Consequences of sexual selection for reproductive and life history traits in *Tribolium castaneum*

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Thesis submitted for the degree of Doctor of Philosophy
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May 2016

In loving memory of Graham G. Godwin 1952-2014

"After all this time?"

"Always"

(Harry Potter and the Deathly Hallows, J. K. Rowling)

Abstract

Sexual selection is a widespread evolutionary force that acts on variation between individuals in reproduction, driving the evolution of traits that increase fertilisation success. Although generally well-studied, there are gaps in our understanding of how sexual selection (1) impacts population-level fitness, and (2) shapes trait divergence at the level of the gamete. Here, I investigate these questions using experimentally evolved populations of the promiscuous flour beetle, *Tribolium castaneum,* which vary only in contrasting strengths of sexual selection across 58+ generations. For population fitness, sexual selection is theorised to act either positively or negatively, depending on its alignment with natural selection, and/or the impact of sexual conflict. I found no evidence for either consistently positive or negative effects of sexual selection history on adult resistance to environmental stresses or intrinsic ageing. However, significant and consistent benefits of strong sexual selection histories were recorded within the ability of individuals to cope with poor nutrition. Furthermore, a history of strong sexual selection improved a population's ability to invade novel, intra-specific competitor populations across multiple generations, demonstrating superior biotic fitness across life history traits. Overall, these results provide evidence for population fitness benefits of sexual selection. At the gamete level, sperm number is known to be a vital predictor of sperm competition success, however, the influence of sexual selection on the huge diversity in sperm morphology remains unclear. I found that strong sexual selection led to the evolution of greater sperm competitiveness in adult males, and this was associated with increasing sperm length divergence, but no effect on length variance. Finally, a study of sex-specific ageing in this promiscuous species revealed that stark sexual dimorphism in reproductive senescence was associated with sexual dimorphism in intrinsic lifespan, supporting ageing theories and suggesting significant differences in the cost of early reproduction for males and females.

Contents

Acknowledgements

Firstly, my sincere thanks go to my primary supervisor Matt Gage for the opportunity to carry out this PhD and more importantly, for the belief he has shown in me, particularly when I have doubted myself. His inexhaustible mental list of potential experiments, scribbled graphs on scraps of paper and good humour have been a pleasure to work with. A huge thank you also to my secondary supervisor Tracey Chapman for her comments and guidance and for being a great role model for women in science.

I could not have completed this PhD without the help of former and current members of Team Tribolium and the Chapman Lab. In particular, Alyson Lumley for 'beetle wrangling' advice and wonderful company during long lab days, Ram Vasudeva for his positivity and humour and the Chapman lab group for invaluable practical advice, helpful critique and statistical knowledge!

It has been an exceptionally difficult 4 years without the small matter of a PhD and I am incredibly grateful to my friends and colleagues for helping me through. Thank you so much to: Janet for therapeutic coffee and cake; to Dani, Amy (and Steve), Aly, and Lucy for highly memorable, laughter filled, 'girls' nights in' involving much pizza and wine and fabulous emotional support; to Phil, Will, Mike and Wayne for awkward hugs and statistical advice; to the 'Cabbage' lab for always being pleased to see me when I randomly appear in the office, and to my non-science friends: Jackie for a long and open friendship and surviving a morning at 30°C and 60% humidity with a raging hangover to help me check beetles, and Suzie for calming walks and her absolute belief in me.

Most importantly my thanks go to my family. Thank you to my sister Sarah for her frank honesty at all times and many remedial glasses of wine. Also, to my Mum for trying her hardest to understand why anyone would want to spend 4 years studying beetles and for her love and support when life was really tough. Finally, I would never have got this far in Science without my Dad. He taught me to be determined and meticulous and to always work hardest at the things I find most difficult. I only wish he were here to read my thesis and to provide his astute questions and constructive comments.

Chapter 1

General Introduction

1.1 Chapter outline

This thesis aims to advance our understanding of sexual selection, a widespread and important evolutionary force that acts on variation between individuals in success within sexual reproduction (Darwin 1859; 1871). Through a series of empirical tests of theoretical ideas, this research seeks to increase understanding of the consequences of sexual selection, at a range of levels from the gamete to the population, and measuring consequences for fitness in a number of ways from fertilisation success, to ageing and death. In particular I make use of experimentally evolved populations of *Tribolium castaneum,* with at least 5 year histories of contrasting strengths of sexual selection, at the commencement of this work.

This chapter first provides an overview of the main theoretical concepts regarding how and why sexual selection operates, to provide general background to inform subsequent chapters where more focused investigations are described (**1.2**). The consequences of sexual selection are also outlined, and chapters in which these are introduced, investigated and discussed in greater detail are highlighted (**1.3**). Having introduced the conceptual background, I then describe the experimental model, *T. castaneum*, including general stock maintenance and practical merits, along with a brief analysis of the opportunity for and strength of sexual selection in this system (**1.4**). I also introduce experimental evolution, its use in studies of sexual selection, the protocol for the experimental evolution of sexual selection here, and a discussion of effective population size and contrasting strengths of selection under each treatment (**1.5**). Finally, general procedures for my approaches with statistical analyses are described (**1.6**).

1.2 Central concepts in sexual selection theory

"Sexual selection…… depends not on a struggle for existence, but on a struggle between the males for possession of females; the result is not death to the unsuccessful competitor, but few or no offspring."

Darwin, 1859

"the advantage which certain individuals have over other individuals of the same sex and species solely in respect of reproduction"

Darwin, 1871

The Darwin-Bateman paradigm

While forming his theory of natural selection, Darwin (1859, 1871) was puzzled by extravagant dimorphisms within species, most often expressed in males, which did not appear to have any survival value. Combined with observations of aggressive rivalry and eager courtship in males, compared to more reluctant behaviour in females, Darwin concluded that these traits and behaviours increased success in competition between rivals or improved the chances of being chosen as a mate, and would therefore persist despite their costs, by ensuring greater numbers of offspring for an individual. He termed this advantage in reproduction, sexual selection (Darwin 1859, 1871; reviewed in Andersson 1994). A series of experiments by Bateman (1948) linked Darwin's ideas about male-male competition and choosey female behaviour to the fundamental difference between males and females in their gamete characteristics. Bateman (1948) showed that i) variance in reproductive success ('the opportunity for selection') is greater in males than females, and ii) male reproductive success increases with number of mates, while female reproductive success is usually maximised with just one, or a few, mates (see section 1.3 for demonstration of the 'Bateman-gradient' in *T. castaneum*). He concluded that this difference in potential meant that sexual selection is more intense in males than females, and suggested that these differences stemmed from anisogamy. Production of vast numbers of tiny sperm causes male potential reproductive success to be high, however, their actual reproductive success is limited by females, who produce fewer, nutrient rich and energetically costly, ova (Bateman 1948). Trivers (1972), consolidated Bateman's ideas about sex differences in gamete production, and suggested that it was the relative 'parental investment', encompassing any action that increased offspring survival, which underlies contrasting potential reproductive rates of males and females. The outcome of this sex difference in parental investment is that males are under stronger selection to compete and gain the greatest quantity of mates, while selection acts on females to increase resources and ensure mates are of the highest quality (Darwin 1871; Bateman 1948; Trivers 1972). The concept of parental investment also explains situations in which the normal sex roles of males and females are reversed. Where males provide greater parental investment, and therefore a higher energetic outlay than females, males will be the choosey sex and females must compete (Trivers 1972), and this situation is borne out (Gwynne 1981). Sexual selection therefore favours a diverse range of signals, including ornaments, weapons, acoustic signals and behaviours, which increase success in intra-sexual competition and inform inter-sexual choice (reviewed in Andersson 1994). The combination of ideas regarding sex differences in reproductive potential and success, gamete investment and mating strategies, is known as the 'Darwin-Bateman paradigm' (Dewsbury 2005), and is the central concept of sexual selection theory.

Post-copulatory sexual selection

The most important addition to this central concept came from the realisation that females often mate with multiple males (polyandry), creating the potential for sperm from rival males to overlap in the female reproductive tract, therefore generating conditions of competition and choice that continue beyond mating for fertilisations (Parker 1970). Sperm competition (Parker 1970) and cryptic female choice (Eberhard 1996) therefore also drive the evolution of peri- and post-copulatory courtship behaviours, genitalic and spermatozoal morphology, and a diverse set of non-sperm ejaculate components which increase male fertilisation success, or allow females to bias paternity towards favoured males (reviewed in Eberhard 2009; Simmons & Fitzpatrick 2012; Rowe *et al.* 2015). However, increased investment in peri- and post-copulatory traits and behaviours, as a result of polyandry, is predicted to trade against male ability to invest in obtaining future mates and fertilisations, and also reduce the reproductive success gained by males at each mating (Parker & Birkhead 2013). These factors decrease the slope of the 'Bateman gradient', and thus reduce the strength of sexual selection acting on males (Parker & Birkhead 2013; Shuster *et al.* 2013). Furthermore, growing empirical evidence reveals both direct and indirect benefits of polyandry for female reproductive success (reviewed in Kvarnemo & Simmons 2013) which could increase the slope of the association between number of mates and offspring production, and also increase the variance in female reproductive success (Kvarnemo & Simmons 2013**;** Shuster *et al.* 2013). However, overall polyandry is predicted to increase competition between males and importantly, shift the emphasis to post-copulatory processes (Parker & Birkhead 2013; Shuster *et al.* 2013). Sexual selection therefore acts as a result of, and upon, variation in reproductive success, via the mechanisms of intra-sexual competition and inter-sexual choice, at both pre- and post-copulatory stages of reproduction.

Post-copulatory sperm competition and cryptic female choice are now not only thought to result in divergence of reproductive traits, but critically to maintain anisogamy (Parker 1982). Increasing sperm size, which could benefit zygote provisioning, is predicted to trade-off against the number of sperm produced, however, theoretical (Parker 1982) and empirical evidence (reviewed in Wedell *et al.* 2002) shows that high numbers of sperm are a vital component of competition success. The extreme small size of sperm means that even a doubling in size is likely to have a trivial advantage, compared to the consequences of halving sperm number for competition. Therefore, the cost, in terms of loss of fertilisations in the face of competition, outweighs the benefit of increased chances of offspring survival, so selection favours numerous, tiny sperm (Parker 1982). Following the evolution of anisogamy, sperm competition is theorised to drive the subsequent ‘sexual cascade’, which culminates in distinct sex roles and pre-copulatory ‘Darwinian’ sexual selection (Parker 2014; Parker & Pizzari 2015). Parker (2014) reasoned that sperm competition is the ancestral form of sexual selection acting on early multicellular organisms and external fertilisers. The evolution of mobility enabled males to search for females and target sperm release, the most extreme example of this being internal fertilisation, in order to reduce sperm competition risk and thus increase reproductive success (Parker 2014; Parker & Pizzari 2015). Decreased sperm competition risk enabled a reduction in investment in gonads and ejaculates, and therefore freed resources to be invested in pre-copulatory traits which culminated in distinct sex roles, the consequences of which are apparent in characteristics of the Darwin-Bateman paradigm (Parker 2014; Parker & Pizzari 2015).

Sexual conflict

A further, significant, consequence of anisogamy, and contrasting selection pressures on males and females, is that traits which benefit the reproductive success of one sex, may be costly to the other, and divergent trait optima often cannot be achieved simultaneously (Parker 1979). Parker (1979) used the term ‘sexual conflict’ where there are differences in the evolutionary interests of males and females. Either one sex must ‘lose’ the conflict, or both must compromise and operate away from their optima (Parker 1979; 2006). Conflict may occur either over the outcome of male-female interactions (inter-locus conflict), or where there are sex-specific optima for trait expression levels controlled by a single locus in both sexes (intra-locus conflict) (Chapman *et al.* 2003). Sexual conflict may arise over any reproductive process, from courtship to parental investment, and bring about evolutionary change in associated traits (Chapman 2006). Mating is a significant source of inter-locus sexual conflict, as male fitness increases with number of mates, whilst female fitness can be maximised with one or a few copulations (Bateman 1948; Parker 1979; Lessells 2006). This situation has led to many examples of sexually antagonistic coevolution of traits in males which aim to control the mating rate of females, versus traits in females which resist male manipulation (reviewed in Arnqvist & Rowe 2005). Sexually antagonistic selection results in a reduction in the relative fitness of one of both sexes known as 'conflict load' (Lessells 2006), leading to an evolutionary tug of war between manipulation and resistance, the outcome of which will depend on which sex is further from their optima and therefore has more to gain (Parker 2006). Sexual conflict is therefore a fundamental aspect of sexual selection, and it alters the relative costs and benefits of sexual selection for individuals and, ultimately, populations.

Summary

Anisogamy underpins the fundamental difference between males and females, and creates contrasting, sometimes conflicting, evolutionary pressures within either sex that drive sexual selection. A high potential rate of reproduction in males, due to the production of large numbers of small sperm, is primarily limited by access to females and their ova. Selection therefore favours male investment in effective competition with rivals to maximise mating and fertilisation success. Conversely, investment in nutrient rich, and thus energetically costly, ova generates selection on females to gain resources and choose the highest quality males for fertilisation. Sexual selection therefore acts both because of, and on, variation in reproductive success. Mechanisms of intra-sexual competition and inter-sexual choice, at both pre- and post-copulatory stages of reproduction, contribute to trait divergence and their consequences for reproductive fitness through each life cycle.

