature, with baselines set to stocks and 30°C, respectively. There was also a fixed effect of elytra length, a linear covariate and a random effect of Line ID (Var < 0.001).

Fixed Effect	Estimate	Standard Error	T value	Pr(>|t|)
Intercept	-2.368	6.086	-0.389	0.698
Line Type (30°C Lines)	-1.093	0.608	-1.800	0.074
Line Type (38°C Lines)	1.086	0.611	1.776	0.078
Temperature: 38°C	-1.089	0.539	-2.020	0.045
Elytra Length	1.546	2.514	0.615	0.539
Temperature (38°C) * Line Type (30°C Lines)	-3.152	1.217	-2.589	0.011
Temperature (38°C) * Line Type (38°C Lines)	-1.847	1.220	-1.514	0.132

Table 5.10. An LMM testing whether PC1 changed in virgin *T. castaneum* ovaries over generations in lines exposed to 38°C. The response variable was PC1 and the explanatory variables were line type (with the stock populations set as the baseline), generation (with generation 1 as the baseline) and elytra length. There was a random effect of line ID (Var = 0.367).

Fixed Effect	Estimate	Standard Error	T value	Pr(>|t|)
Intercept	12.011	7.696	1.561	0.121
Line Type	-2.255	0.837	-2.694	0.056
Generation	0.088	0.256	0.343	0.732
Elytra Length	-4.940	3.333	-1.482	0.140
Line Type * Generation	0.645	0.421	1.532	0.127

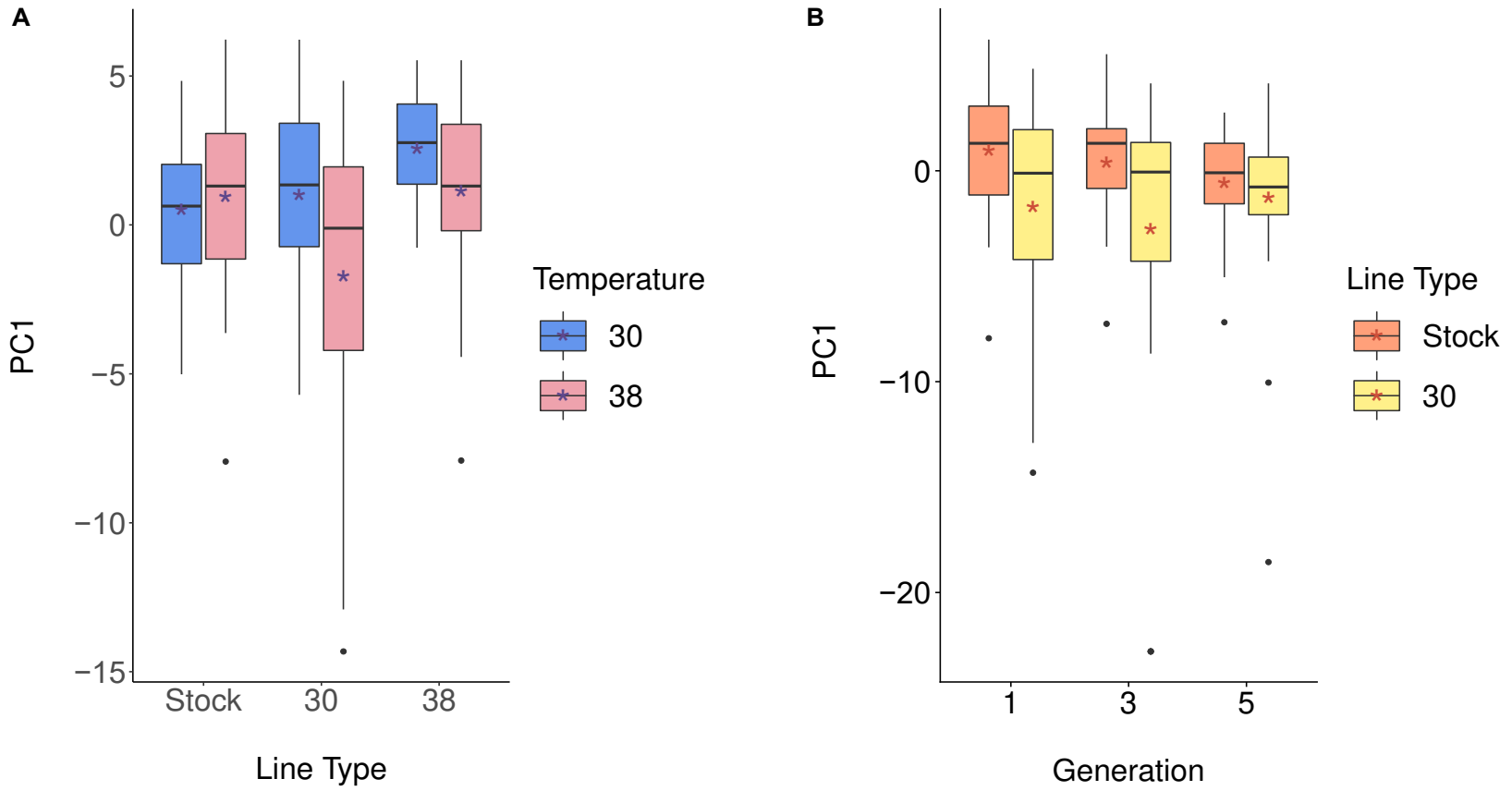

Figure 5.7. PC1 of *T. castaneum* ovaries A. across different lines (diverse stocks, control thermal lines and high temperature thermal lines) and thermal regimes (30°C and 38°C) in generation 1 and **B.** over five generations of exposure of stock populations and 30°C thermal lines to 38°C. Mean values are represented by *. PC1 was lower at high temperature (equating to more 'blockage' of the ovaries) with a stronger effect in the 30°C lines. There was no significant change in PC1 over five generations of exposure to high temperature in stocks or 30°C lines.

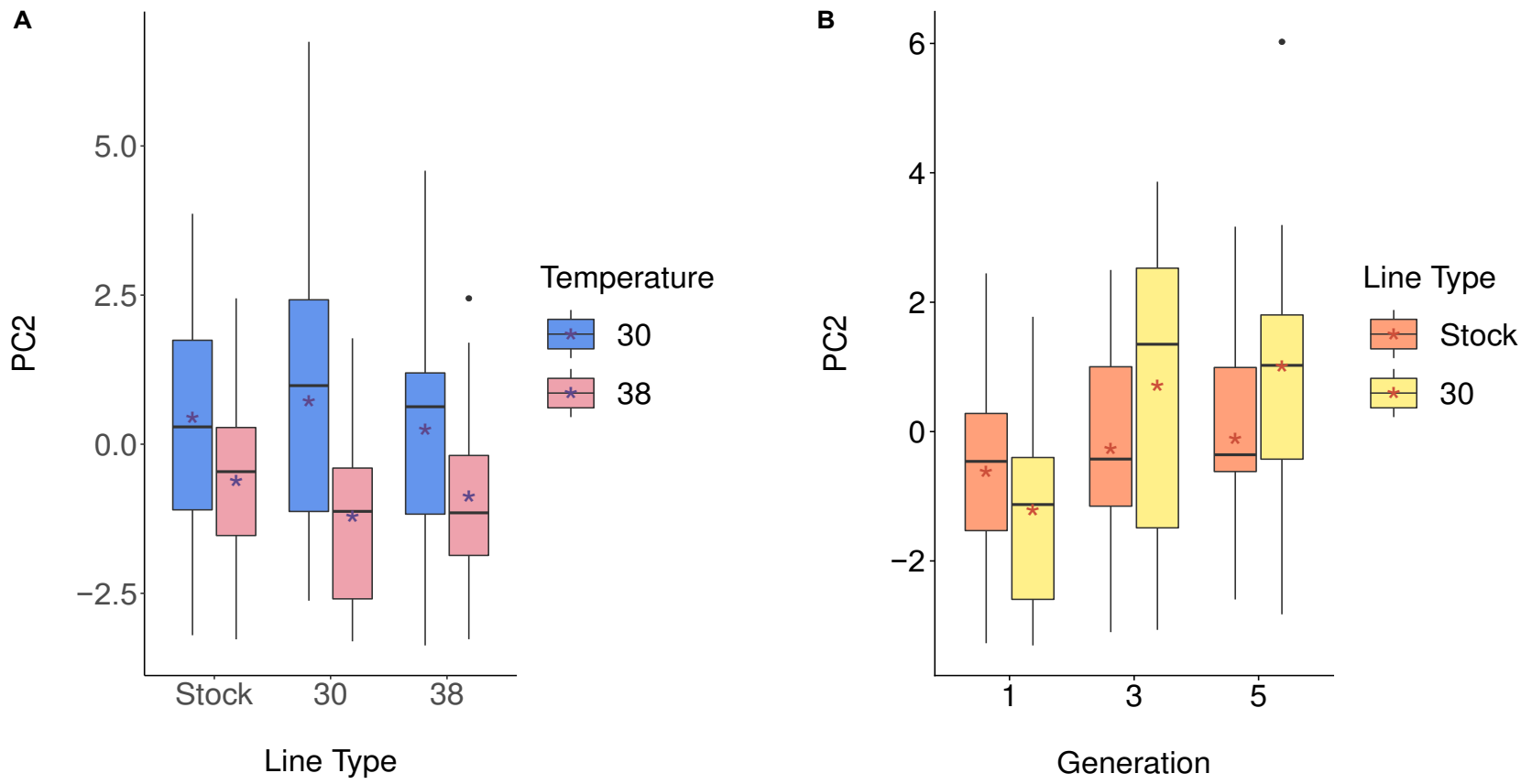

Figure 5.8. PC2 of *T. castaneum* ovaries A. across different line types (diverse stocks, smaller control thermal lines and high temperature thermal lines) and thermal regimes (30°C and 38°C) in generation 1 and **B.** over generations of exposure of stocks and 30°C thermal lines to 38°C. Mean values are represented by *. Development at high temperature resulted in a clear reduction in PC2 (equating to increased ovary 'blockage'). There was no significant change in PC2 over generations of exposure to 38°C, but there was a larger change in PC2 in the 30°C lines than in the KSS stock.

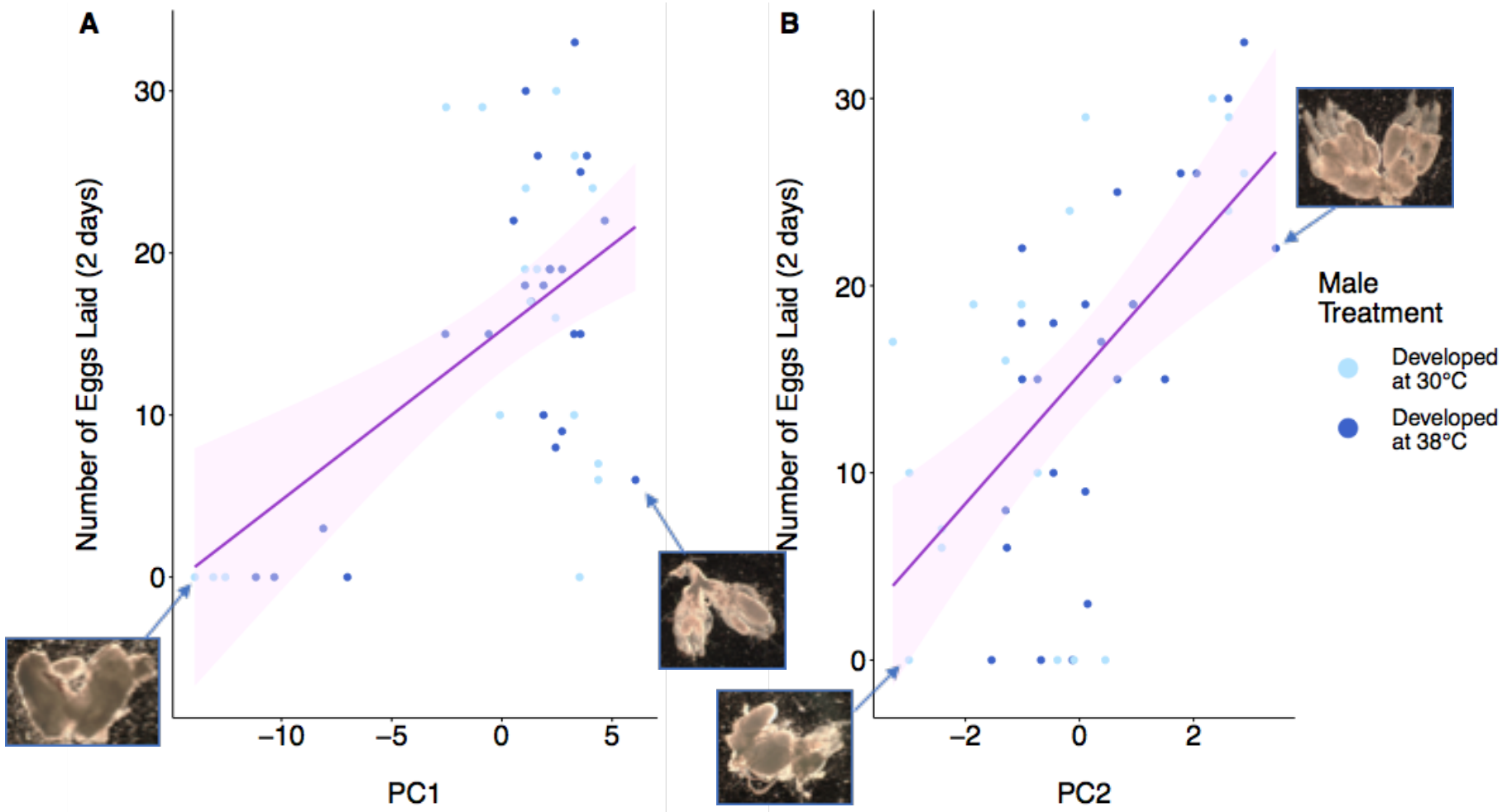

Figure 5.9. Correlations of principal components with the number of eggs laid by female *T. castaneum*. Plots show **A.** PC 1 and **B.** PC 2 (R_A = 0.56, p_A = 0.018; R_B = 0.6, p_B = 0.013), with points coloured by male treatment (development at 30°C - pale blue or 38°C - dark blue), which had no effect on fecundity. Inset photos show the ovaries with most extreme **A.** PC1 values and **B.** PC2 values.

Table 5.11. An LMM testing how PC2 of *T. castaneum* ovaries varies across different types of populations and different rearing temperatures. PC2 was the response variable, with a fixed effects of line type and temperature, with baselines set to stocks and 30°C, respectively. There was also a fixed effect of elytra length, a linear covariate and a random effect of Line ID (Var = 0.036).

Fixed Effect	Estimate	Standard Error	T value	Pr(>|t|)
Intercept	-1.640	3.126	-0.525	0.601
Line Type (30°C Lines)	- 0.148	0.365	- 0.405	0.700
Line Type (38°C Lines)	- 0.233	0.366	- 0.637	0.548
Temperature: 38°C	- 1.292	0.275	- 4.702	< 0.001
Elytra Length	0.928	1.290	0.719	0.473
Temperature (38°C) * Line Type (30°C Lines)	-0.792	0.629	-1.259	0.210
Temperature (38°C) * Line Type (38°C Lines)	-0.016	0.630	-0.025	0.980

Table 5.12. An LMM output testing whether PC2 of *T. castaneum* ovaries varies over generations in lines exposed to 38°C. The response variable was PC2 and the explanatory variables were line type (with the stock set as the baseline), generation (with generation 1 as the baseline) and elytra length. There was a random effect of line ID (Var < 0.001).

Fixed Effect	Estimate	Standard Error	T value	Pr(>|t|)
Intercept	-9.331	2.856	-3.267	0.001
Line Type	0.497	0.255	1.947	0.053
Generation	0.186	0.096	1.934	0.055
Elytra Length	3.480	1.238	2.811	0.005
Line Type * Generation	0.355	0.158	2.254	0.026

Table 5.13. A summary of the loadings of the PCA of non-virgin *T. castaneum* ovary morphology, following development at high temperature (38°C), in a single generation. Five variables were input into the analysis.

	PC 1	**PC 2**	**PC 3**	**PC 4**	**PC 5**
Proportion of Variance	0.902	0.097	< 0.001	< 0.001	< 0.001
Standard Deviation	5.184	1.700	0.102	0.057	0.018
Correlation to number of eggs in ovaries per individual	- 0.551	0.832	- 0.065	0.021	- 0.001
Correlation to mean egg length per individual	- 0.004	-0.010	- 0.383	0.839	0.386
Correlation to mean egg width per individual	- 0.002	- 0.007	- 0.173	0.345	- 0.922
Correlation to number of eggs found in calyces per individual	- 0.833	- 0.543	0.095	0.033	< 0.001
Correlation to proportion of eggs found in calyces per individual	- 0.046	- 0.111	- 0.900	- 0.419	0.013

Table 5.14. A linear model used to model the effect of PC1, PC2 and body size (using elytra length as a proxy) on the number of eggs laid by mated *T. castaneum* females over two days following development at high temperature. The number of eggs laid was the response variable, with fixed effects of PC1, PC2 and elytra length, all coded as linear covariates.

	Estimate	**Standard Error**	**t value**	**p**
Intercept	-2.309	26.365	-0.088	0.931
PC1	1.067	0.173	6.150	< 0.001
PC2	3.463	0.524	6.613	< 0.001
Elytra Length	7.483	11.225	0.667	0.509

5.4 Discussion

In this study, I investigated thermal adaptation over five generations of exposure to high temperature. I observed an increase in fitness over these five generations, evident as an increase in the reproductive output of pairs. In males, I saw no overall effect of temperature on the morphological characteristics of the reproductive organs (testes), but in females, development at high temperature generally resulted in a change in ovary morphology. I have been able to directly link the ovary phenotypes observed to fecundity differences, suggesting that development at high temperature is detrimental to fitness.

I measured reproductive fitness by monitoring reproductive output of pairs over one week. At control temperature, there was no change in reproductive output over the five generations of exposure, however, at high temperature there was an increase in reproductive output in all lines over the five generations. This indicates adaptation may have occurred over this relatively short time at high temperature. Very rapid rates of adaptation have been observed in several organisms (e.g. Costas *et al.*, 2008; Christie *et al.*, 2012), including *T. castaneum* (Agashe *et al.*, 2011). Although I observed a significant increase in reproductive output over time, it is important to note that there were large fluctuations between generations. These fluctuations appeared to be fairly consistent across populations, which indicates a wider environmental effect, as opposed to a genetic effect or stochastic fluctuations. Although these populations were reared in a controlled temperature environment, there is a possibility that these fluctuations were caused by uncontrolled environmental factors, such as air pressure or micro-climatic fluctuations. There is limited evidence that small variations in such factors could affect *T. castaneum* fitness and population dynamics, especially over relatively short time scales, although some studies have found them susceptible to extreme low pressure, particularly at high temperature (Kučerova *et al*., 2013; Donahaye, 1990).

After controlling for differences in body size, the mean follicle size of the males' testes and the within-individual variance in follicle showed only weak and inconsistent differences between populations and over generations. However, from previous research (see Chapter 4) it is clear that that development at high temperature does have a detrimental effect on male fertility. The lack of strong signal here suggests that this effect may be due to sperm morphology rather than testes morphology, as I was unable to detect large changes in testes morphology beyond the follicle number. Previous research has shown that *T. castaneum* sperm morphology and function can be affected by a number of variables including long-term temperature, short-term heatwaves and sexual selection regime (Vasudeva *et al.*, 2019; Sales *et al.*, 2018; Godwin *et al.*, 2017, respectively).

One clear effect from this study was an increase in the number of follicles present on the testes in control temperature lines (thermal lines and stocks) over five generations of exposure to high temperature, which may be an evolutionary response. Little is known about changes in testis follicle number in response to developmental stress. However, insect testes have previously been found to differ in the number of follicles between closely related species, within some species, and even between the two testes of a single male (Kuznetsova *et al.*, 2019), suggesting that follicle number is highly variable (Emeljanov *et al.*, 2001). When dissecting testes, it was difficult to avoid damaging the delicate structures and individual follicles can tear off or disintegrate. Therefore, it is possible that an increase in follicle number could be due to increased experience in dissecting by the generation five dissections. However, this seems unlikely because in generation one, where all lines were sampled, the high-temperature thermal lines had more testis follicles. An alternative explanation, therefore, is that adaptation to high temperature causes the testes to become less fragile, possibly through a thickening or strengthening of the walls of the follicles, although it is unclear what benefit a strengthened follicle wall could confer in high temperature. Although the vast majority of the *T. castaneum* testes were formed of six follicles, there were several testes observed consisting of seven follicles. This may suggest that the increase in follicle number detected here is an indication of a true

increase in the number of follicles on the testes, from six to seven, due to transgenerational heat exposure. However, as the number of individuals with seven intact follicles is so low, I do not have statistical power to detect any change in this over five generations. Kuznetsova *et al*. (2019) suggested that follicle number may vary with body size, although I found no evidence of this.