1.3 Consequences of sexual selection and thesis outline

Using the central concepts of sexual selection theory, regarding how and why this evolutionary force operates, this thesis aims to further our understanding of the consequences of sexual selection for gametes, individual reproductive and life history traits, and their impacts upon population fitness. These consequences, along with gaps in understanding and unresolved questions which form the motivation for this thesis, are summarised below and links to subsequent chapters are given.

Consequences for secondary reproductive traits

> *"That these characters are the result of sexual and not of ordinary selection is clear, as unarmed, unornamented, or unattractive males would succeed equally well in the battle for life and in leaving numerous progeny, if better endowed males were not present."*
>
> Darwin 1871

Without sexual selection, the world would be a far less colourful, noisy and complex place (Gage 2012). The primary outcome of sexual selection, and the stimulus for Darwin's secondary theory of evolution, is a diverse range of secondary sexual traits that increase success in intra-sexual competition and inform inter-sexual choice (Darwin 1859, 1871). Following the insight that polyandry facilitates ongoing competition and choice beyond copulation (Parker 1970), these traits are often divided into those that aid pre-copulatory mating success, and peri-/post-copulatory traits which increase the probability of sperm being used for fertilisation over those of a rival. Pre-copulatory secondary sexual traits include weapons, ornaments, colouration, acoustic signals, chemical signals, size dimorphism and mating tactics, many of which are elaborate, conspicuous and familiar morphologies and behaviours (reviewed in Andersson 1994). Post-copulatory competition will favour traits which either aid males where females have already mated to displace and outcompete rival sperm, including sperm number and morphology, or reduce the risk of subsequent inseminations through traits such as mating plugs, mate guarding and a number of non-sperm ejaculate components which manipulate female mating rate (Parker 1970; reviewed in Eberhard 2009). Adaptations which enable cryptic female choice to occur include morphology of sperm storage organs, and molecular compatibility of sperm with the egg and/or ovarian fluid (reviewed in Eberhard 2009).

How does sexual selection shape trait divergence at the level of the gamete?

The evolution of secondary sexual traits at the level of the individual has been the focus of sexual selection research and is well established. However, although sexual selection is also predicted to influence the evolution of gamete traits, our understanding of the pattern of selection, and adaptive significance of, these traits remains unclear. The consequences of sexual selection, in particular sperm competition, for the evolution of sperm traits are introduced and discussed in detail in **chapter 4.** Theoretical (Parker 1982) and empirical studies (reviewed in Wedell *et al.* 2002) suggest sperm number to be a vital component of sperm competition success, with high sperm competition risk predicted to drive the evolution of increased investment in sperm number. However, a number of qualitative aspects of sperm form, including length, longevity and mobility, could also influence the outcome of competition, and far less is understood about how these traits respond to sexual selection (Snook 2005). The role of sperm length in competition success is discussed, along with patterns of selection, directional or stabilising, created by conditions of high sperm competition.

Sexual selection and speciation

In addition to increasing mating and fertilisation success, divergences in secondary sexual traits are implicated in the evolution of reproductive isolation and subsequent speciation. Secondary sexual traits play a vital role in mate/species recognition, and often show the greatest divergence of any traits among closely related species which has caused sexual selection (Lande 1981; West-Eberhard 1983), and sexual conflict (Rice 1998; Gavrilets 2000) to be theorised as significant drivers of speciation. Divergence in secondary sexual traits is predicted to either independently, or in association with ecological divergence, aid the formation of reproductive barriers (reviewed in Panhuis *et al.* 2001; Ritchie 2007). Evidence of reproductive isolation is also looked for in **chapter 4**. However, the time-scale over which speciation is predicted to occur makes experimental investigation of this process impractical and often inconclusive (Kawecki *et al.* 2012). The extent of the roles of sexual selection and conflict in speciation are current, and significant areas of evolutionary research.

Consequences for life history traits and population fitness

> *"sexual selection will give its aid to ordinary selection, by assuring to the most vigorous and best adapted males the greatest number of offspring"*
>
> Darwin, 1859

Sexually selected signals are energetically costly to develop and maintain and, in addition, can have negative impacts on survival (reviewed in Andersson 1994). It has also been predicted that extreme ornamentation could drive populations over an 'extinction threshold' (Lande 1980), an often considered example of which is the extinct Irish elk, in which males had hugely developed antler ornamentation (Gould, 1974; discussed in Kokko & Brooks 2003). Where resources are allocated to one trait, they must become unavailable for others (Stearns 1992), therefore investment in mating and fertilisation success is predicted to trade against other components of fitness investment, and sexual selection is often viewed as working in opposition to natural selection (Lande 1980; Kirkpatrick & Ryan 1991; Houle & Kondrashov 2001). However, mating and fertilisation success are not simply determined by secondary sexual traits, but also by a broad range of underlying physiology and behaviour, combined with the ability to develop and maintain costly secondary sexual traits (Andersson 1994; Whitlock & Agrawal 2009). Therefore, mating and fertilisation success are predicted to be ultimately dependent on condition, and consequently determined by a wide range of naturally selected loci across the genome (Rowe & Houle 1996). Condition-dependence of sexually selected traits could lead to alignment between these evolutionary forces and, as a result, sexual selection may benefit a wider range of life history and non-reproductive traits, and not simply those closely related to mating and fertilisation success (Rowe & Houle 1996; Lorch *et al.* 2003). Furthermore, as a result of heightened selection from sexual selection for optimal condition, sexual competition and choice within populations are predicted to increase the purging of deleterious mutations, and aid the fixation of beneficial alleles (Whitlock 2000; Whitlock & Agrawal 2009). This could generate significant population fitness benefits, especially where alleles exist at loci with individually small fitness effects, which could sum to a significant load effect. Natural selection may struggle to shape individual loci with small effects, but sexual selection could be more sensitive, and therefore more effective in its actions on genetic load (Agrawal & Whitlock 2009). Empirical evidence regarding whether sexual selection has a net positive or negative influence on population fitness remains limited and inconclusive. Therefore, this remains a notable gap in our understanding of this widespread and important evolutionary force, with potentially significant implications for population viability.

Does sexual selection act mainly on reproduction or also on survival?

The consequences of sexual selection for a wider range of life history traits and population fitness are introduced, tested and discussed in detail in **chapters 2 and 3.** Intrinsic ageing, environmental stress resistance, and population invasion ability are used as phenotypic indicators of population fitness, and compared between populations that have experimentally evolved under weak versus strong sexual selection. Ageing is theorised to be a result of mutations with deleterious effects in later-life (Medawar 1952; Williams 1957), therefore, if sexual selection aids the purging of genetic load, deleterious alleles which would otherwise accumulate or persist could be removed, thus reducing ageing and increasing lifespan. In addition, if sexual selection creates populations containing individuals with enhanced condition and reduced mutation load, this is predicted to result in greater resilience to environmental stress (Lorch *et al.* 2003; Agrawal & Whitlock 2012). Finally, if sexual selection benefits a wide range of life history traits, and not simply those closely linked to mating and fertilisation, I predict an improved ability to invade and introgress a novel competitor population across multiple generations, because invasion success under these circumstances requires improvement across a range of fitness components in the face of demands from both biotic and abiotic challenges.

Is sexual dimorphism in ageing explained by sex-specific investment in reproduction?

In addition to questions regarding the influence of sexual selection on intrinsic ageing and lifespan, this thesis examines sex-specific differences in ageing, in relation to the dynamics of male and female fertility and reproductive potential as either sex gets older in *Tribolium castaneum.* Differential costs in gamete and reproductive investment could lead to sex-specific trade-offs between reproduction and survival (Stearns 1992; Bonduriansky *et al.* 2008). Intra-sexual competition is theorised to increase the level of extrinsic ageing in males, leading to reduced longevity compared to females in many species (reviewed in Bonduriansky *et al.* 2008). However, fewer studies have measured differences in intrinsic ageing between the sexes in relation to reproductive output through time, or considered species where female longevity is reduced compared to males as a result of reproductive investment. In **chapter 5,** sex-specific reproductive and intrinsic ageing are described and discussed with reference to the contrasting reproductive strategies of males and females within the promiscuous mating system of *T. castaneum*.

Chapter contributions

This thesis was funded by the University of East Anglia under the supervision of Professor Matt Gage (principal investigator) and Professor Tracey Chapman (secondary supervisor). Data demonstrating the Bateman gradient in chapter 1 were collected by A.J. Lumley and re-analysed by the author. All experiments in chapters 2 to 5 were performed by the author with contributions to population maintenance for chapter 3 from R. Vasudeva and K. Sales, and data collection in chapter 4 from M.J.G. Gage.

1.4 The model, *Tribolium castaneum*

The red flour beetle, *Tribolium castaneum,* is a globally pervasive and economically significant pest of grain and dried food stores. Precise knowledge of the geographic origin and ancestral habitat of *T. castaneum* is lacking as *Tribolium sp.* have been associated with human food stores since early agricultural development (Sokoloff 1972; Dawson 1977). It is therefore reasonable to assume that this is now their natural ecology, and that *Tribolium castaneum* has been shaped by this food storage environment for 10,000 years. Anthropogenic movement has been assumed as the cause of their global distribution, although recent evidence suggests adult flight also contributes significantly to active dispersal (Ridley *et al.* 2011). High fecundity, rapid larval development, and fast maturation make them excellent colonisers (Dawson 1977; Fedina & Lewis 2008) and these traits, along with adaptations to arid environments and a natural history as scavengers of decaying plant material (Good 1936), may have pre-adapted *Tribolium sp.* to take advantage of human stored products (Dawson 1977).

T. castaneum has been used in biological research for almost a century (Brown *et al.* 2009). A short lifecycle, high fecundity and ease of rearing and manipulation make them an excellent laboratory model (Sokoloff 1972). The development of sophisticated genetic and genomic tools, including the *Tribolium* genome (Richards *et al.* 2008), has increasingly made *T. castaneum* a rival to *Drosophila sp.* for understanding the evolution and genetic control of development (reviewed in Brown *et al.* 2003 and Denell 2008). *Tribolium sp.* have also been extensively used as a research model in population dynamics (reviewed in Sokoloff 1974), and host-parasite co-evolution and speciation, while their negative agricultural and economic impact has driven applied research into pest management and pesticide resistance (reviewed in Brown *et al*. 2009).

Tribolium sp. are also frequently used in studies of sexual selection (Fedina & Lewis 2008; Zuk *et al.* 2014) as they are highly promiscuous (Sokoloff 1972; Lumley *et al.* 2015) , and evidence for post-copulatory sperm competition and cryptic female choice is plentiful (reviewed in Fedina & Lewis, 2008). Recent evidence suggests pre-copulatory processes also guide post-copulatory mechanisms, with male paternity share being higher in the presence of a rival, compared to when competition is limited to sperm competition (Sbilordo & Martin 2014). Females are thought to use selective sperm storage, spermathecal morphology and re-mating receptivity to bias paternity, while ejaculate size, seminal fluid proteins and manipulation of rival ejaculates may enable males to increase their paternity share (reviewed in Fedina & Lewis 2008).

Beetle stocks and culture

Populations of *Tribolium castaneum* have been maintained at UEA since 2004. Stocks originated from the Richard Beeman Lab (US Department of Agriculture, Agricultural Research Service) and were maintained at 500-1000 individuals. Beetles were reared on *ad libitum* standard medium containing 90% organic plain white flour, 10% powdered brewer's yeast and a thin layer of organic oats to aid locomotion of the adults. Temperature was controlled at 30°C, with 60% relative humidity. Under these conditions *T. castaneum* follows the following life cycle: egg (~3 days), larva (~20 days), pupa (~4 days), juvenile imago (~5 days) and sexually mature imago (1-2 years).

The commonly used 'wild type' strain Georgia 1 (GA1) and a mutant strain with distinctive swollen antennae, 'Reindeer' (Rd), were used throughout this PhD project. The Rd mutation is dominant and homozygous within the Rd population, therefore all offspring sired by a Rd male have swollen antennae, while offspring of the focal males have 'wild-type', filiform antennae (Figure 1.1).

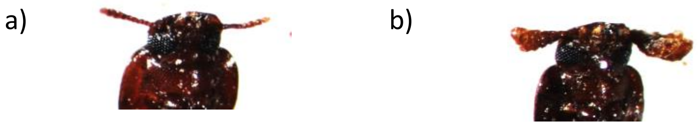


Figure 1.1 | Head and antennae of *Tribolium castaneum* adults. a) Georgia 1 (GA1) 'wild-type' filiform antennae and b) Reindeer (Rd) swollen antennae.

Opportunity for sexual selection and the Bateman gradient in *T. castaneum*

Sexual selection acts on variation in reproductive success (Darwin 1871). Measures of the strength of sexual selection are calculated from a standardised measure of variance in reproductive success, known as the 'opportunity for selection' (I), which is calculated for each sex as (Shuster & Wade 2003):

$$I = \frac{variance\ in\ total\ reproductive\ success}{(mean\ total\ reproductive\ success)^2}$$

The greater the variance in fitness the stronger the force of selection (Shuster & Wade 2003). Bateman (1948) was the first to empirically show that variance in reproductive success is greater in males than females. In addition, he revealed that male reproductive success increases with number of mates, while female reproductive success does not change after the first one or few mates, the 'Bateman gradient' (Bateman 1948; Dewsbury 2005). These pieces of evidence led Bateman (1948) to conclude that males produce more sperm than there are eggs available to fertilise, and therefore selection will drive males to compete for fertilisation opportunities while females will be under selection to gain resources and high quality mates. In order to inform understanding of the strength of sexual selection*,* data were collected to apply Bateman's work in *T. castaneum*.