I observed strong, immediate effects of temperature on the ovary morphology of females in all lines. Development at high temperature consistently led to lower values of PC2 and, to a lesser extent, PC1, which were associated with more eggs in the calyces and fewer in the ovarioles. To confirm that this variation in morphology was representative of reproductive fitness, I correlated the number of eggs laid by a cohort of non-virgin females with their ovary morphology. This confirmed that my measures of ovary morphology are were related to fecundity, perhaps because of "blockages" in the ovaries that prevent eggs from being released. Previous work by Park (1935) has shown that development at high temperature resulted in impaired fecundity in the same way in *T. castaneum,* albeit on a much smaller scale. In studies of insects and climate change, female reproduction is poorly understood relative to male reproduction, probably due to the perception that male fertility is more sensitive than female fertility (David *et al.*, 2005; Sales *et al.*, 2018). However, these results, among others (Janowitz & Fischer, 2011), highlight the importance of understanding how temperature can affect female insects.

Despite the clear effect of temperature on ovary morphology, I did not see any differences between the experimental lines. Previously, I have shown that the high temperature lines produce more offspring than control lines following development at high temperature (see Chapter 4), and the results presented here show that reproductive output is higher in the high temperature lines after one, but not five, generations. This suggests that the experimental design implemented here was unable to fully capture the phenotypic variation underlying adaptation in the high temperature lines, which may be affecting traits in addition/other than the ovary

morphology. I have shown here that the measures used do correspond to fecundity, and would therefore expect it to be adaptive.

This study has yielded some unexpected results that require extra investigation. Firstly, it would useful to further explore the apparent ovary blockage that occurs following development at high temperature, including the underlying cause, and to what extent it is heritable as a trait. I observed no obvious differences in egg size between treatments, despite previous research on this system showing that egg size exhibits temperature-dependent plasticity (Vasudeva *et al.*, 2019). As the effect I have found here is observed only following development at high temperature, not in adults exposed to high temperature, I suspect that it is a change in the structure of the ovaries rather than the egg themselves, as has been found in a previous study (Xu *et al*., 2009). I conducted preliminary investigations into this to identify the cellular structure of the ovaries using cryosection and it seems that, with some work, a methodology could be developed to see this more clearly (Appendix C). More generally, further work using this system could help to elucidate the mechanisms of thermal adaptation in this species, which may be applicable in wider biological contexts as global warming progresses. This work has highlighted the need for further investigation into the temperature-dependence of female reproduction, particularly in invertebrates, as this could have severe consequences for population viability at increased temperatures.

General discussion

6.1 Thesis overview

The overarching objective of this thesis is to advance our knowledge of thermal adaptation. I approached this using experimental evolution of *Tribolium castaneum* populations to identify how adaptation to high temperature occurs. There were two main objectives: 1) to test the efficacy of genetic rescue techniques on thermal adaptation in long-term selection lines, and 2) to identify effects of high temperature during development and quantify how quickly phenotypic traits can change under intense selection.

My first goal was to quantify the extent to which adaptation had occurred in the high temperature thermal lines, with focus on adult reproduction and survival (Chapter 2). Compared with adults of the control thermal lines, the high temperature thermal lies did not produce many offspring, which suggested that either the lines had not adapted, or that they had adapted in a way that I had not measured. In response to this result, I attempted to facilitate adaptation in these lines, using genetic rescue techniques (Chapter 3). The population sizes were scaled up and migration events carried out for ten generations, with the aim of increasing the available genetic diversity on which natural selection could act. I observed no evidence of increased adaptation, but instead found that the increase in population size had a detrimental effect on reproductive output at high temperature, and that experimental migration had no effect. Further investigations in Chapter 3 revealed that the thermal lines were not suffering from severe inbreeding depression due to small population size.

The first step towards addressing the second objective was to test how males and females responded to thermal stress during development. I found strong negative effects in both males and females, affecting fertility and fecundity, respectively (Chapter 4) and further investigation into these effects in Chapter 5 revealed morphological differences in the reproductive organs of *T. castaneum*. There was also evidence of changes in both reproductive output and morphology, occurring in as little as five generations of exposure to elevated temperature, indicating that thermal

adaptation can be rapid. In this discussion I pull together and discuss the general findings from across the chapters, and suggest some potential avenues for future research.

6.2 General findings

38°C is a stressful temperature for *T. castaneum*, but adaptation is possible.

Many studies of thermal stress using *T. castaneum* focus on pest control (e.g. Dowdy, 1999; Mahroof *et al.*, 2005; Tilley *et al.*, 2007). Research into using heat as a pest control measure is common, and these studies usually aim for a temperature that results in either fatalities or sterilisation of the beetles (e.g. Mahroof *et al.*, 2005 used 50°C). I was not aiming for lethality or sterilisation, so opted for a lower, but still stressful temperature of 38°C. This approximate temperature has been used as a relatively mild thermal stress in other studies (e.g. Park 1935; Scharf *et al.*, 2015). Throughout this thesis it has been clear that 38°C is indeed a stressful temperature for *T. castaneum*. Exposure to this temperature consistently results in a decrease in reproductive output, an effect observed in every chapter of this thesis. Although I have identified that exposure to 38°C during development has the strongest effect, exposure during adulthood also causes a reproductive decline. This effect is also evident in the high temperature thermal lines, which had been exposed to high temperature for 60 - 80 generations before the experiments performed here. It seems that regardless of adaptation, *T. castaneum* perform better in terms of survival and reproductive output at 30°C.

I have also shown that *T. castaneum* can adapt to prolonged exposure to 38°C. This adaptation takes effect during development, enabling reproductive function, although not quite at 'normal' levels. Without adaptation, development at high temperature results in reduced female fecundity and male fertility, as shown in Chapter 4. Although I did not see differences in ovary morphology between the long-term control and high temperature lines, I did find that the level of ovary blockage was directly linked to fecundity and, across all lines, the ovaries were consistently more blocked after

development at high temperature. This supports the observation from Chapter 4, that female fecundity is reduced following developmental stress.

In males, I did not pick up any specific phenotypic differences in testes from different lines, but over the generations of exposure to high temperature, I observed an increase in average follicle size and an increase in follicle number, possibly due to a reduction in testis fragility. With the data I have collected, it is not clear if/how these changes are related to male fertility. As the reproductive output of pairs from these populations increased over time at high temperature, it is likely that these changes do affect fertility, but explicit links cannot be made with the existing data.

Evidence of adaptation is not necessarily apparent throughout the life history.

Another key finding from this thesis is that evidence for adaptation can be hidden if only one life stage is studied. In Chapter 2, I did not observe evidence of adaptation when I tested adult beetles from different lines, without considering juvenile life stages. Holometabolous insects undergo complete metamorphosis during their development, so each developmental stage can be resilient or vulnerable to different stressors. In fact, Chapter 4 of this thesis showed that, in *T. castaneum* females, the pupal stage is more vulnerable to high temperature (with regards to later life productivity) than any other stage. A previous study has found this effect in pupae, and although they saw a stronger effect in females, it was present in both sexes (Mahroof *et al.*, 2005), which is likely because the pupal stage is when the majority of the maturation of the ovary and spermatheca occurs (Sokoloff, 1974). It is possible that the reason I did not see a similar effect in males was that the experimental design used 5-day blocks, rather than focussing on isolating specific developmental stages for heat exposure. Therefore, most individuals were exposed to high temperature for only part of their pupation. Given the opportunity, I would redesign this experiment accordingly in the future.

The research I conducted into reproductive morphology yielded unexpected results. Although I was anticipating that development at high temperature would cause some morphological differences in reproductive organs, I did not expect to see ovary 'blockage'. I predicted that the ovaries may be smaller, under developed or empty, as observed in other studies of insect development in stressful conditions (e.g. Xu *et al.*, 2009; Everman *et al.*, 2018), so it is interesting that, in this case, the ovaries do appear to be producing eggs, and that the impact on fecundity seems to be due to mature eggs not being released. I began to look at ovary morphology at the cellular level (see appendix C), but again this requires further work.

I saw relatively minor immediate effects on testis morphology following development at high temperature (Chapter 5). Previous work from this laboratory has shown that stress (dietary and thermal) reduces testis size (Godwin *et al.*, 2017 and Sales, 2018, respectively) and that thermal stress specifically, reduces sperm viability (Sales *et al.*, 2018), so I was expecting to see more of an effect here. However, over five generations of exposure to high temperature, the average follicle size and the number of follicles increased which may indicate adaptive change. Although not detected in this study, previous work has shown a decrease in follicle size following exposure to high temperature (Sales, 2018). Therefore, it is possible that the increase in follicle size that I observed over the five generations may be evidence of adaptation to development at high temperature.

Increasing population size and genetic diversity does not always facilitate adaptation.

Chapter 3 provides evidence that genetic diversity does not always affect adaptation in the way we expect. From population genetic theory, I would expect that larger populations would be more adaptable than small ones and that introducing migrants should further increase adaptive potential (Whiteley *et al.*, 2015). However, I showed that increasing population size can have a detrimental effect on survival and reproduction.

I was aware that the long-term high temperature lines had undergone a bottleneck when they were first established. Therefore, my assumption was that this would result in a lack of genetic diversity and a lack of adaptive potential. Chapters 3 and 4 demonstrate that in these particular populations, adaptation has been able to occur despite the suspected low genetic diversity, and in fact outcrossing provided no benefit. This may suggest that the initial bottleneck was due to strong selection, leaving only those most capable of surviving and reproducing at 38°C to contribute to the next generation. Outbreeding in this scenario would only serve to dilute the beneficial effects of adaptive genetic variation, which is a form of outbreeding depression (Monson & Sadler, 2010). It is a concern for conservation as attempts to induce genetic rescue through crossing between populations can result in reduced fitness in vulnerable species (Tallmon *et al.*, 2004). It was therefore interesting that adding diverse migrants to the high temperature thermal lines did not affect fitness, either adversely or favourably.

Another unexpected effect of high genetic diversity was that it was associated with slower rates of adaptation. In the first five generations of exposure to a selective pressure (in this case, high temperature), outbred stock populations had reduced levels of reproductive success compared to the small, less diverse control lines (see Chapter 5). This effect was present in measures of ovary morphology and reproductive output. Literature suggests that more diverse populations should be associated with higher adaptive potential than smaller, homogenous populations (Willi *et al.*, 2006; Raghwani *et al.*, 2016).

Generation effects & repeatability

It has become apparent, through completing this research, that the reproductive output values I have been measuring are highly variable between generations (Chapter 5). Previous studies have recognised seasonal differences in productivity of *T. castaneum* (Turaki *et al.*, 2007 & Campbell *et al.*, 2010), but I would not expect to see

such differences in the controlled temperature rooms used for this research. Furthermore, fluctuations in reproductive output between generations, rather than experiments, would not be explained by seasonality, as each generation is only one month apart. Instead, these may be explained by potential short-term microclimatic changes invoked by variables such as the position of containers in incubators and the frequency of doors to the warm room opening. Natural populations can adapt to very specific microclimates (Nevo *et al.*, 1998), so a small shift in temperature or humidity caused by a variation within an incubator could have large effects on reproduction. Incubators are also prone to slight fluctuations in temperature and certain areas within a population enclosure may be more resistant to this than others. Anecdotally, there tend to be fewer individuals on the surface in the high temperature environment than the control temperature, which may be due to microclimates within a container.

6.3 Future research suggestions

Although this thesis has generated new information about the effects of heat on *T. castaneum* reproduction, and how they adapt to this, it has also highlighted some further questions that need to be addressed. In response to the inconsistency in reproductive output over generations, I would like to conduct a concurrent reproductive output experiment in a range of incubators, to ensure that this effect is not due to equipment unpredictability. If the results were consistent across incubators, it would suggest that the cause may be a wider environmental variable. In addition to this, it would be interesting to monitor the temperature of different locations within an enclosure, buried in the flour compared to on the surface, to see whether populations in larger containers, with a larger volume of flour are better insulated from any environmental fluctuations than small populations.

In Chapter 4, I exposed developing beetles to high temperature at various stages of development, in 5-day blocks. I detected an effect in females, but not in males, in that the pupal stage was more vulnerable to high temperature. Previous studies have found evidence of this pupal sensitivity in both sexes (Mahroof *et al.*, 2005). In my

experiment, the heat treatment was applied to 5-day intervals. I did not check for the frequency of pupae present in the population at that time. From experience, we know that *T. castaneum* generally pupate approximately 21 days after hatching, but there is natural variation in this. The 5-day intervals were not designed around typical pupation times, but I could identify that certain treatments would have a higher frequency of pupae experiencing the heat treatment than others. These treatments would likely also contain some final-instar larvae and some newly eclosed adults. This natural variation may explain why my study did not identify a stage-specific effect in males. I would be keen to redesign this experiment, ensuring that each block does correspond precisely to a developmental stage, to confirm whether or not there is a sex-specific difference in pupal sensitivity to high-temperature, as the current results indicate.

The effect of development at high temperature on ovary structure merits additional study. Here, I highlighted a visible effect, but did not identify why this occurs. My preliminary work in investigating this has shown that using cryosection and staining techniques to examine cross sections of ovaries is feasible, and clearly shows the cellular structure of the ovary (Appendix C). A useful continuation of this research would be to examine ovaries in this way at various points in development to identify any differences in heat-stressed beetles from control beetles. There are many other avenues that further research into this effect could follow, including: investigations into the heritability of 'blockage resistance'; identification of any genes responsible for this response; and research into the reason for the blockage. It is possible that the blockage is a behavioural response; a reluctance to release eggs into an unfavourable environment in case it improves. Alternatively, it could be a physiological effect and the eggs are too large to leave the ovaries. This could arise if, in response to developmental thermal stress, either the oviducts (the tube through which the eggs are released) become smaller or underdeveloped, or if the eggs grow larger in response to elevated temperature. Previous research (Vasudeva *et al*., 2019) has shown that egg size is a plastic trait and even after a short time of adult exposure to high temperature (2 days), egg size does increase.

The genetic rescue techniques studied in Chapter 3 did not aid the populations in adapting and actually reduced their fitness. This suggests that using genetic rescue as a conservation technique could be detrimental to already struggling populations. Therefore, without further study into why this occurs, genetic rescue could be a high-risk approach to population management. To minimise this risk, there are several big questions which should be addressed in this field. When does genetic rescue help or hinder populations? How long do any benefits of genetic rescue last? Why does genetic rescue sometimes not work? Ideally, test parameters must be developed, to enable us to calculate a likelihood of success for genetic rescue in conservation. An experimental approach could be useful here, accepting the fact the life history of insects is dissimilar to many species of conservation concern. On the other hand, observational studies of genetic rescue lack replication, and using a laboratory insect model it would be possible to develop a highly replicated set of inbred populations, which could be 'rescued' by varying numbers of migrants from different genetic backgrounds. It would also be useful to monitor these experiments for several generations following the rescue event, to identify the duration of genetic rescue benefits.

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Appendices

Appendix A. – Lewis & Pointer *et al.*, 2020

Received: 31 July 2020 | Accepted: 6 August 2020

DOI: 10.1002/ece3.6742

Ecology and Evolution Open Access WILEY

ORIGINAL RESEARCH

Polyandry provides reproductive and genetic benefits in colonising populations

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Funding information
Biotechnology and Biological Sciences Research Council, Grant/Award Number: BB/N011759/1

Abstract

Polyandry, when females mate with more than one male, is theorised to play an important role in successful colonisation of new habitats. In addition to possible benefits from sexual selection, even mild polyandry could facilitate colonisation by protecting against inbreeding and reducing the costs of mating with incompatible or infertile males. Here, we measure the importance of mild polyandry for population viability and reproductive fitness following experimental founder events into a higher-temperature regime. Using colonisation experiments with the model beetle *Tribolium castaneum*, in which females can produce offspring for up to 140 days following a single mating, we founded more than 100 replicate populations using single females that had been given the opportunity to mate with either one or two males and then tracked their subsequent population dynamics. Following population viability and fitness across 10 generations, we found that extinction rates were significantly lower in populations founded by females given polyandrous opportunities to mate with two males (9%) compared to populations founded by monogamous females (34%). In addition, populations founded by females that had been provided with opportunities to store sperm from two different males showed double the median productivity following colonisation compared to monogamous-founded populations. Notably, we identified short-term and longer-term benefits to post-colonisation populations from double-mating, with results suggesting that polyandry acts to both protect against mating with incompatible males through the founder event, and reduce inbreeding depression as the colonisation proceeds for 10 generations. Our results therefore show that even mild polyandry provides both reproductive and genetic benefits for colonising populations.

KEYWORDS

extinction, inbreeding depression, population dynamics, sexual selection, *Tribolium*

Rebecca C. Lewis and Michael D. Pointer contributed equally to this work.

1 | INTRODUCTION

Populations colonising a new habitat often face an array of challenges, including Allee effects, inbreeding depression, and loss of genetic diversity as a result of small founder population size (Dlugosch & Parker, 2008). Alongside this, colonising populations often need to respond and adapt to a new environment (Sax & Brown, 2000). Understanding the behavioural, ecological, and genetic processes that occur in colonising populations is important for understanding—and ultimately predicting—their establishment probability and is therefore of interest to a range of fields, from pest science to conservation biology (Bouzat, 2010; Fagan, Lewis, Neubert, & van den Driessche, 2002).

One way that populations may be buffered against the challenges posed by colonising new environments is through female multiple mating or polyandry (Candolin & Heuschele, 2008; Parrett & Knell, 2018). In addition to the direct benefits that polyandry may provide to females and their offspring (e.g., increased resources from males) (Fedorka & Mousseau, 2002), multiple mating can provide genetic benefits (Zeh & Zeh, 2001). Polyandry facilitates sexual selection, in which females may encourage genetic benefits for their descendants by skewing paternity towards specific males (Andersson & Iwasa, 1996). Sexual selection can therefore improve population fitness through a range of mechanisms (reviewed in Yasui, 1998), including offspring inheriting "good" or "compatible" genes as a result of polyandry (Neff & Pitcher, 2005) or through selection for increased genetic diversity in offspring, which may increase adaptability to fluctuating or novel environments (García-González, Yasui, & Evans, 2015).

Polyandry may also provide benefits to individuals and populations in the absence of sexual selection; one recognised mechanism for this is via bet-hedging (Watson, 1991). If mate-choice is unreliable or costly, multiple mating may be an effective strategy to protect against unsuitable males. It is still debated whether the fitness gains derived from bet-hedging are sufficient to drive the evolution of polyandry (Holman, 2016). However, bet-hedging may play an important role in the dynamics of small colonising populations, where the consequences of mating with a single male who happens to be of low quality or compatibility are expected to be particularly severe (Yasui & Garcia-Gonzalez, 2016).

Finally, polyandry may play a key role in colonisation through reductions in inbreeding. Cornell and Tregenza (2007) developed a model showing that, because offspring of polyandrous females contain half-sibs, inbreeding depression in future generations will be significantly reduced by even mild polyandry, improving the probability of colonisation success. This theory received empirical support in a study of seed beetles (*Callosobruchus maculatus*) in which populations founded by polyandrous females had increased fitness after five generations, compared to monogamous females (Power & Holman, 2014). Interestingly, despite the benefits of polyandry for individual fitness, Power and Holman (2014) found no effect of mating treatment on extinction rates, which were low throughout the experiment. It is therefore not yet known how strong or widespread these benefits are across different species and environmental conditions. Given that the strength of inbreeding depression can be dependent on environmental conditions (Armbruster & Reed, 2005), this is an important area for future investigation.