Protocol

To measure Bateman gradients, all adults were virgin and 10-12 days post-eclosion. Males were isolated 3 days prior to the experiment to prevent confounds introduced by homosexual mounting. Focal males (n = 20) were presented with a single female every 30 min for a total of 20 females. Following mating opportunity bouts, females were transferred to a petri dish of fresh medium and left to oviposit for 10 days before being removed. Focal females (n = 5 per treatment) were presented with treatments of between 1 and 20 males (20 treatments in total, n = 100 total females). In parallel to the male-focused condition, single males were presented sequentially to females every 30 minutes. Following presentation of the final male of the treatment, females were transferred to a petri dish of fresh medium and left to oviposit for 10 days before being removed. Although females can produce offspring for much longer than 10 days, output over this period correlates well (r = 0.31) with maximum offspring production following a single mating bout and accounts for ~25% of the potential total offspring production from such a mating period (Gage & Dickinson unpubl. data). All petri dishes containing eggs/larvae were left to develop into adults and counted.

Statistical analysis

The opportunity for selection (I), was calculated using total offspring production from 20 mates in both sexes (males; n = 20, females; n = 5). To compare offspring production with increasing number of mates between males and females, a non-linear regression model was constructed with a 3-parameter asymptotic exponential function and separate estimates of variance to account for unequal sample size.

Results

The opportunity for sexual selection, was much higher in males (0.39) than in females (0.07). Furthermore, the estimates of the non-linear regression model reveal important differences in the rate of increase in offspring production with number of mates between males and females. Rate does not differ significantly from zero for females (t = -1.65, p = 0.10), but does differ significantly in males (t = -5.80, $p < 0.001$) (Figure 1.2).

Discussion

This data demonstrates the huge divergence in potential reproductive rates between males and females and consequently the high intensity of sexual selection operating in this system. Greater variance in the reproductive success of males, and a steeper gradient for the association between offspring production and number of mates (Figure 1.2), indicates that the strength of sexual selection is stronger on males than females in *T. castaneum*. These data were collected in the absence of competition and choice, which can obviously alter the benefit of increasing mate number for both sexes (Parker & Birkhead 2013; Shuster *et al.* 2013). Future work could assess the impact of rivals on male reproductive success with increasing number of mates. Likewise, the impact of female choice should be assessed which could be achieved by presenting males in groups of increasing size rather than sequentially.

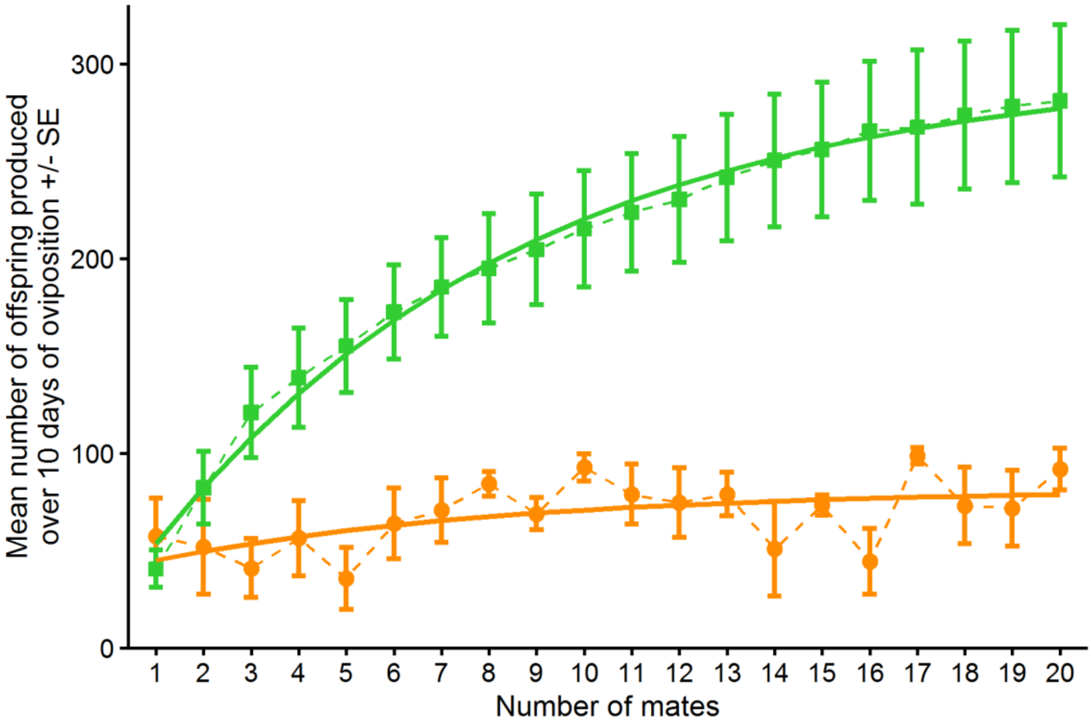


Figure 1.2 | Evidence of the 'Bateman gradient' in *Tribolium castaneum.* Male (green squares, n = 20) reproductive success increases with number of mates where female (orange circles, n = 5 per treatment) reproductive success is maximised with one or a few mates. Raw data (dashed lines) overlaid with non-linear regression model fit (solid lines) (Lumley, Gage & Godwin unpubl. data).

1.5 Experimental evolution of sexual selection

Experimental evolution

Evolutionary biology aims to explain the functional diversity and adaptive significance of biological traits. Much empirical evidence comes from the comparative approach, which identifies patterns in morphology, physiology, behaviour, genetics or development, across taxa, and provides information about selective forces during the evolutionary past of groups under comparison (Harvey & Purvis 1991). Mathematical modelling has also advanced evolutionary understanding by providing tests of the logic of theoretical ideas and assumptions (Gavrilets 2004; Servedio *et al.* 2014). More recently, experimental evolution, in which the investigator directly manipulates traits or selective pressures in experimental populations under controlled conditions while they pass through multiple generations, has become widespread in evolutionary biology (Garland & Rose 2009; Kawecki *et al.* 2012). This approach allows evolutionary processes to be observed and measured as they are happening, and at a micro- rather than macro-evolutionary scale (Bennett & Lenski 1999; Kawecki *et al.* 2012). In addition, the ability to replicate treatments to investigate whether an outcome is consistent, and to provide a control as a direct comparison with the experimental treatment, allows rigorous testing of evolutionary hypotheses (Futuyma & Bennett 2009). Experimental evolution is often divided into two approaches: artificial selection, in which breeding individuals are chosen based on a particular trait; and laboratory natural selection, where some aspect of the abiotic or biotic environment is consistently manipulated over successive generations (Bennett & Lenski 1999; Garland and Rose 2009). Laboratory natural selection acts on the whole phenotype, and therefore provides the most realistic response to the imposed selection pressure (Futuyma & Bennett 2009). ‘Experimental evolution’ is most often used to refer to ‘laboratory natural selection’ (Bennett & Lenski 1999; Snook *et al.* 2009) and was the approach I have used throughout much of this thesis.

Experimental evolution has been used extensively in studies investigating the primary target of sexual selection. The evolution of traits and behaviours which increase mating and fertilisation success have been studied, including: pre-copulatory courtship displays (Snook *et al.* 2005; Crudgington *et al.* 2010); ornaments (Snook *et al.* 2013); mate recognition (Chenoweth *et al.* 2008); mating behaviour (Bacigalupe *et al.* 2008; Michalczyk *et al.* 2011). In addition, traits associated with post-copulatory sexual selection have been studied such as: reproductive physiology (Hosken & Ward 2001; Reuter *et al.* 2008; Simmons & Garcia-Gonzaez 2008); genital morphology (Cayetano *et al.* 2011; Gay *et al.* 2011); sperm and ejaculate traits (Pitnick *et al.* 2001; Linklater *et al.* 2007; Firman & Simmons 2010). Experimental evolution has also uncovered sexual conflict and antagonistic coevolution of traits and behaviours, including: male harm and female resistance (Holland & Rice 1999; Wigby & Chapman 2004; Crudgington *et al.* 2010); physiological dimorphism (Bedhomme *et al.* 2011); female fecundity (Tilszer *et al.* 2006; Nandy *et al.* 2014); and both inter-locus (Stewart *et al.* 2005), and intra-locus conflict (Morrow *et al*. 2008; Gay *et al.* 2011). In addition, experimental evolution has provided empirical evidence regarding the role of sexual selection in a wider range of evolutionary questions and processes including: reproductive isolation and speciation (Martin & Hosken 2003; Wigby & Chapman 2006); immunity (McKean & Nunney 2008; Hangartner *et al.* 2015); ageing (Maklakov *et al.* 2007; Maklakov *et al.* 2009); adaptation (Fricke & Arnqvist 2007); and population viability (Rundle *et al.* 2006; Hollis *et al.* 2009; Lumley *et al.* 2015).

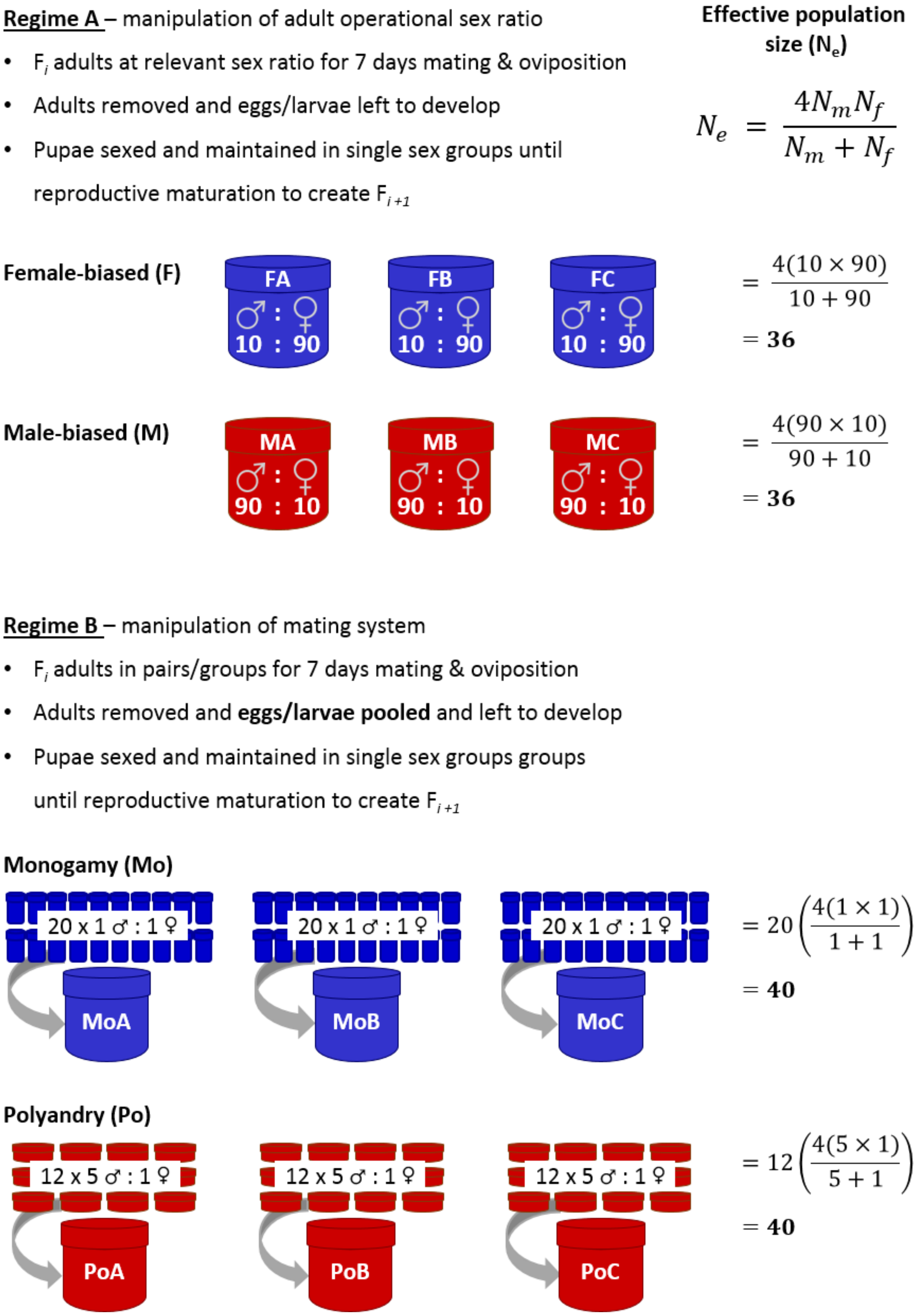
Comparison of enforced monogamy with control or promiscuous populations is a common methodology for investigating sexual selection and conflict (Holland & Rice 1999; Hosken *et al.* 2001; Pitnick *et al.* 2001b; Fricke & Arnqvist 2007; Hollis *et al.* 2014). Enforced monogamy not only removes both pre- and post-copulatory competition and choice, but also ensures the fitness of each sex relies on the other and thus removes the cause of sexual conflict (Holland & Rice 1999). Alternatively, manipulation of the operational sex ratio (OSR) can create contrasting levels of male-male competition and opportunity for female choice (Wigby & Chapman 2004; Reuter *et al.* 2008; Michalcyzk *et al.* 2011; Lumley *et al.* 2015). Population density is expected to influence mating systems (discussed in Kokko & Rankin 2006) and has also been used to manipulate the frequency of sexual interactions in experimentally evolved populations (Martin & Hosken 2003, 2004), although such approaches can be confounded by ecological density-dependent effects.

Sexual selection regime protocol

All experiments described in this thesis were carried out after 58+ generations of experimental evolution of contrasting strengths of sexual selection. Experimental evolution regimes were set up in 2005 by M. Gage, O. Martin and L. Michalczyk to compare contrasting strengths of sexual selection within populations.

Regime A makes use of divergent operational sex ratios to create a female-biased (10 males to 90 females) 'weak' sexual selection treatment in which there is little competition between males and little choice for females. This regime contrasts with the male-biased treatment (90 males to 10 females) which creates a 'strong' sexual selection force where males must compete repeatedly for mating and fertilisation with the limited number of females, who have greater opportunity to exert choice over mating and paternity of their offspring. **Regime B** complements this by contrasting enforced monogamy (1 male to 1 female) with polyandry (5 males to 1 female) treatments to create divergent sexual selection forces. Genetic drift will cause populations to diverge over time, therefore experimental treatments require independent population level replicates to show that population divergences are greater between than within treatments (Kawecki *et al.* 2012). Three independent replicate populations of all treatments are maintained.

To create the regimes, GA1 stock beetles were randomly allocated to treatments. For each population, at every generation, pupae are sexed to ensure virginity and to manipulate the sex ratio. Adults are placed in fresh medium for 7 days for mating and oviposition. Adults are then removed and eggs/larvae left to develop. To ensure equal effective population sizes in regime B, at each generation 20 monogamous pairs and 12 polyandrous groups are set up and, once mating has taken place in the adult stage, the eggs/larvae from these are pooled to form a single population (summarised in Figure 1.3). Inconsistent effective population size (N_e) between treatments could lead to unequal genetic drift, loss of genetic variation, mutation accumulation and genetic hitchhiking, which could confound treatment effects (Wigby & Chapman 2004; Snook *et al* 2009). The calculation and impact of effective population size are discussed further below. The shared genetic background of these populations, and subsequent evolution under standardised conditions, provides a powerful tool to investigate the impact of sexual selection on traits and behaviours.



Regime A – manipulation of adult operational sex ratio

- F_i adults at relevant sex ratio for 7 days mating & oviposition
- Adults removed and eggs/larvae left to develop
- Pupae sexed and maintained in single sex groups until reproductive maturation to create F_{i+1}

Effective population size (N_e)

$$N_e = \frac{4N_mN_f}{N_m + N_f}$$

Female-biased (F)

$$= \frac{4(10 \times 90)}{10 + 90} = \mathbf{36}$$

Male-biased (M)

$$= \frac{4(90 \times 10)}{90 + 10} = \mathbf{36}$$

Regime B – manipulation of mating system

- F_i adults in pairs/groups for 7 days mating & oviposition
- Adults removed and **eggs/larvae pooled** and left to develop
- Pupae sexed and maintained in single sex groups groups until reproductive maturation to create F_{i+1}

Monogamy (Mo)

$$= 20\left(\frac{4(1 \times 1)}{1 + 1}\right) = \mathbf{40}$$

Polyandry (Po)

$$= 12\left(\frac{4(5 \times 1)}{5 + 1}\right) = \mathbf{40}$$

Figure 1.3 | Experimental evolution of the strength of sexual selection protocols for regimes A and B. Contrasting intensities of weak (blue) versus strong (red) sexual selection were imposed upon each generation of adult reproduction, while equalising effective population size within a regime, and allowing genetic mixing of juveniles within the independent lines.

Effective population size

An important consideration for experimental evolution regimes is the effective population size (N_e) which is the size of an 'ideal' population, with random mating, no selection, equal sex ratio and constant adult population size, that would lose genetic variation at the same rate as the actual population (Wright 1931). Smaller populations suffer increased effects of genetic drift which can lead to loss of genetic variation, mutation accumulation and genetic hitchhiking (Charlesworth & Charlesworth 2010), thus limiting the response to selection and significantly affecting the rate of evolution (reviewed in Lanfear *et al.* 2014). Inconsistent N_e between experimental evolution treatments could therefore influence the response to selection and confound observed effects, for example by reducing individual reproductive fitness due to inbreeding, rather than a period of enforced monogamy (Wigby & Chapman 2004; Snook *et al.* 2009). Experimental evolution regimes A and B were designed to ensure consistent theoretical N_e in contrasting sexual selection treatments (Michalczyk *et al.* 2011; Lumley *et al.* 2015). Number of effective breeders, which takes into account a sex ratio which deviates from 1: 1, was used to calculate theoretical estimates of N_e using equation (1) (Wright 1931; Falconer & Mackay 1996), where N_f is number of breeding females and N_m is number of breeding males:

$$N_e = 4\, N_m\, N_f\ /\ N_m + N_f \tag{1}$$

This calculation assumes random mating, however, a fundamental outcome of sexual selection is that some individuals are favoured over others and gain greater reproductive success (Andersson 1994), causing a skew which could therefore influence the effective population size (Wright 1931). In *T. castaneum* the last male to mate will gain the greatest paternity share (last male precedence), which can be incorporated into N_e estimates using equation (2) (Rice & Holland 2005), where P_i^2 is the proportion of offspring is sired by male *i*:

$$N_e = 4\, N_f\, N_f\ /\ N_f\ \sum_1^i P_i^2 +\ N_f \tag{2}$$

Estimates of last male precedence are variable and sometimes high (> 0.6) in *T. castaneum* (reviewed in Fedina & Lewis 2008). A value of 0.87, estimated in original studies carried out by Schlager (1960) was incorporated into the calculation of N_e for male-biased and polyandrous populations in order to assess the impact of high male-male competition (Table 1.1). This method lowers the estimate of N_e in strong sexual selection populations which, therefore, might be expected to experience negative fitness effectives of genetic drift and inbreeding (Snook *et* *al.* 2009). If this is the case, observed fitness benefits of strong sexual selection are likely to be conservative if the effects are being dampened by drift and inbreeding.

Table 1.1 | Estimates of effective population size (N_e) for contrasting sexual selection treatments in experimental evolution regimes A and B.

	No. effective breeders: (Wright 1931) $\boldsymbol{N_e = 4\, N_m\, N_f\, /\, N_m + N_f}$	**Last male sperm precedence:** (Rice & Holland 2005) $\boldsymbol{N_e = 4\, N_f\, N_f\, /\, N_f \sum_{1}^{i} P_i^2 + N_f}$
Female-biased	$\frac{4\,(10\ \times 90)}{10+90} = \mathbf{36}$	-
Male-biased	$\frac{4\,(90\ \times 10)}{90+10} = \mathbf{36}$	$\frac{4\,(10\ \times 10)}{10\,(0.87^2+0.13^2)+10} = \mathbf{23}$
Monogamy	$20 \times \left(\frac{4\,(1\ \times 1)}{1+1}\right) = \mathbf{40}$	-
Polyandry	$12 \times \left(\frac{4\,(5\ \times 1)}{5+1}\right) = \mathbf{40}$	$12 \times \left(\frac{4\,(1\ \times 1)}{1\,(0.87^2+0.13^2)+1}\right) = \mathbf{27}$

However, in our experimental evolution populations, last male sperm precedence is unlikely to influence estimates of effective population size, because *T. castaneum* is highly promiscuous and re-mates continuously (Michalczyk *et al.* 2011 and Figure 1.2). Therefore, although the last male to mate may achieve high precedence, where females are free to re-mate, this precedence will quickly be usurped by the next male to mate. Thus, the estimate of N_e in our system is more likely to be dependent on the numbers of males that mate and achieve some fertilisations which, given the promiscuous mating pattern, is likely to be almost all males within each adult population. Furthermore, despite theoretical concerns regarding consistency of N_e between contrasting sexual selection treatments, measures of heterozygosity and allelic richness across 13 microsatellite loci, showed almost identical heterozygosity between contrasting sexual selection treatments within regimes (Lumley *et al.* 2015). Therefore, contrasting sexual selection treatments are expected to have equal potential to respond to selection and divergence in traits and fitness are a reliable response to the strength of sexual selection.

Contrasting strength of sexual selection in treatments

The operational sex ratio (OSR), the ratio of fertilisable females to sexually active males, is a commonly used measure of the strength of sexual selection that estimates the degree of monopolisation of mates, and therefore the potential level of competition (Emlen & Oring 1977). Manipulation of the OSR is used to create contrasting female-biased and male-biased sexual selection in regime A. In the male-biased treatment (90 ♂ : 10 ♀), males are unable to monopolise females and must theoretically compete with 89 other males for mating opportunities with the females. In contrast, in the female-biased (10 ♂ : 90 ♀) treatment, males are theoretically able to monopolise females and face much reduced competition, although multiple mating by the 10 females will reduce this monopolisation and maintain some level of sexual selection as a result of the promiscuous model system.

Data regarding the potential mating rate of males provides further evidence for the extent of the contrast in the strength of sexual selection in female-biased versus male-biased treatments. Males from the ancestral GA1 stock population, which was used to initiate the sexual selection regimes, were able to successfully mate and produce offspring with a mean of 50 females over 7 days (males presented with 6 females, every 12 hours, for 7 days n = 84 females in total) (Lumley *et al.* 2015). This high mating frequency suggests that in the male-biased (90 ♂ : 10 ♀) treatment, during the 7 days that adults are placed together for mating and oviposition (see Figure 1.3), 90 males are capable of mating 4,500 times in total, which equates to 450 matings per female (4500 mating opportunities / 10 females). Data from the demonstration of the Bateman gradient (see section 1.4) show that females can mate with at least 20 males without negative impacts on offspring production. Therefore, in the male-biased regime, both pre-copulatory male-male competition for mating and post-copulatory sperm competition for fertilisations are predicted to be extremely high, along with opportunities for female choice. In contrast, in the female-biased (10 ♂ : 90 ♀) treatment, during 7 days mating and oviposition, 10 males are capable of mating 500 times in total and consequently each female could mate 5-6 times (500 mating opportunities/90 females). Therefore, although male-male/sperm competition is not absent in the female-biased treatment, it is predicted to be substantially lower than in male-biased populations, and these estimates do not account for the possibility that higher male mating rates may have evolved under stronger sexual selection. Thus, the potential mating rate of males indicates that variance in reproductive success of males in the male-biased regime will far exceed that of female-biased males creating highly contrasting levels of male-male competition between treatments.

In regime B, enforced monogamy (1 ♂ : 1 ♀), prevents male-male completion and removes the opportunity for female choice. In contrast, in the polyandrous (5 ♂ : 1 ♀) treatment, the potential mating rate of males, combined with the absence of negative effects of multiple mating on female offspring production, suggests females are likely to mate with each of the 5 males. This will result in high levels of sperm competition and the opportunity for cryptic female choice and creates a strong contrast with enforced monogamy.

Previous findings

Previous work using these experimentally evolved populations has revealed responses in mating behaviour, female tolerance of sexual conflict, and male reproductive success under competition, to high male-male competition and opportunity for female choice. Males from male-biased populations mounted more readily, more frequently and for longer, and gained a greater share of paternity when in competition with a rival, than males from female-biased populations. Females from male-biased populations were resistant to conflict and produced the same number of offspring regardless of number of mates they were exposed to. However, offspring production of females from female-biased populations decreased with increasing number of mates, indicating that sexual conflict exists in *T. castaneum,* and that females usually maintain traits to defend against this phenomenon, which they quickly lose when sexual conflict is removed (Michalczyk *et al.* 2011). Similar responses were also found for males and females with a history of polyandry compared to monogamy. When in competition with rivals, polyandrous males were quicker to copulate and sired more offspring than monogamous males, while polyandrous females faced with a choice of mate delayed their first copulation and produced higher numbers of offspring, compared to monogamous females (Demont *et al.* 2014). Immune system activity has also been shown to be higher in polyandrous females compared to monogamous females and males from both treatments, which is thought to be a response to higher risk of infection and injury as a result of mating with multiple males (Hangartner *et al.* 2015).

In a study making use of both experimental evolution regimes, Lumley *et al*. (2015) examined evidence for relative differences in genetic load and fitness, in contrasting sexual selection treatments. By exposing load via inbreeding, populations with a history of high male-male competition and opportunities for female choice, showed subsequent resilience to extinction through inbreeding, and maintained fitness better than populations with histories of weak or no sexual selection. Results therefore suggested a historic reduction in mutation load where populations had experienced strong inter-sexual competition and intra-sexual choice (Lumley *et al.* 2015). There is therefore ample evidence that experimental evolution under sexual selection has caused significant changes in our selection lines.

1.6 Data analysis and presentation

All analyses were conducted in 'R' (R Core Team 2015) and associated packages. Throughout this thesis, means are presented ± 1 standard error (mean ± SE). 'plyr' (Wickham 2015) was used for data exploration and manipulation and 'pastecs' (Grosjean *et al.* 2014) for descriptive statistics including tests for skew and kurtosis. The Shapiro Wilk test was used to test for departures from normality ('stats' (R Core Team 2015)), Levene's test to investigate homogeneity of variance ('car' (Fox *et al.* 2015)), and residual plots were created to look at error distribution. Unless otherwise stated, data met the assumptions of parametric analysis.