The red flour beetle, *Tribolium castaneum*, is an ideal model system to test experimentally how polyandry influences colonisation success. A pest of stored products, the ecology of *T. castaneum* is characterised by continued colonisation of empty habitats (e.g. grain stores), presumably often by a small number of founders (Dawson, 1977). Females can mate polyandrously and then store sperm to enable offspring production without males for more than 100 days postmating (Michalczyk, Martin, Millard, Emerson, & Gage, 2010). Experimental studies in this species have shown that founder effects have pronounced costs as a result of genetic and demographic effects and that colonising populations are able to rapidly adapt to novel environments (Szucs, Melbourne, Tuff, & Hufbauer, 2014; Szucs, Melbourne, Tuff, Weiss-Lehman, & Hufbauer, 2017). Further, this species is promiscuous, and experimental evolution studies have shown that a history of strong sexual selection results in decreased risk of extinction under inbreeding and improved invasion into competitor populations (Godwin et al., 2018; Lumley et al., 2015). Moreover, matings and fertility often appear to fail in this species (Tyler & Tregenza, 2013), and there is some evidence to suggest that these costs are reduced when females mate multiply (Pai, Bennett, & Yan, 2005).

Using the *T. castaneum* system, here we test how mild polyandry impacts upon colonisation success when foundresses enter a challenging thermal environment. Following mating opportunities with either one or two males, we placed single females into an empty habitat at 38°C, a temperature which we know is stressful for *T. castaneum* (Dickinson, 2018), and then tracked population dynamics and extinction rates for 10 generations (1 year). We tested the hypotheses that populations founded from polyandrous females (a) were less likely to go extinct and (b) maintained larger sizes due to increased reproductive fitness and then identified the behavioural, ecological, and genetic drivers behind colonisation success. Note that our aim here is not to test explicitly how temperature affects colonisation success, but rather to test how mating patterns affect colonisation success in an environment that is known to be challenging. We discuss our finding in the context of how mating strategy and inbreeding interact to affect subsequent colonisation dynamics.

2 | MATERIALS AND METHODS

2.1 | Experimental protocols

All beetles used were from our outbred Krakow Super Strain (KSS), which are reared under standard conditions of 30°C and 60% humidity (Dickinson, 2018). Beetles were maintained both before and throughout the experiment on a fodder medium consisting of 90% organic strong white bread flour mixed with 10% Brewer's yeast and topped with a layer of oats for traction.

The overall experimental design is outlined in Figure 1. Founding females and their mates were reared separately and mated under standard conditions as above. To allow matings to occur, pairs were placed into small (7 ml) screw-top vials containing 1.5 g of fodder. All females received two mating opportunities, each lasting 24 hr. In the first round of pairings, virgin females were randomly paired with virgin males (aged ~7 days posteclosion). In the second round of pairings, half of the females were paired with a second male who had previously been paired with a different female for 24 hr (hereafter referred to as the "polyandrous" treatment). The remaining females were assigned to a "monogamous" treatment, in which they were re-paired for 24 hr with the same male who, for consistency between treatments, was briefly removed from the dish before being replaced. Thus, all females were paired with single males across two 24-hr mating periods, either with different males ($N = 55$, polyandry treatment) or with the same male twice ($N = 53$, monogamy treatment). We note that with our experimental design we cannot be sure that all polyandrous females mated twice and that this may result in reduced power to distinguish the true effects of polyandry.

Following the above mating treatments, we then allowed populations to become established in a challenging thermal environment for the remainder of the experiment (Figure 1). Specifically, after 48 hr of mating opportunities with either one or two males, individual females were transferred alone to a population container (100-ml PVC screw-cap containers, with the caps pierced for ventilation, containing 70 ml fodder) and allowed to oviposit for 7 days in a warmer thermal regime of 38°C and 60% humidity, after which they were removed and offspring left to develop. This temperature is at the upper limit at which *T. castaneum* can reproduce and presents a stressful and demanding environment for survival and reproduction (Howe, 1960). All population containers postmating were marked only with a randomised ID number so that experimental treatment was unknown by researchers during subsequent handling and counting. Twenty-eight days after females were removed, the first generation of offspring were separated from the fodder by sieving, the fodder was discarded, and the container and sieve cleaned with ethanol between replicates. The number of live adults was counted and placed into fresh fodder to seed the next generation. If >100 adults were present in a population, 100 were used to seed the next generation, and the remainder discarded after counting (in order to minimise density-dependent effects). This next new generation of adults was then allowed to mate and oviposit in the fresh fodder for 7 days, after which adults were removed by sieving and the offspring again left to develop into adults for 28 days. This process was repeated for 10 generations, all at 38°C.

2.2 | Statistical analyses

All analyses were carried out using R version 3.3.3 (R Development Core Team, 2011). We separately modelled how the experimental mating treatment affected (a) the probability of extinction over 10 generations and (b) changes in population size over the same period. For the extinction analysis, we used Cox proportional hazards models, implemented in the Survival package (Therneau, 2020) in R. Because some populations went extinct in the first generation, possibly as a result of failure to mate, fertilise, or develop, we ran the survival models both with and without populations that went extinct in the first generation.

To model how population size changed over time, we used generalised linear mixed models, implemented using the glmmADMB package (Fournier et al., 2012) in R. For this analysis, we only

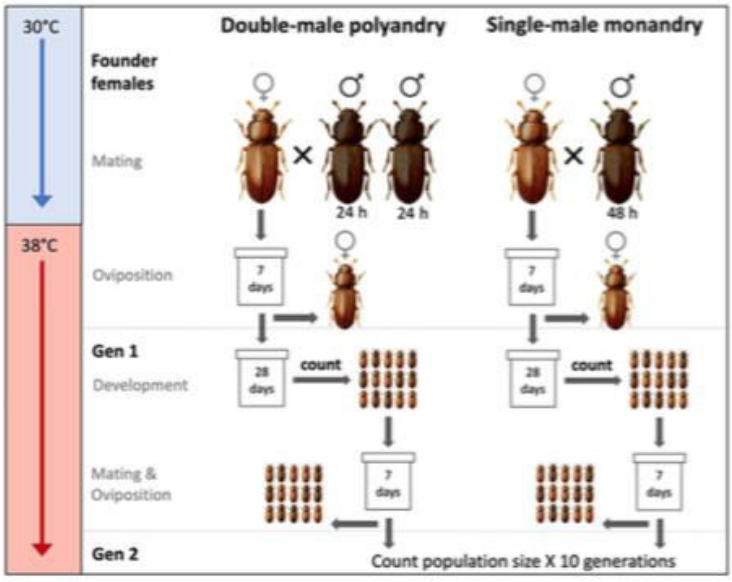

FIGURE 1 Experimental design for *Tribolium castaneum* colonisations. Individual females were mated with either one or two males, then introduced into a challenging thermal environment to lay eggs. Offspring were counted and used to found subsequent generations. We tracked a total of 108 populations for 10 generations

included population counts above zero. Offspring number per generation was modelled as a response variable with a negative binomial error distribution, and generation and experimental treatment (monogamous vs. polyandrous founder) were fitted as explanatory variables, alongside the interaction between treatment and generation. To account for potential nonlinear changes in population size over time, we fitted changes in offspring numbers over generations as (a) a continuous variable and (b) a third-order polynomial. We fitted random slopes models, which allowed variation among individual populations over generations. Finally, we tested for a difference in population size between experimental treatments in the first and last generations, using two separate generalised linear models (as above but with no random effects), implemented using the MASS package (Venables & Ripley, 2002) in R.

3 | RESULTS

We tracked the dynamics of 53 monogamy-founded and 55 polyandry-founded *T. castaneum* populations at high temperature for 10 generations or until extinction, with overall dynamics shown in Figure 2. Although there was a general trend for increasing population size postcolonisation, there were substantial fluctuations over some generations, with decreases in population size between generations three and four, and generations six and seven, possibly due to density-dependent crashes (Figure 2). Despite this variation, we observed a clear and consistent trend for larger adult population sizes in populations founded by polyandrous-treatment compared to monogamous-treatment females (Figure 2, tested below). Across all generations, the median size of polyandry-founded populations founded was 162 (interquartile range = 58–308), compared to 85 (interquartile range = 2–216) for monogamy-founded populations.

For statistical comparison, we separately tested for differences in extinction rates between mating pattern treatments and population size changes over time. In the first colonisation generation, six populations founded by monogamous females went extinct (11%), while no populations founded by polyandrous females went extinct. By generation 10, 18 monogamous populations (34%) had gone extinct, but only five polyandrous populations (9%) were no longer producing offspring (Figure 3a). The effect of treatment on time to extinction was significant (Cox proportional hazards; hazard ratio = 0.256; 95% CIs = 0.102, 0.642; $p = .004$). This effect remained significant after removal of populations that went extinct in the first generation (hazard ratio = 0.361; 95% CIs = 0.137, 0.950; $p = .039$).

We next tested how founder mating regime affected subsequent population fitness and growth trajectories. Excluding extinctions, we found no significant difference in the number of offspring produced by monogamous or polyandrous females in the first generation (GLM, $p = .503$), suggesting that mating pattern per se did not directly influence offspring production at the initial colonisation event. Considering all generations, however, we found that populations founded from polyandrous females had larger overall population sizes than populations founded by monogamous females (Figure 3b, Table 1). When generation was modelled as a linear continuous variable, population size increased over time, but there was no interaction between treatment and generation (Figure 3b, Table 1). The effect of experimental treatment was also significant when generation was modelled as a third-order polynomial ($p = .019$). Finally, considering only populations that survived all 10 generations, population size in polyandrous-founded populations in generation 10 was significantly larger than monogamous-founded populations (GLM, $p = .004$).

4 | DISCUSSION

Because of the recognised costs to females of mating with multiple males (when a single male can provide full fertility), the widespread evolution and maintenance of polyandry is an evolutionary puzzle (Simmons, 2005). Here, we reveal substantial fitness benefits from polyandry for colonising populations, even when the opportunity for precopulatory sexual selection is experimentally reduced.