Linear mixed-effects models (LMMs) and generalised linear mixed-effects models (GLMMs) were fitted using 'lme4' (Bates *et al.* 2015). GLMMs were also fitted using 'glmmADMB' (Fournier *et al.* 2012) and 'R2admb' (Bolker *et al.* 2015) where a negative binomial distribution was required to account for overdispersion. The use of mixed-effects models allowed for random effects to be included, which can account for nesting in the data. Structuring of the data is provided in each chapter. Maximal models were fitted using maximum likelihood (ML) and stepwise deletions were made to reach a minimum adequate model. During deletions, the model with the lowest Akaike's Information Criterion (AIC) score was selected (Bolker *et al.* 2009; Crawley 2013). Minimum adequate models were established using likelihood ratio tests to compare models with and without the variable of interest (Crawley 2013).

Figures were created using 'ggplot2' (Wickham & Chang 2015), with boxplots used through much of the thesis to clearly show the distribution of the data (Weissgerber *et al.* 2015). In 'ggplot2', boxplots follow the Tukey method, with the box showing the interquartile range, the median displayed as a horizontal line, the whiskers describing the data within 1.5 IQR, and any data not included in the box and whiskers shown as outliers (Wickham & Chang 2015). An additional point was added to the box to show the mean. Where error bar plots are used, these represent the mean (point) ± 1 SE (error bars). Survival curves were drawn using 'survival' (Therneau 2015). Throughout chapters 2, 3 and 4 which make use of populations experimentally evolved under contrasting strengths of sexual selection, low male-male competition (female-biased and monogamous treatments) are presented in blue, and high male-male competition (male-biased and polyandrous treatments) in red. These opposite ends of a thermal colour spectrum are used to represent divergent forms and strengths of sexual selection in contrasting treatments.

Chapter 2

Does sexual selection improve fitness in the face of ageing and environmental stress?

Abstract

Sexual selection is traditionally considered to work in opposition to natural selection due to the cost of maintaining sexually-selected signals. However, genic capture theory states that mating success, especially in the face of competition, is ultimately determined by condition, which will be shaped by a large number of naturally selected genes. As a result of condition-dependence, sexual selection may therefore augment natural selection by improving the purging of deleterious mutations and the fixation of beneficial ones, which could translate into population fitness benefits. The aim of this study was to test how histories of contrasting sexual selection have impacted on population fitness through phenotypic measures of intrinsic ageing and resistance to environmental stress, using experimentally evolved populations of the model insect *Tribolium castaneum.* A history of beneficial purging of mutation load via genic capture under heightened sexual selection was predicted to correspond to increased intrinsic lifespan, however, there was no evidence for differences in survivorship across 2 years of ageing between individuals from populations subjected to contrasting sexual selection treatments. Individuals from histories of strong sexual selection were also predicted to show increased resistance to abiotic stress, however, responses to hot and cold thermal stress, and nutritional deprivation through development, did not differ between treatments. However, there was a highly significant response by adults from histories of strong sexual selection showing an increased resistance to starvation which was augmented following development under nutritional stress; this improved starvation response was replicated independently under two different regimes applying strong versus weak sexual selection. Increased trait variance under nutritional stress was also seen in low sexual selection populations, consistent with higher genetic load. Therefore, ageing and resistance to environmental stress did not show general improvements following strong sexual selection, although the ability to cope with adult starvation did. These findings suggest that investment in sexual selection does not trade-off against the ability to cope with environmental stress or resist ageing, and can benefit particular traits in relation to specific stress (starvation).

2.1 Introduction

Sexual selection is responsible for many extravagant sexual dimorphisms and displays which, despite adverse effects on survival, persist due to their positive impact on intra-sexual competition and inter-sexual choice, thus increasing reproductive success (Darwin 1859). The cost of developing and maintaining these elaborate traits, in terms of survival and resources, has resulted in sexual selection often being viewed as working against natural selection (summarised in Andersson 1994). However, in his original description, Darwin (1859) suggested that '*sexual selection will give its aid to ordinary selection, by assuring to the most vigorous and best adapted males the greatest number of offspring'*. This original perspective implies that sexual selection may have a wider evolutionary role than often considered, in augmenting the natural selection of traits associated with condition and survival, as these fundamental qualities will also be vital for reproduction. Mating success is not simply determined by elaborate secondary sexual traits, but also by a broad range of underlying physiology and behaviour, combined with the ability to perform these tasks and maintain these traits (Andersson 1994; Whitlock & Agrawal 2009). Search effort (Lane *et al.* 2009), courtship intensity/tactics (Shine *et al.* 2004; Shamble *et al.* 2009) and endurance (Reichard *et al.* 2008; Higham *et al.* 2011) have all been empirically shown to increase mating success. Successful expression of these behaviours will depend on individual condition, defined as the resources available for allocation to fitness-enhancing traits (Rowe & Houle 1996). Finite resources must be allocated strategically such that trade-offs will occur between fitness components, however, an individual in better condition will be able to invest in mating success without experiencing costs to survival (Stearns 1992; Rowe & Houle 1996). Elaborate secondary sexual traits, the 'traditional' targets of sexual selection, have long been hypothesised to be an 'indicator' of condition, as only individuals in high condition will have the resources available to express costly traits (Fisher 1915; Williams 1966; Zahavi 1975). Condition can therefore impact on mating and reproductive success through a number of interconnected paths and, as a result, sexual selection could augment the natural selection of high condition individuals (Lorch *et al.* 2003; Whitlock & Agrawal 2009).

The association between condition and sexually selected traits has provided a simple resolution to the complex problem of the maintenance of genetic variation in traits closely linked to reproductive fitness (Rowe & Houle 1996; Tomkins *et al.* 2004). Variation in traits associated with mating success is expected to be quickly eroded as a result of persistent, directional mate choice (Barton & Turelli 1989; Charlesworth & Charlesworth 2010). This 'lek paradox' (Borgia 1979) is particularly apparent in species where females gain no direct benefits from their choice of male and are therefore predicted to gain indirect genetic benefits. Condition is expected to be underpinned by genes at many loci, therefore, if mating and reproductive success are condition-dependent, genetic variance of traits will be high (Rowe and Houle 1996). Rowe and Houle (1996) used the term 'genic capture' for the involvement of large parts of the genome in the expression of sexually selected traits due to covariance between condition and the trait. The significance of genic capture is that it provides an explicit mechanism whereby sexual selection can also enhance non-mating traits and, consequently, population fitness (Lorch *et al.* 2003; Whitlock & Agrawal 2009). Additional modelling has shown that genic capture can create synergy between natural and sexual selection and drive the evolution of rapid and extensive adaptations for increased condition (Lorch *et al.* 2003). Further theoretical work suggests because many new mutations will be deleterious to condition, sexual selection, via competition and choice of high condition individuals, will result in those with a lower genetic load gaining greater mating success. Consequently, sexual selection could reduce the fixation of deleterious alleles and aid the purging of mutation load from a population (Whitlock 2000; Whitlock & Agrawal 2009). Recessive deleterious mutations, and those with small individual effects, can accumulate and persist in populations as their phenotypic effects are not individually harmful enough to be removed by natural selection; however, the cumulative effect can be a substantial reduction in fitness, the so-called genetic or mutation load (Haldane 1937; Agrawal & Whitlock 2012). Sexual selection is theorised to be a particularly efficient filter of mutation load, because it acts on variance between competing individuals within a species, which can become highly skewed when sexual selection intensifies (Agrawal 2001; Siller 2001). Accordingly, populations with higher levels of competition and choice might be predicted to show increased relative fitness due to lower mutation load (Whitlock & Agrawal 2009). The extent of these population fitness benefits are theorised to mitigate the 'two-fold cost of sex' and therefore explain the maintenance of sexual reproduction (Agrawal 2001; Siller 2001).

To gain empirical evidence for the removal of deleterious alleles by sexual selection, a number of studies have introduced mutations and shown that male-male competition and female choice enable the fitness of subsequent generations to recover more rapidly compared to where monogamy is enforced (Radwan 2004; Hollis *et al.* 2009; Almbro & Simmons 2014). However, other studies using the same approach have found either no benefit (Arbuthnott & Rundle 2012), or negative effects (Hollis & Houle 2011) of sexual selection on the removal of mutations. An alternative approach is to consider the response of populations to inbreeding, which is expected to increase homozygosity and consequently expose deleterious alleles carried as mutation load (Charlesworth & Charlesworth 1987). Sexual selection was shown reduce the proportion of populations that went extinct as a result of inbreeding, suggesting a reduced rate of deleterious mutation accumulation (Jarzebowska & Radwan 2010; McGuigan *et al.* 2011). Most recently, Lumley *et al.* (2015) examined evidence for relative differences in genetic load and fitness, in populations under long-term experimental evolution of high versus low sexual selection. By exposing load via inbreeding, populations with a history of strong male-male competition and opportunities for female choice showed subsequent resilience to extinction through inbreeding, and maintained fitness better than populations with histories of weak or no sexual selection. Results therefore suggested a historic reduction in mutation load where populations had experienced strong inter-sexual competition and intra-sexual choice (Lumley *et al.* 2015).

In addition to purging mutation load, sexual selection is theorised to offer an alternative route to increased population fitness, through the fixation of beneficial alleles within successful reproducers in the face of competition and choice (Proulx 1999; Whitlock 2000), which could be of particular value during adaptation to new and changing environments (Lorch *et al.* 2003; Candolin & Heuschele 2008; Whitlock & Agrawal 2009). Mathematical modelling by Lorch *et al.* (2003) showed synergy between natural and sexual selection can accelerate rates of adaptation. However, experimental evolution in which populations were allowed to adapt to a novel abiotic environment have proved inconsistent with some reporting that sexual selection increased rate of adaptation (Fricke & Arnqvist 2007) and prevented extinction (Plesnar-Bielak *et al.* 2012), but others finding no benefit (Holland 2002; Rundle *et al.* 2006) or even antagonism (Chenoweth *et al.* 2015).

Inconsistent findings regarding the impact of sexual selection on genetic load and mutation accumulation may be caused by a number of factors. Manipulation of sexual selection will accordingly alter the strength of sexual conflict; antagonism between males and females preventing one or both achieving an optimum (Parker 1979). Theoretical and empirical studies indicate negative effects of sexual antagonism on female fitness (Arnqvist & Rowe 2005), however the impact of this on population fitness remains unclear (reviewed in Holman & Kokko 2013). If sexual conflict has an adverse impact on population fitness, the benefits of sexual selection acting via genic-capture may be reduced or even outweighed (Whitlock & Agrawal 2009). In populations close to their 'adaptive-peak', where deleterious mutations are in low numbers, successful males are predicted to sire less fit daughters due to the impact of sexually antagonistic alleles. However this antagonism may be irrelevant where populations are 'off-peak' and mutation load is high (Long *et al.* 2012). As well as the extent of the genetic load, the nature of deleterious mutations may also determine the extent of the benefits from sexual selection. Mutations with severely deleterious effects may be purged more rapidly than many low level mutations, as they will have the greater effect on fitness and therefore be more apparent to selection (Jarzebowska & Radwan 2010). Alternatively, high numbers of deleterious alleles may increase the opportunity for synergistic epistasis such that the combined negative effects are greater than when acting in isolation, and selection then becomes more efficient by removing multiple deleterious alleles (Agrawal & Whitlock 2012). Furthermore, the time scale of many studies may be a further limitation as a small number of generations for sexual selection to operate may not provide enough evolutionary time for mutation load to reach a new equilibrium (Whitlock & Agrawal 2009). Thus, the empirical evidence for genome wide effects of sexual selection is inconclusive, and further work is needed to clarify the effects.

The aim of this study is to gather empirical evidence regarding whether there are population fitness benefits of sexual selection, by examining how naturally selected phenotypic traits have responded to potential differences in condition and genetic load within populations subject to contrasting strengths of sexual selection via male-male completion and opportunity for female choice. In addition to mating success, which is driven by sexual selection, survivorship, senescence and fecundity are additional key components of fitness, and are usually considered to evolve under natural selection (Arnqvist & Rowe 2005). However, as a result of genic-capture, sexual selection may also contribute to the evolution of these traits (Whitlock & Agrawal 2009). Senescence or ageing is the increase in intrinsic mortality and decrease in fecundity with increasing age, and is theorised to be caused by mutations with deleterious effects in later life (Stearns 1992). Natural selection to remove deleterious alleles is predicted to be less efficient with increasing age as extrinsic causes of mortality, predation, disease and accidents, are more likely to cause death and prevent future contributions to fitness, thus decreasing the strength of selection on mutation removal as an individual ages (Haldane 1941; Medawar 1952; Williams 1957; Hamilton 1966; Charlesworth 1994). The consequence of this unidirectional reduction in the strength of selection is that deleterious mutations could accumulate ('mutation accumulation', Medawar 1952) or genes with pleiotropic early life benefits but later life costs could persist ('antagonistic pleiotropy', Williams 1957) within a population. Which of these specific models (which are unlikely to be mutually exclusive) best explains the evolution of ageing remains controversial, however, the general theory that ageing evolves due to a reduction in the strength of selection is widely accepted (reviewed in Partridge & Gems 2002). Increases in genetic drift, and consequently mutation load, experienced by small populations, has also been shown to lead to increased rate of ageing and reduction in lifespan (Lohr *et al.*

2014). If high male-male competition and female choice in a population increase the strength of selection against deleterious mutations, and consequently reduce their accumulation or persistence, this could be reflected in the evolution of increased intrinsic lifespan of its individuals. In addition, experimental evolution has recently shown that although high random extrinsic mortality weakens natural selection and leads to the evolution of shorter lifespans, as predicted by theory (Williams 1957), high condition-dependent mortality selects the best all-round survivors and can result in increased longevity (Chen & Maklakov 2012). Therefore, if strong sexual selection generates high condition individuals, intrinsic survival is again predicted to increase in such populations.