Potential indirect benefits of polyandry include: (a) enabling sexual selection, (b) protection via bet-hedging, and (c) reducing inbreeding load. Perhaps the best-studied way in which females can increase their fitness through polyandry is via bet-hedging (Yasui & Garcia-Gonzalez, 2016). By mating with multiple males, females may

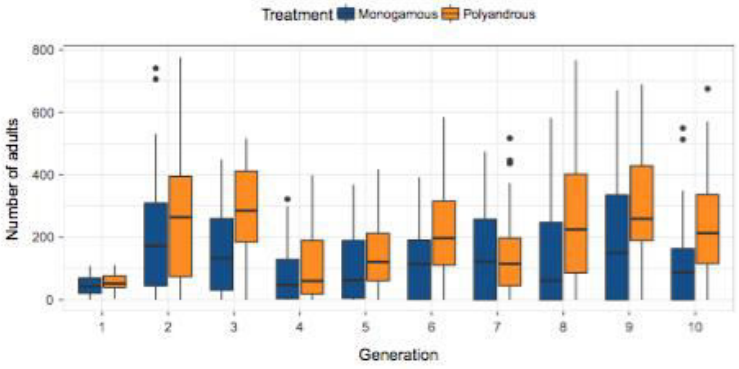

FIGURE 2 Colonisation dynamics of experimental *Tribolium castaneum* populations founded from monogamous or polyandrous females

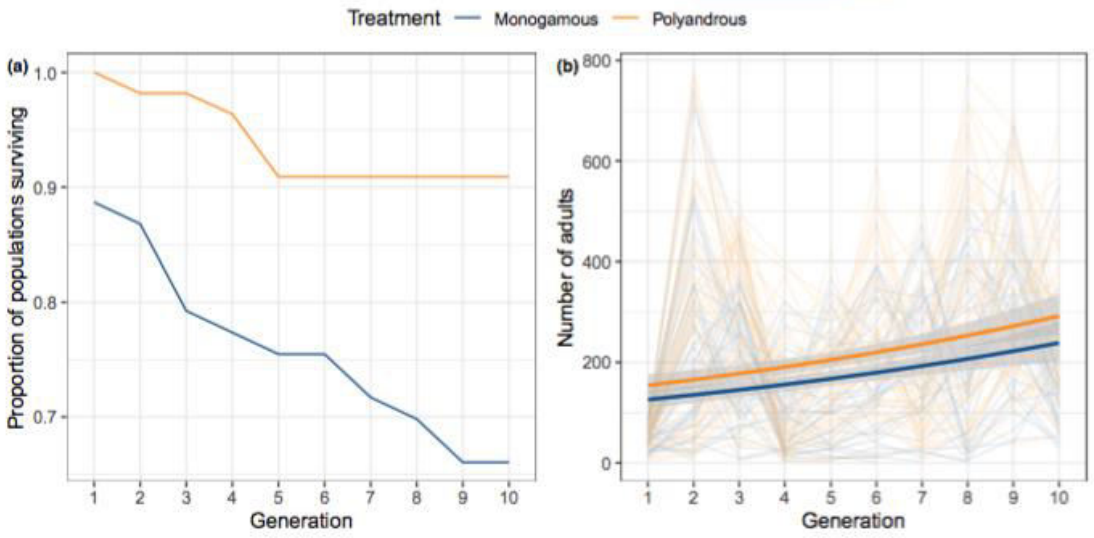

FIGURE 3 Extinction and population dynamics of experimental *Tribolium castaneum* populations founded from monogamous or polyandrous females. (a) Proportion of populations surviving over time; (b) number of adults in experimental populations. In b, thin lines represent individual populations, while the thick lines represent fitted values from a negative-binomial GLM

TABLE 1 Summary of results from a generalised linear mixed model of population dynamics of experimental *Tribolium castaneum* populations founded from monogamous or polyandrous females. Here, the "treatment" estimate refers to the effect of polyandrous relative to monogamous females, and generation was modelled as a linear effect. As random effects, we modelled a random intercept of population ID (Var = 0.103, *SD* = 0.32) and a random slope of generation with population ID (Var = 0.0008, *SD* = 0.028)

	Estimate	*SE*	*p*
(Intercept)	4.719	0.083	<.001
Treatment	0.229	0.091	.012
Generation	0.063	0.011	<.001
Treatment × Generation	0.007	0.021	.756

reduce the risk of mating failures or being fertilised by an unsuitable male and therefore increase reproductive and/or offspring fitness. Bet-hedging is likely to be most beneficial when (a) there is a substantial proportion of unsuitable (e.g., infertile) males in the population and (b) the population is small (Yasui & Garcia-Gonzalez, 2016). In our study, populations were founded by a single female, and as such there is clear potential for polyandry to provide benefits. We found that 11% of monogamous females produced no offspring in the first colonisation generation, while all polyandrous females produced offspring. The low number of extinctions here means that caution is required when interpreting this result. Nonetheless, these percentages are broadly consistent with a situation in which a failure to produce offspring is the result of a failure to mate or due to infertile or incompatible males, from which we can expect only 1.28% of random pairs of males in the double-mating treatment to both be infertile or incompatible. Previous research in *T. castaneum* has found that a substantial proportion of matings fail to result in offspring production (Pai et al., 2005; Tyler & Tregenza, 2013), and across insects, male infertility or reproductive failure has been observed in the wild (García-González, 2004). It is therefore likely that multiple mating is one important mechanism for increasing short-term establishment probability in newly colonised populations through the simple mechanism of gaining successful insemination of functional and compatible spermatozoa.

Another potential mechanism through which polyandry can benefit colonising populations is by reducing levels of inbreeding in subsequent generations (Cornell & Tregenza, 2007). Consistent with this hypothesis, we found significantly lower population sizes and higher extinction rates in monogamy-founded populations over the full duration of our experiment. Population sizes fluctuated substantially over the course of our experiment, likely a result of density-dependent processes which are well-documented in *T. castaneum* (Mertz, 1972). Although the higher population sizes in polyandry-founded populations were generally consistent over time, it is notable that the difference between treatments was highest when population sizes were high (i.e., in generations 2, 3, 8 and 9) and lowest when population sizes were reduced (i.e., generations 4 and 7). It is possible that a scenario akin to bet-hedging could explain these longer-term benefits of polyandry if there was substantial variation in fitness among fertile males, as multiple-mating would increase the chances of mating with at least one suitable male (Yasui & Garcia-Gonzalez, 2016). However, this scenario is unlikely to explain our results, as we found no difference in population size between mating pattern treatments in the first colonisation generation, but these became obvious when considering later generations. Similarly, if postcopulatory sexual selection explained some of the differences

observed between our experimental treatments, we would expect to observe at least some differences in offspring fitness in the first generation. We therefore suggest that in *T. castaneum* and similar systems, polyandry will benefit colonising populations through two main routes: (a) insuring against male infertility and enabling initial establishment and (b) reducing inbreeding and enabling longer-term population persistence.

Our results are broadly consistent with a recent study in *C. maculatus*, in which the increased fitness in polyandrous-founded populations was observed in F4 and F5 generations, but not F1–F3, generations (Power & Holman, 2014). However, and in contrast to our study, Power and Holman (2014) found no effect of mating treatment on extinction, likely because their experimental environment was relatively benign or because there was insufficient time for extinctions to occur. Here, through a longer-term experiment on colonisation success in a stressful thermal habitat, we demonstrate that the benefits of polyandry persist for longer periods of time and show that they are likely to be important when populations enter challenging environments. Future climate change is expected to result in species shifting their ranges and undergoing changes in population size, and there is increasing realisation that evolutionary processes need to be incorporated into predictive models of population and species responses to climate change (Lavergne, Mouquet, Thuiller, & Ronce, 2010). We recommend that the multiple, interacting benefits of polyandry should be incorporated into such models in order to improve predictive power.

ACKNOWLEDGMENTS

This research was funded by a BBSRC Future Leader fellowship to LGS (BB/N011759/1), a UEA-funded PhD studentship to RCL, BBSRC NRP DTP-funded PhD studentships to MDP and JB.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Rebecca C. Lewis: Conceptualization (supporting); Investigation (lead); Writing-original draft (supporting); Writing-review & editing (equal). **Michael D. Pointer:** Conceptualization (supporting); Investigation (lead); Writing-original draft (supporting); Writing-review & editing (equal). **Lucy A. Friend:** Investigation (equal); Writing-review & editing (equal). **Ramakrishnan Vasudeva:** Investigation (equal); Writing-review & editing (equal). **James Bemrose:** Investigation (equal); Writing-review & editing (equal). **Andreas Sutter:** Conceptualization (supporting); Formal analysis (supporting); Methodology (supporting); Visualization (supporting); Writing-review & editing (equal). **Matthew J. G. Gage:** Conceptualization (equal); Project administration (equal); Supervision (equal); Writing-original draft (supporting); Writing-review & editing (equal). **Lewis G. Spurgin:** Conceptualization (equal); Formal analysis (lead); Funding acquisition (lead); Investigation (equal); Project administration (lead); Supervision (lead); Validation (lead); Visualization (lead); Writing-original draft (lead); Writing-review & editing (lead).

DATA AVAILABILITY STATEMENT

The data and code to reproduce all analyses in this manuscript are available on Github (https://github.com/lgs85/TriboliumSexCol).

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How to cite this article: Lewis RC, Pointer MD, Friend LA, et al. Polyandry provides reproductive and genetic benefits in colonising populations. *Ecol Evol*. 2020;10:10851–10857. https://doi.org/10.1002/ece3.6742

Appendix B - Body Size Analyses.

Table B.1. An LMM modelling differences in male elytra length of male *T. castaneum* between treatments in generation one. The response variable was the elytra length, which was used as a proxy for body size. The explanatory variables were line type (with baseline set as stock populations) and rearing temperature (baseline set to control temperature, 30°C). There was a random effect of line ID (Var < 0.001).

Fixed Effect	Estimate	Standard Error	T value	Pr(>|t|)
Intercept	2.377	0.019	126.698	< 0.001
Line Type (30°C Lines)	- 0.045	0.024	- 1.868	0.111
Line Type (38°C Lines)	0.010	0.024	0.417	0.691
Temperature	- 0.083	0.016	- 5.323	< 0.001
Temperature * Line Type (30°C Lines)	- 0.043	0.037	- 1.146	0.254
Temperature * Line Type (38°C Lines)	- 0.110	0.037	- 2.931	0.004

Table B.2. Summary of an LMM modelling changes in male *T. castaneum* elytra length in 30°C thermal lines and stock populations exposed to 38°C over five generations. Elytra length was the response variable and the explanatory variables were line type (stocks set as baseline) and generation. There was a random effect of Line ID (Var = 0.001).