A further phenotypic indicator of the impact of sexual selection on condition and/or genetic load is predicted to be the fitness of individuals experiencing stressful environments. A stressful environment is one which causes a reduction in fitness relative to average conditions (Bijlsma & Loeschcke 2005; Martin & Lenormand 2006). If sexual selection drives the evolution of high condition, individuals are predicted to be more efficient at gaining, and making use of, a resource pool to allocate to fitness (Rowe & Houle 1996; Whitlock & Agrawal 2009). Where individuals are able to make more efficient use of resources, this will be advantageous where stress is a result of direct resource limitation, for example nutritional stress. Furthermore, if improved acquisition increases resource availability, these can be allocated to physiological response pathways, and allow individuals to show greater resilience to stress, without incurring costs to reproduction. Variation in the amount and type of nutritional resources available during development has been shown to alter adult thermal stress tolerance in invertebrates (Sisodia & Singh 2012). In addition to the benefits from increased condition, if sexual selection reduces mutation load but maintains genetic variation though selection on condition, this could provide an important source of standing genetic variation (Lorch *et al.* 2003), which is predicted to be crucial for species’ responses to environmental stress (Barrett & Schluter 2008). Novel and fluctuating environments move populations away from their adaptive peak and consequently alter the fitness effects of alleles such that neutral or even maladaptive alleles may become beneficial (Martin & Lenormand 2006; Agrawal & Whitlock 2010). A history of sexual selection is also predicted to be beneficial in these environments as populations occupy a broad phenotypic space, often amplified by sexual conflict, across a fitness landscape which allows greater resilience to environmental fluctuations (Bonduriansky *et al.* 2011). Here, I use both thermal and nutritional stress treatments to investigate differences in survivorship and fitness following strong versus weak sexual selection in replicate populations. Adult survivorship under acute stress, extreme high and low temperatures and starvation, were tested, along with developmental fitness (egg to adult development) in an environment with reduced nutritional quality.

To gather further empirical evidence regarding the impact of sexual selection on naturally selected traits, this study makes use of experimentally evolved populations of the promiscuous flour beetle *Tribolium castaneum*, which varied only in their contrasting levels of sexual selection during the adult stage over 75+ generations. The common ancestry and long-term experimental evolution in these populations make them an invaluable resource to investigate the impact of sexual selection history on population fitness. Two independent regimes were maintained, both generating contrastingly strong versus weak sexual selection. Manipulation of the operational sex ratio was used to create male-biased (90 males : 10 females) versus female-biased (10 males : 90 females) populations with opposing levels of male-male competition and female choice. In addition, populations with polyandrous (5 males : 1 female) versus monogamous (1 male : 1 female) mating systems provided an additional and alternative form of contrasting sexual selection, allowing the consequences of complete removal of sexual selection though enforced monogamy to be considered. The recent work by Lumley *et al.* (2015) using these populations provided evidence that histories of high male-male competition and female choice in male-biased and polyandrous lines had acted to reduce mutation load. This was evident as higher relative fitness and resistance to extinction under inbreeding in male-biased and polyandrous treatments, compared with female-biased and monogamous, respectively. The aim of this new study is to examine how naturally selected phenotypic traits have responded to these differences in genetic load and condition following strong versus weak sexual selection. Here, I used intrinsic ageing and lifespan, and response to abiotic stresses, as phenotypic indicators of underlying genetic load. Individuals with a history of high male-male competition (male-biased and polyandrous sexual selection treatments), where previous empirical evidence suggests a reduction in mutation load (Lumley *et al.* 2015), were predicted to show increased intrinsic survivorship and greater resistance to abiotic stress, compared to populations with a history of low male-male competition (female-biased selection) or no sexual selection (enforced monogamy).

2.2 Methods

The following assays were carried out using replicate lines which have been experimentally evolved under contrasting strengths of sexual selection using female-biased versus male-biased adult population structures (Regime A), and a monogamous versus polyandrous mating system (Regime B). For details of stock maintenance and experimental evolution see General Introduction 1.5 and Figure 1.2. Analyses were carried out independently in Regime A and B.

2.2.1 Intrinsic ageing and lifespan

Intrinsic ageing and lifespan of individuals from male-biased and female-biased sexual selection treatments were compared following 68 generations of experimental evolution and polyandrous and monogamous sexual selection treatments after 58 generations. To control for potential transgenerational effects of sexual selection treatment on condition and survivorship, equal sex ratio populations (50 males : 50 females) were created from each independent line (n = 3 per treatment) for one generation before taking experimental individuals to measure ageing.

All adults were virgin, and 10-12 days post-eclosion. Extrinsic impacts on mortality were equalised and minimised by isolation of individuals in vials containing *ad libitum* control food medium and oats to aid locomotion. Individuals were checked weekly for deaths, and food medium/oats were refreshed every 4 weeks. N = 50 males and 50 females from each of the 3 independent lines were measured, so that ageing was determined in a total of 1200 individuals, 300 per sexual selection treatment across four treatments (two weak and two strong).

Statistical analysis

Intrinsic survivorship of males and females was compared using the ‘survival’ package (Therneau 2015). A parametric accelerated failure time (AFT) model was used which allows an appropriate error distribution to be specified (Crawley 2013). Event times are positively skewed such that a normal distribution is inappropriate for survival data, therefore, a number of distributions were compared using AIC values, along with visual assessment of log-log plots and raw data overlaid with model fits (Crawley 2013). A Weibull error distribution was used for analysis of regime A, however, non-proportional hazards shown in the log-log plot for regime B made this inappropriate, therefore a logistic distribution was used. Censoring was not required as time of death of all experimental individuals was known. Lack of independence between nested replicates, was accounted for using a generalised estimating equation approach which includes a cluster term in the model (Therneau 2015; Lumley *et al.* 2015). A Kaplan-Meier object was created with sexual selection (SS) treatment (female-biased versus male-biased, or monogamous versus polyandrous) and sex (female versus male) as fixed effects. Independent line (A, B, C) was entered as a cluster indicator to account for the hierarchical design.

2.2.2 Thermal stress

Adult survivorship under acute heat and cold stress was assessed in individuals from male-biased and female-biased selection treatments following 73 generations of experimental evolution. All adults were virgin and 10-12 days post-eclosion. Individuals were randomly allocated to within line, single sex groups (20 individuals), and placed in *ad libitum* control medium and oats to aid locomotion. 100 males and 100 females from each independent line (n = 200 individuals x 3 lines = 600 individuals per sexual selection treatment per thermal stress treatment) were placed in pre-set incubators for the allotted time. Following a 1 day recovery period, the number of living and dead beetles were counted and the proportion surviving was calculated.

Heat stress

Previous work using the ancestral GA1 strain of *T. castaneum* showed ~25% survival of males exposed to 43°C over a 5 day heatwave scenario (Godwin 2010 unpubl. data), therefore this treatment was used to assess differences in heat stress resistance between individuals with contrasting sexual selection backgrounds.

Cold stress

A wild strain of *T. castaneum* exposed to 0°C showed 20% survival after 4 days (Wijayaratne & Fields 2010), however a pilot study using GA1 stock adults suggested higher resistance to 0°C with ~70% survival after 4 days and ~40% survival after 6 days exposure. Therefore, resistance to cold stress was measured here as the proportion of individuals surviving 0°C for 6 days.

Statistical analysis

Proportion data are strictly bounded between 0 and 1, with non-normal errors and non-constant variance, making parametric analysis inappropriate therefore the binomial distribution was used to describe the data. Proportions were compared by constructing generalised linear mixed models (GLMMs) in 'lme4' (Bates *et al.* 2015) with a binomial error structure which incorporates a 'logit' link function to apply an inverse logistic transformation (Crawley 2013; Bates *et al.* 2015). The response variable was entered as a paired variable containing the number of 'successes' and 'failures' to retain information on sample size within the model (Crawley 2013). Models were assessed for overdispersion, using 'overdisp_fun()' (Bolker *et al.* 2008) and, where necessary, this was accounted for using a negative binomial error distribution in the 'glmmADMB' (Fournier *et al.* 2012) and 'R2admb' packages (Bolker *et al.* 2015). GlmmADMB has two options for fitting a negative binomial model which both incorporate an additional parameter into the mean, variance relationship to account for overdispersion; i) nbinom: where variance = mean(1+mean/dispersion parameter) or ii) nbinom1: where variance = mean*scale parameter (Bolker *et al.* 2008; Fournier *et al.* 2012). Each option was tried and the model with the lowest AIC was selected.

Maximal models were fitted independently for heat and cold stress assays with sexual selection treatment (female-biased versus male-biased) entered as the fixed effects and line (A, B, C) entered as a random effect to account for the hierarchical design. Two incubators were used simultaneously in the heat stress treatment therefore incubator (1 or 2) was also included in the random effects of the heat stress model. However, this was found to have no significant effect on the model and subsequently removed.

2.2.3 Nutritional stress

Resistance to nutritional stress was assessed in individuals from male-biased and female-biased sexual selection treatments following 69 generations of experimental evolution, and monogamous and polyandrous sexual selection treatments, after 60 generations. The impact of a reduced nutritional value medium (poor diet) was compared to control medium. Control medium contained 90% organic plain white flour and 10% yeast. The poor diet medium contained 100% organic plain white flour and 0% yeast.

Viability – egg to adult development

All adults were virgin and 10-12 days post-eclosion. Males and females were randomly allocated to within-line pairs (n = 10 pairs per independent line) for 48 hours to mate, then the male was removed. Females were left in *ad libitum* control medium until peak oviposition, 10-14 days after mating (Michalczyk 2008), to gain the maximum number of eggs with the least variation in female age. On day 10 after mating, females were transferred to fresh control medium and left to oviposit for 48 hours. 13-20 eggs per female were isolated and those from 5 females per line were placed in *ad libitum* control medium and eggs from the other 5 females per line were placed in *ad libitum* poor medium. Females were transferred to more fresh control medium and left to oviposit for a further 48 hours. An additional 13-20 eggs per female were isolated and placed in the alternate medium (13-20 eggs x 10 females x 3 lines x 2 nutritional treatment for each sexual selection treatment). Under standard conditions development takes eggs ~3 days, larvae ~20 days, pupae ~4 days and immature adult ~5 days. On day 26 after oviposition, checks for eclosed adults were made and repeated daily for 10 days. Day of eclosion was recorded and the proportion of eggs successfully reaching full adult maturity (>5 days post-eclosion), and the number of days required for development, were calculated.

Survivorship - adult starvation resistance

Immediately following eclosion, all adults were isolated in empty eppendorphs which were checked daily for deaths as evidenced by immobility, and the number of days survival was recorded.

Statistical analysis

Viability – egg to adult development

The proportions of eggs developing to fully mature adults were compared by constructing a GLMM with a binomial error structure (proportion data were treated as for the thermal stress analysis (above)). A maximal model was fitted with sexual selection (SS) treatment (female-biased versus male-biased or monogamous versus polyandrous) and nutritional (N) treatment (control versus poor) entered as fixed effects and pair (1 to 10) nested within line (A, B, C) entered as random effects to account for the hierarchical design and non-independence between eggs (eggs from the same pair were split between both diet treatments).

Development time was reciprocally transformed to remove positive skew and meet the assumptions of parametric analysis. A linear mixed effects model was constructed with sexual selection (SS) treatment (female-biased versus male-biased or monogamous versus polyandrous) and nutritional (N) treatment (control versus poor) entered as fixed effects and pair (1 to 10) nested within line (A, B, C) entered as random effects to account for the hierarchical design and non-independence between eggs.

Survivorship - adult starvation resistance

Subsequent adult survival time under starvation conditions was compared using a parametric accelerated failure time (AFT) model with a log-logistic error distribution (see intrinsic ageing analysis for details of survival analysis). Sexual selection (SS) treatment (female-biased versus male-biased or monogamous versus polyandrous) and nutritional (N) treatment (control versus poor) were entered as fixed effects. Pair (1 to 10) nested within line (A, B, C) was entered as a cluster indicator to account for the hierarchical design and non-independence between eggs.

Variance in traits

Theoretical and empirical evidence suggests variance increases in stressful compared to control conditions (Martin & Lenormand 2006). Therefore, within sexual selection treatments a Levene's test was used to compare the variance in the above measures of resistance to nutritional stress in control and poor nutritional conditions.

2.3 Results

2.3.1 Intrinsic ageing

T. castaneum adults lived for up to 2 years. No difference in intrinsic mortality was found between individuals from contrasting sexual selection treatments, however a significant effect of sex was found, with males living longer than females in all treatments. Males in female-biased and male-biased populations live 54% longer than females, while in monogamous and polyandrous populations, survival of males is 32% longer (Table 2.1, Figure 2.1 and Figure 2.2).

Table 2.1 | Summary of intrinsic survivorship and parametric accelerated failure time (AFT) analysis of individuals from contrasting sexual selection (SS) treatments.

Sexual selection	**Age at Death (mean ± SE weeks)**		**Outcome AFT model (z)**	
	Females	**Males**	* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$	
			Weibull distribution	
Female-biased	44.65 (± 4.59)	71.58 (± 5.04)	SS treatment	- 0.84
			Sex	**21.22** ***
Male-biased	44.97 (± 2.22)	68.95 (± 3.51)	SS treatment * Sex	- 0.22
			logistic distribution	
Monogamous	43.43 (± 0.47)	55.23 (± 4.07)	SS treatment	0.45
			Sex	**4.81** ***
Polyandrous	45.77 (± 3.78)	55.97 (± 6.69)	SS treatment * Sex	-0.02

2.3.2 Thermal stress

The proportion of individuals surviving heat stress did not differ significantly between contrasting male-biased (0.33 ± 0.11) and female-biased (0.37 ± 0.11) sexual selection treatments (z = -0.41, p = 0.68) (Figure 2.3a). However, following cold stress, the proportion of individuals that survived was significantly higher from a female-biased background (0.25 ± 0.10) than from a male-biased background (0.10 ± 0.07) (z = -2.29, p = 0.02) (Figure 2.3b).

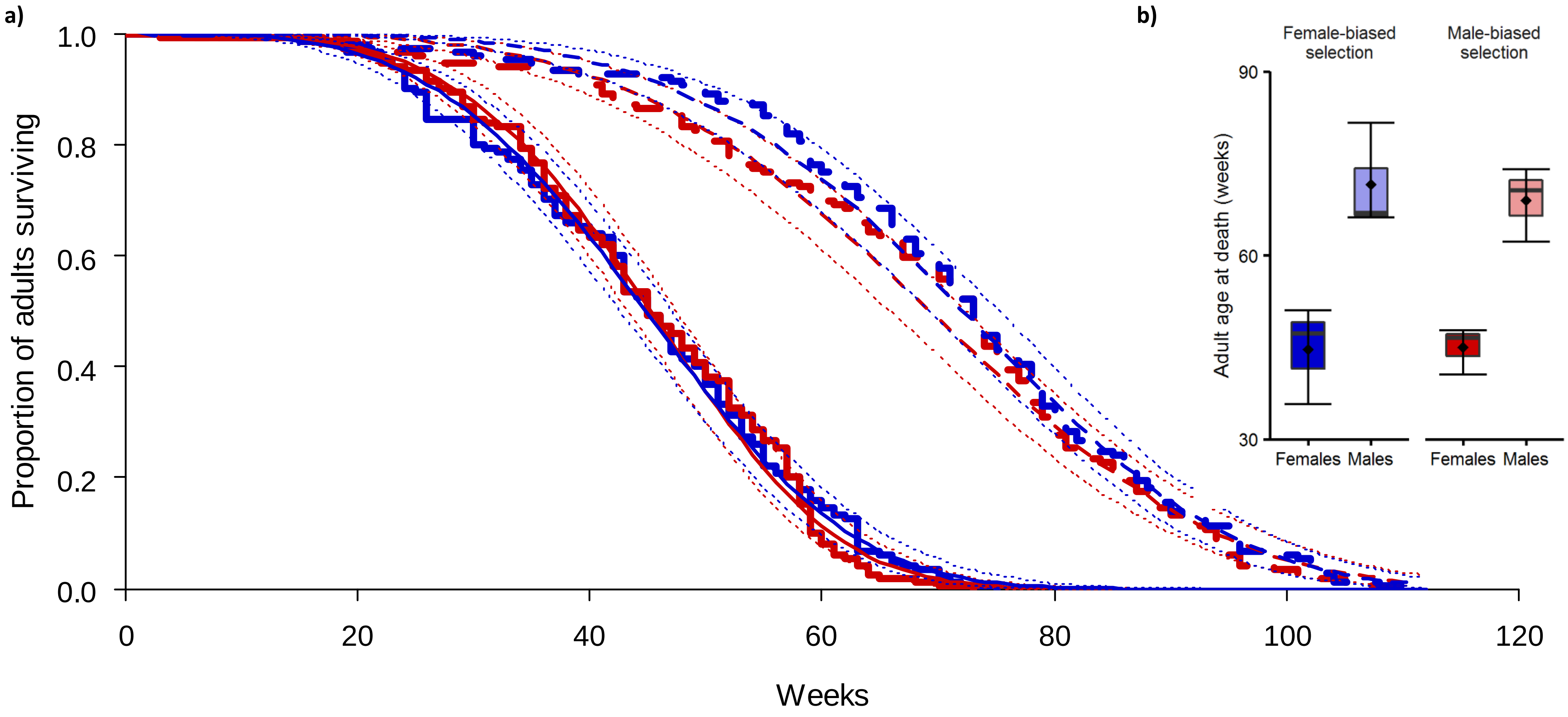


Figure 2.1 | Intrinsic survivorship of females (solid lines) and males (dashed lines) from contrasting female-biased (blue) and male-biased (red) sexual selection treatments. Data grouped by sexual selection treatment (n = 3 independent lines, n = 50 ♂ and 50 ♀ per line). a) Survivorship curves of raw data (thick lines) overlaid with AFT (Weibull hazard distribution) model fit (mean (thin lines) and 95% confidence intervals (thin dashed lines)). No effect of sexual selection treatment but a significant effect of sex ($z = 11.1$, $p < 0.001$) b) Boxplot to show mean and median adult age at death.

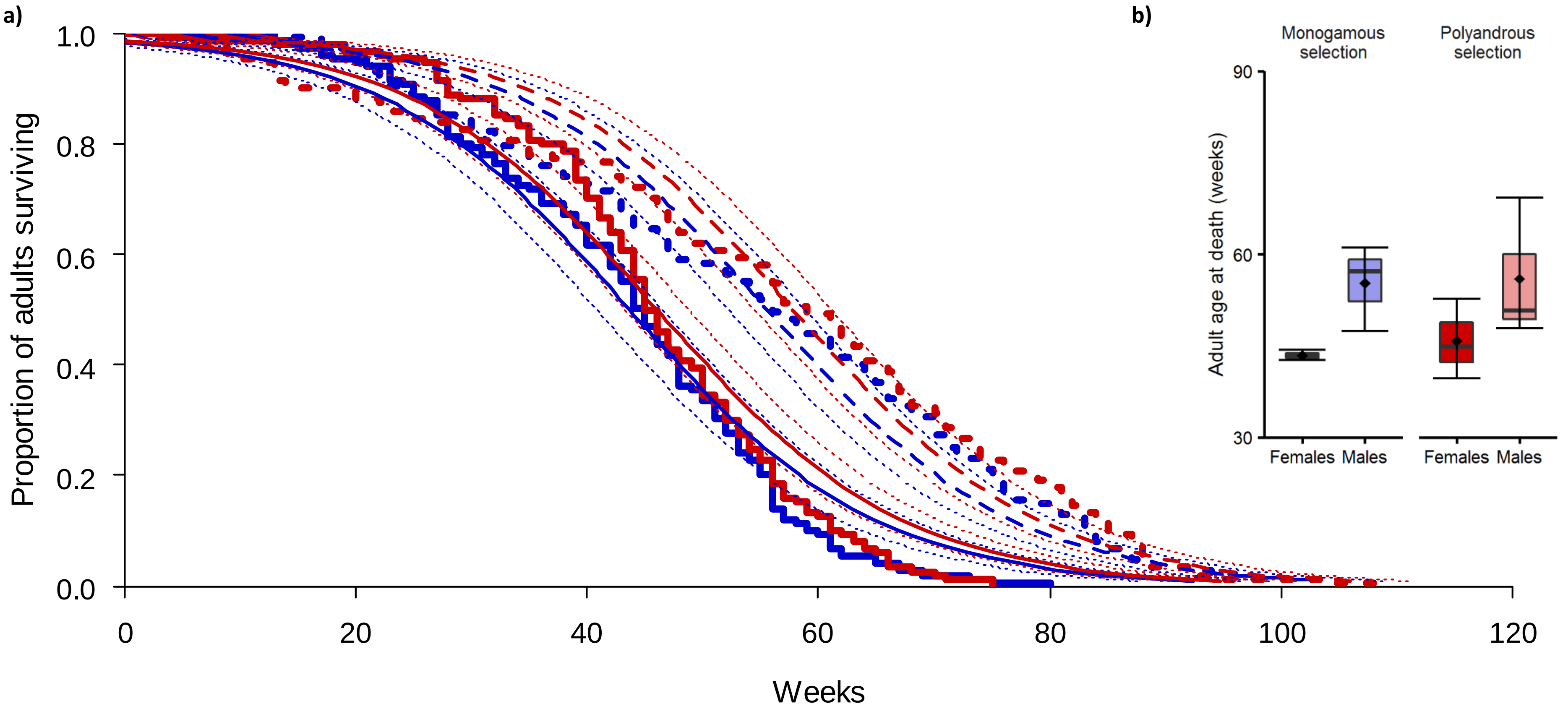


Figure 2.2 | Intrinsic survivorship of females (solid lines) and males (dashed lines) from contrasting monogamous (blue) and polyandrous (red) sexual selection treatments. Data grouped by sexual selection treatment (n = 3 independent lines, n = 50 ♂ and 50 ♀ per line). a) Survivorship curves of raw data (thick lines) overlaid with AFT (log-logistic hazard distribution) model fit (mean (thin lines) and 95% confidence intervals (thin dashed lines)). No effect of sexual selection treatment but a significant effect of sex ($z = 4.81$, $p < 0.001$) b) Boxplot to show mean and median adult age at death.

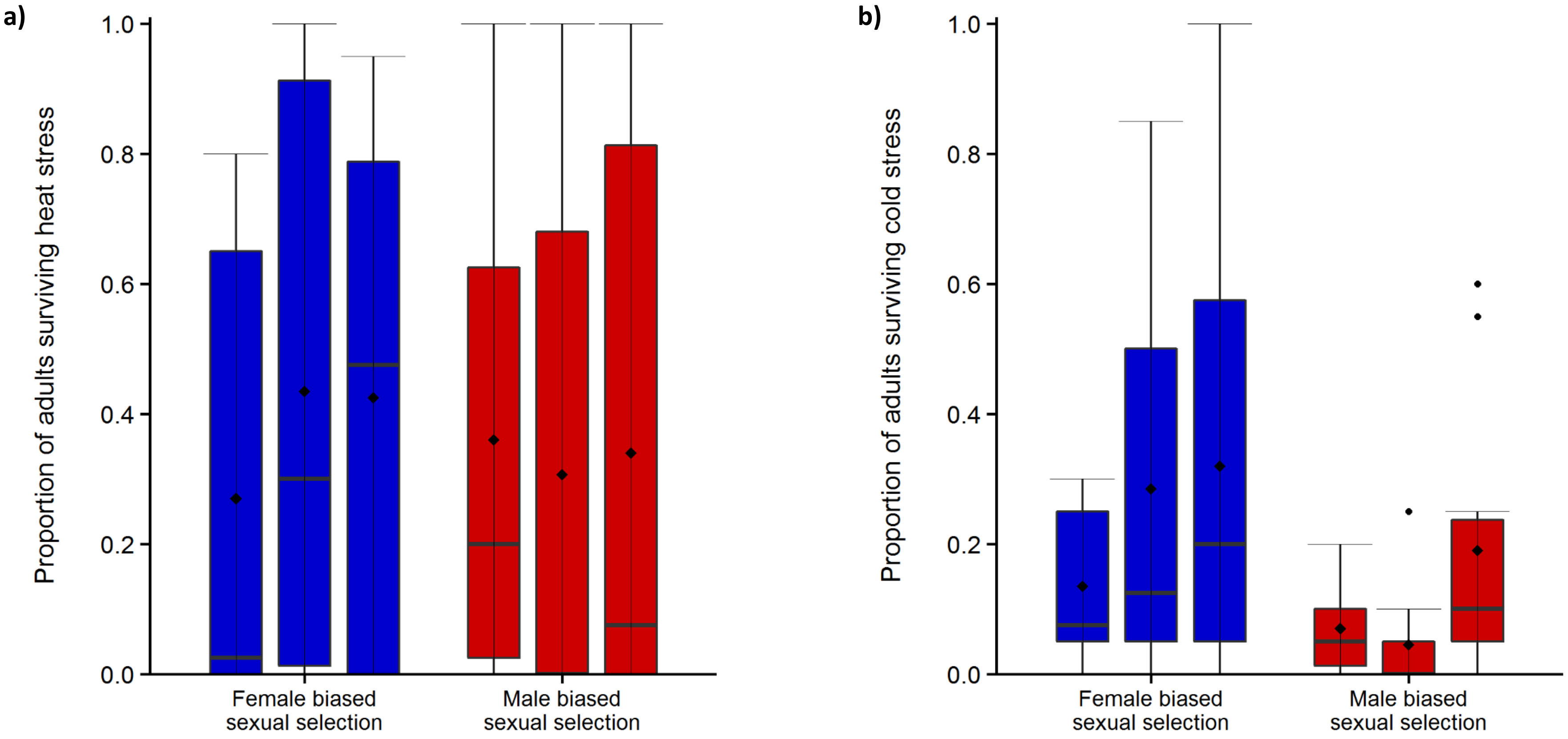


Figure 2.3 | Proportion of adults surviving acute thermal stress treatments. Data grouped by independent line (n = 200 adults per line). a) Heat stress 43°C for 5 days + 1 day recovery. Mean proportions (± SE) are 0.37 (± 0.11) for female-biased selection and 0.33 (± 0.11) for male-biased selection. This difference was not significant (z = -0.41, p = 0.68). b) Cold stress 0° C for 6 days + 1 day recovery. Mean proportions (± SE) are 0.25 (± 0.10) for female-biased selection and 0.10 (± 0.07) for male-biased selection. This difference was significant (z = -2.29, p = 0.02).