	Estimate	Standard Error	T value	Pr(>|t|)
Intercept	2.262	0.026	85.562	< 0.001
Line Type	-0.024	0.032	-0.749	0.496
Generation	0.047	0.005	9.828	< 0.001
Line Type * Generation	0.016	0.009	1.657	0.099

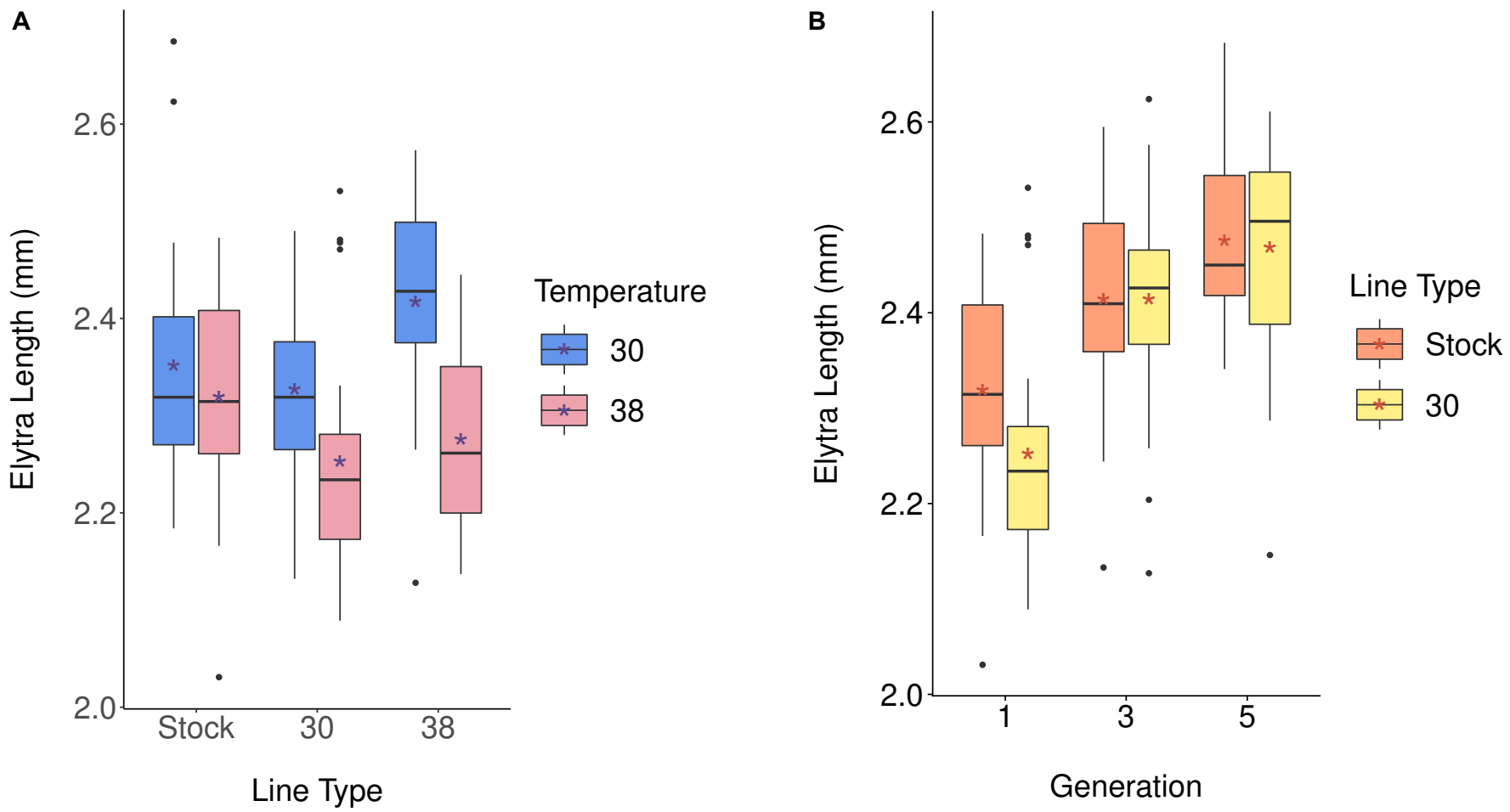

Figure B.1. Elytra length (a proxy for body size) of *T. castaneum* males A. between treatments in generation one and **B.** over generations of exposure to high temperature in the stock populations and 30°C thermal lines.

Table B.3. An LMM modelling differences in female *T. castaneum* elytra length across different treatments (line types and rearing temperatures). The response variable was elytra length. There were explanatory variables of line type and temperature (with baselines of stock populations and 30°C) and a random effect of line ID (Var < 0.001).

	Estimate	**Standard Error**	**T value**	**Pr(>|t|)**
Intercept	2.413	0.020	122.785	< 0.001
Line Type (30°C Lines)	-0.029	0.026	-1.121	0.306
Line Type (38°C Lines)	0.008	0.026	0.308	0.769
Temperature	-0.084	0.015	-5.628	< 0.001
Temperature * Line Type (30°C Lines)	-0.104	0.035	-2.986	0.003
Temperature * Line Type (38°C Lines)	-0.075	0.036	-2.092	0.038

Table B.4. An LMM modelling changes in female *T. castaneum* elytra length over five generations. Elytra length was the response variable, with generation as an explanatory variable modelled as a linear covariate. The line type was also an explanatory variable, with the baseline set as the stock populations. There was a random effect of line ID (Var = 0.001).

	Estimate	**Standard Error**	**T value**	**Pr(>|t|)**
Intercept	2.295	0.027	86.537	< 0.001
Line Type	- 0.006	0.032	- 0.187	0.861
Generation	0.045	0.005	9.842	< 0.001
Line Type * Generation	0.026	0.009	2.871	0.005

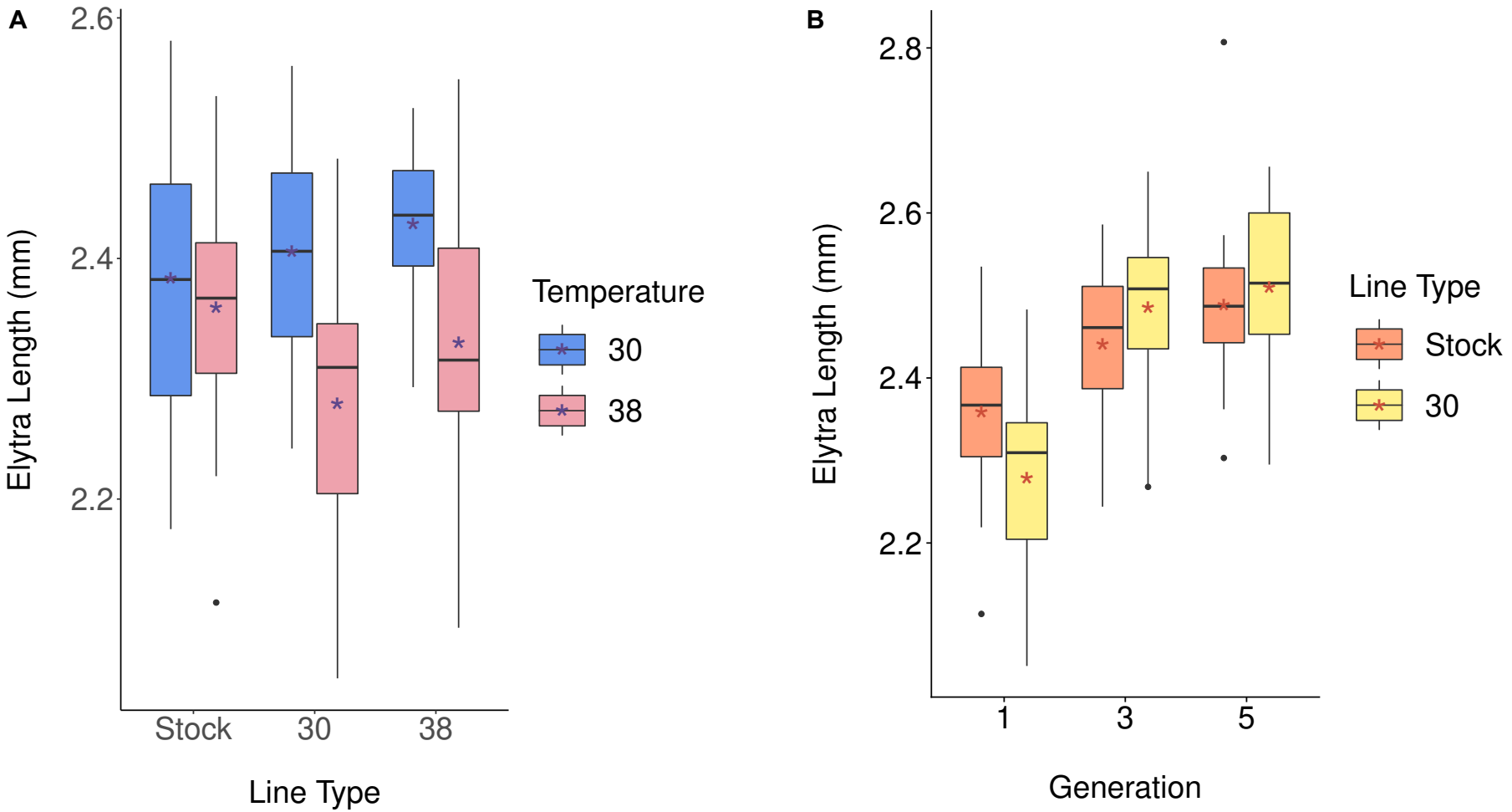

Figure B.2. Elytra length of *T. castaneum* females A. across different treatments in generation 1, and **B.** over five generations of exposure of the tock populations and 30°C thermal lines to high temperature.

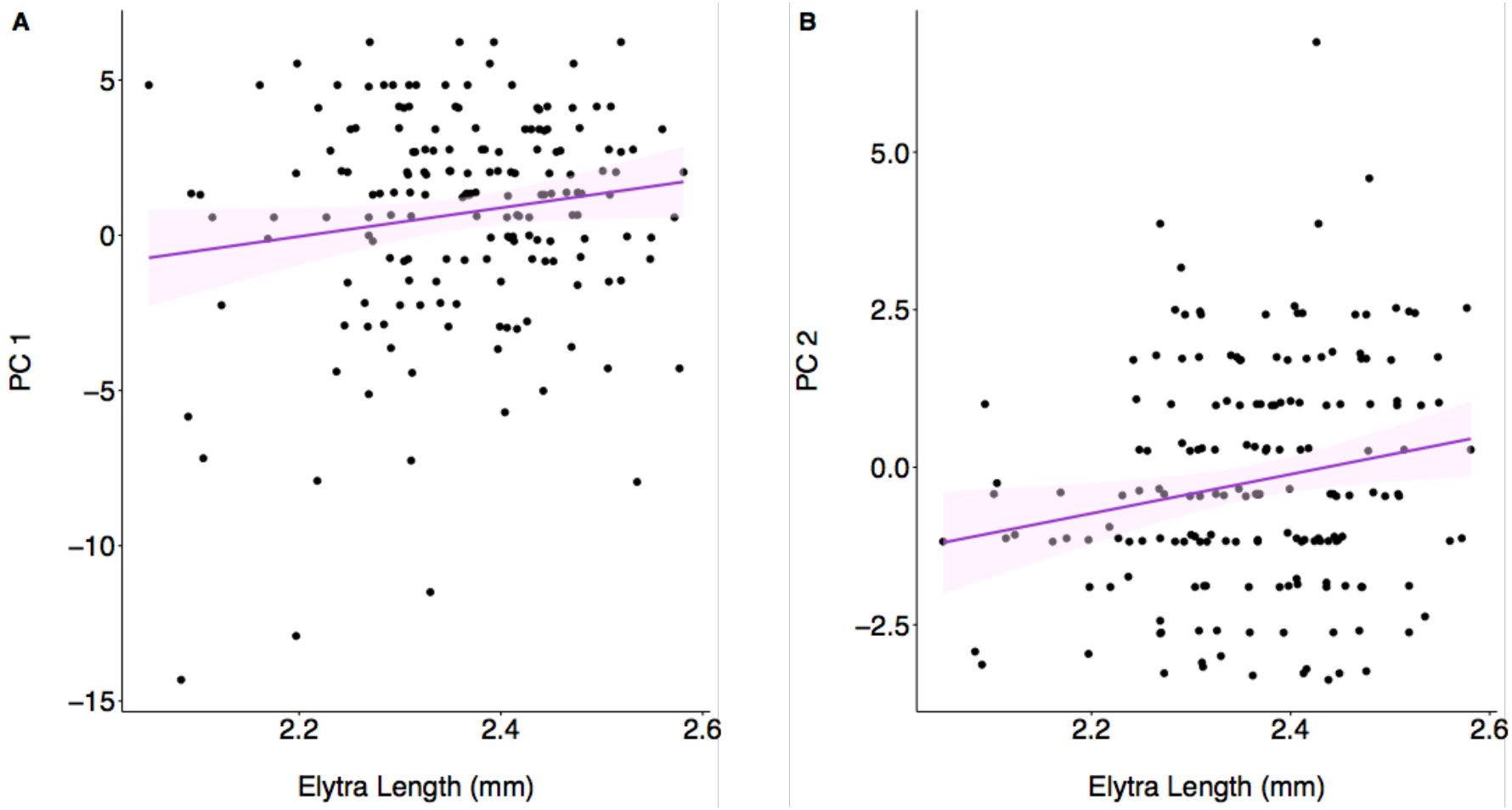

Figure B.3. The correlations between elytra length and ovary morphology in virgin *T. castaneum*. A. Elytra length with PC1 (R = 0.15, p = 0.054) and **B.** Elytra length with PC2 (R = 0.19, p = 0.013) in females from various lines (stocks, 30°C thermal lines and 38°C thermal lines) and reared at different temperatures (30°C and 38°C).

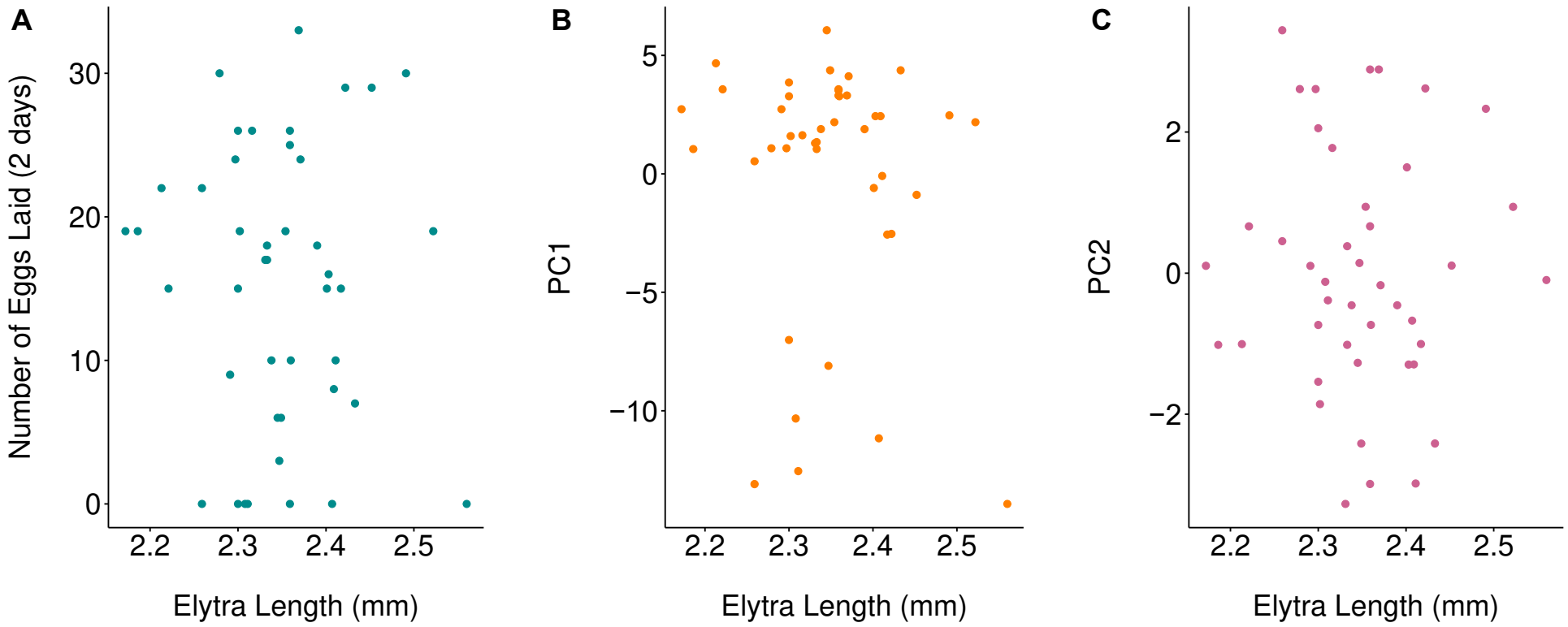

Figure B.4. The correlations between *T. castaneum* elytra length, ovary morphology and fecundity. **A.** number of eggs laid (R = - 0.014, p = 0.93), **B.** PC1 (R = - 0.058, p = 0.71) and **C.** PC2 (R = - 0.066, p = 0.67) in females from the stock population following development at high temperature (38°C)

Appendix C – Ovary Imaging

To trial the protocol for detailed ovary imaging, I collaborated with Professor Andrea Munsterberg and Dr. Johannes Wittig. After dissecting the ovaries, they were soaked in paraformaldehyde solution (PFA) overnight, followed by sucrose, and then were frozen in gelatin. Using a cryostat, the ovaries in gelatin were sliced and each slice placed onto a slide. The gelatin was removed from the slide, using a water bath and phosphate buffer solution (PBS) before the ovary slides were soaked in solution (5g bovine serum albumin, 5ml goat serum and 95ml PBS). The slides were stained with i) 4',6-diamidino-2-phenylindole (DAPI), which binds to A-T rich regions of DNA, and ii) Phalloidin, which binds to actin filaments. Stained ovaries were photographed and images processed using Fiji/ImageJ, and are displayed in Figure C.1.

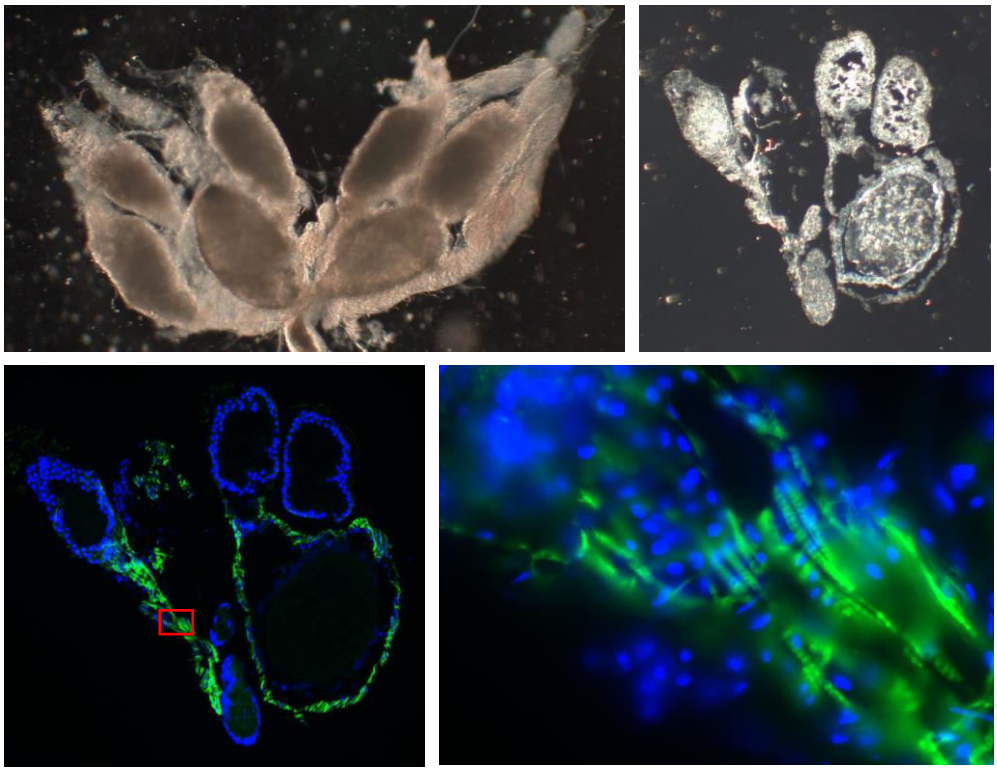

Figure C.1. A trial of *T. castaneum* ovary imaging using cryosection and staining. **A.** The ovary immediately after dissection. **B**. One cross section of the ovary following staining and cryosection. **C**. Fluorescent imaging of the ovary section shown in B. Blue shows DAPI stained regions (nuclei) and green shows Phalloidin staining (actin filaments) **D**. Zoomed in cellular structure of the area shown in the red box in C. Images for indicative purposes only.