2.3.3 Nutritional stress

Viability – egg to adult development

The proportion of eggs that developed to adult did not differ between contrasting male-biased and female-biased sexual selection treatments, under either control or poor nutritional treatments. Variance in the response also did not differ (Table 2.2 & Figure 2.4a). However, a significant interaction between sexual selection treatment and nutritional treatment was found in the proportion of eggs developing to adult in monogamous and polyandrous lines (SS*N, $z = 2.72$, $p = 0.01$). In monogamous lines the proportion of eggs that developed dropped by 10% in nutritionally poor medium. No difference in variance was found (Table 2.3 & Figure 2.4b).

Mean development time did not differ between contrasting male-biased and female-biased, or monogamous and polyandrous sexual selection treatments, although, development was significantly slower for individuals experiencing poor nutrition compared to a control diet (male-biased and female-biased; $t = -13.1$, $p < 0.001$, Table 2.2 & Figure 2.5a), (monogamy and polyandry; $t = -6.69$, $p < 0.001$, Table 2.3 & Figure 2.5b). However, an increase in the variance in development time was found in under nutritional stress compared to control in monogamous ($F = 5.98$, $p = 0.02$) and female-biased ($F = 3.37$, $p = 0.07$), although this was non-significant, lines, which was not seen in polyandrous and male-biased populations (Table 2.2 & 2.3).

Survivorship - adult starvation resistance

Following development in control medium, individuals from male-biased lines showed significantly greater resistance to starvation and survived 10% longer, than those from female-biased lines ($z = 7.13$, $p < 0.001$). Development in nutritionally poor medium caused survivorship to increase significantly ($z = 4.19$, $p < 0.001$), and a significant interaction between sexual selection treatment and nutritional treatment ($z = 3.53$, $p < 0.001$) revealed individuals with a history of male-biased selection resisted starvation 17% longer than individuals from a female-biased background (Table 2.2 & Figure 2.6).

Although no difference in starvation resistance was seen between monogamous and polyandrous individuals that had developed in control conditions, development in nutritionally poor medium again caused a significant increase in survivorship ($z = 3.36$, $p < 0.001$) and a significant interaction between sexual selection treatment and nutritional treatment ($z = 2.31$, $p < 0.05$) showed individuals from polyandrous populations survived starvation conditions for 7% longer than those from monogamous populations (Table 2.3 & Figure 2.7).

Table 2.2 | Summary of analysis of nutritional treatments on individuals from contrasting female-biased *versus* male-biased sexual selection treatments (• $p < 0.1$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

	Sexual selection treatment (SS)	**Nutritional treatment (N)** (mean ± SE)		**Comparison of variance (F)**	**Outcome of 2-way model**		
		Control	**Poor**				
Viability	**female-biased**	0.68 (± 0.10)	0.68 (± 0.10)	0.00	Binomial GLMM (z)		
					SS	-0.51	
proportion of eggs that developed to adult					N	0.55	
	male-biased	0.72 (± 0.10)	0.72 (± 0.10)	0.43	SS*N	0.05	
Viability	**female-biased**	28.14 (± 0.15)	30.28 (± 0.29)	**3.37** •	LMM (t)		
					SS	-0.80	
egg to adult development time (days)					**N**	**-13.10**	***
	male-biased	28.51 (± 0.25)	30.14 (± 0.34)	0.76	SS*N	-1.80	
Survivorship	**female-biased**	12.23 (± 0.22)	12.29 (± 0.37)	**34.47** ***	AFT model (z)		
					SS	**7.13**	***
adult resistance to starvation (days)					**N**	**4.19**	***
	male-biased	13.48 (± 0.37)	14.42 (± 0.53)	**27.47** ***	**SS*N**	**3.53**	***

Table 2.3 | Summary of analysis of nutritional treatments on individuals from contrasting monogamous *versus* polyandrous sexual selection treatments (• p < 0.1, * p < 0.05, ** p < 0.01, *** p < 0.001).

	Sexual selection treatment (SS)	**Nutritional treatment (N)** (mean ± SE)		**Comparison of variance (F)**	**Outcome of 2-way model**	
		Control	**Poor**			
Viability	**monogamy**	0.63 (± 0.11)	0.53 (± 0.11)	0.00	Binomial GLMM (z)	
					SS	0.00
proportion of eggs that developed to adult					N	1.52
	polyandry	0.57 (± 0.11)	0.58 (± 0.11)	1.02	**SS*N**	**2.72** **
Viability	**monogamy**	30.53 (± 0.42)	31.68 (± 0.63)	**5.98** *	LMM (t)	
					SS	0.81
egg to adult development time (days)					**N**	**-6.69** ***
	polyandry	30.15 (± 0.07)	31.39 (± 0.11)	0.04	SS*N	0.38
Survivorship	**monogamy**	12.54 (± 0.15)	12.86 (± 0.30)	**8.10** **	AFT model (z)	
					SS	0.88
adult resistance to starvation (days)					**N**	**3.36** ***
	polyandry	12.67 (± 0.28)	13.62 (± 0.44)	**36.57** ***	**SS*N**	**2.31** *

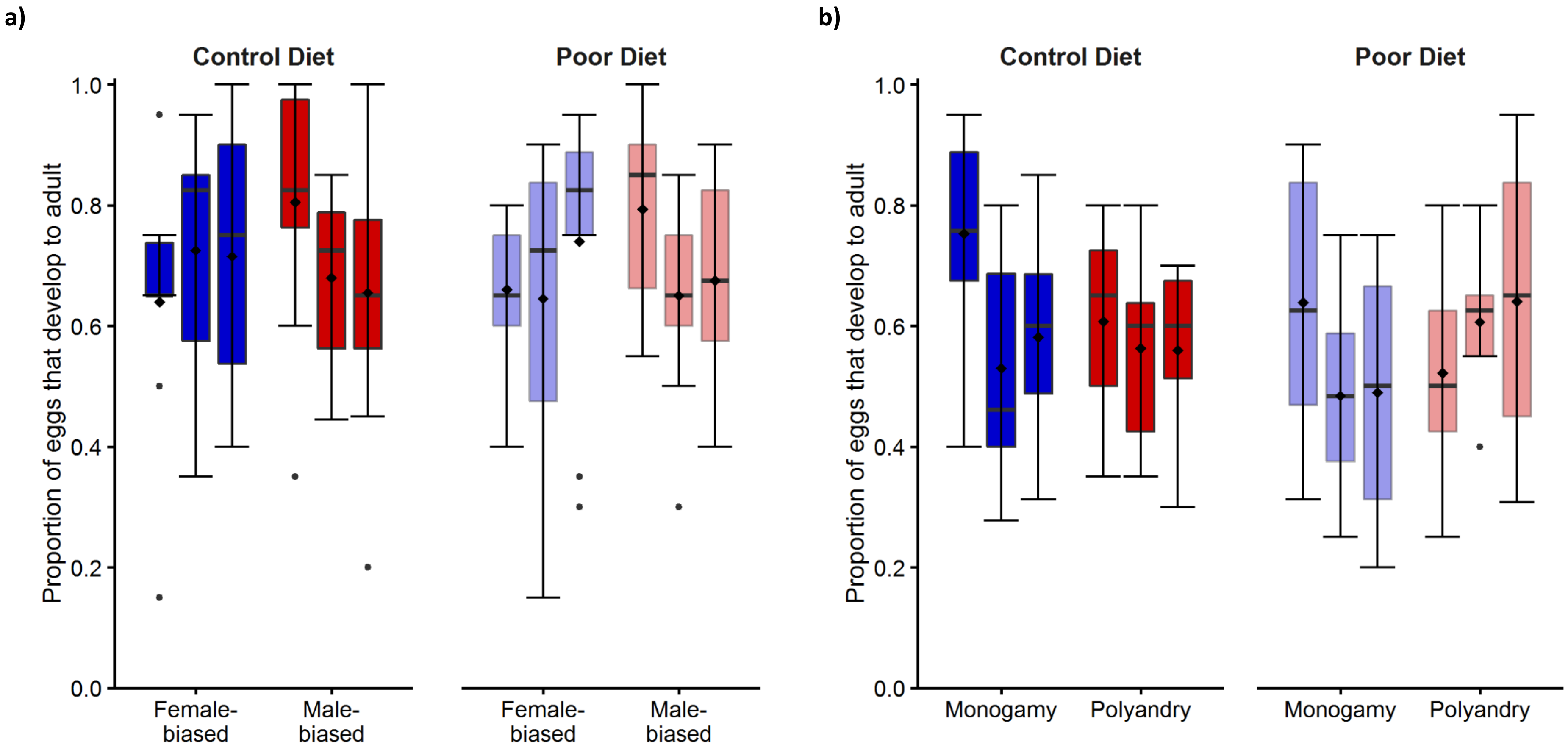


Figure 2.4 | Proportion of eggs reaching adult maturity from contrasting sexual selection treatments, under control and poor diets. a) Regime A, female-biased versus male-biased selection, b) Regime B, monogamous versus polyandrous selection. No significant difference between sexual selection or nutritional treatments in either regime. Data grouped by independent line (n = 13-20 eggs x 10 females line per nutritional treatment per sexual selection treatment).

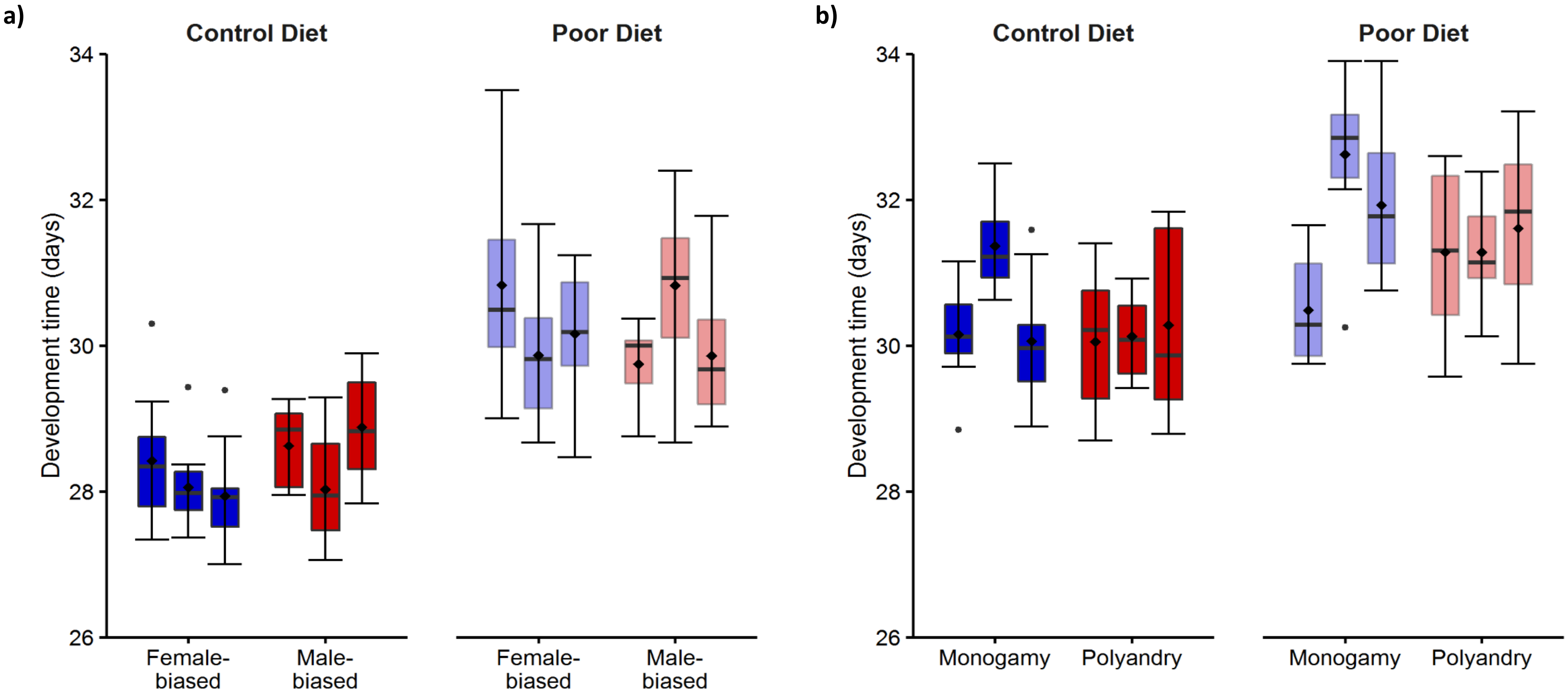


Figure 2.5 | Development time from egg to eclosion for individuals from contrasting sexual selection treatments, under control and poor diets. a) Regime A, female-biased versus male-biased selection, b) Regime B, monogamous versus polyandrous selection. A significant effect of nutritional treatment but not sexual selection treatment in both regimes (Regime A, $\chi^2_{(5)} = 91.79$, $p < 0.001$, Regime B, ($\chi^2_{(5)} = 37.12$, $p < 0.001$). Data grouped by independent line (n = 73 - 161 individuals per line per nutritional treatment per sexual selection treatment).

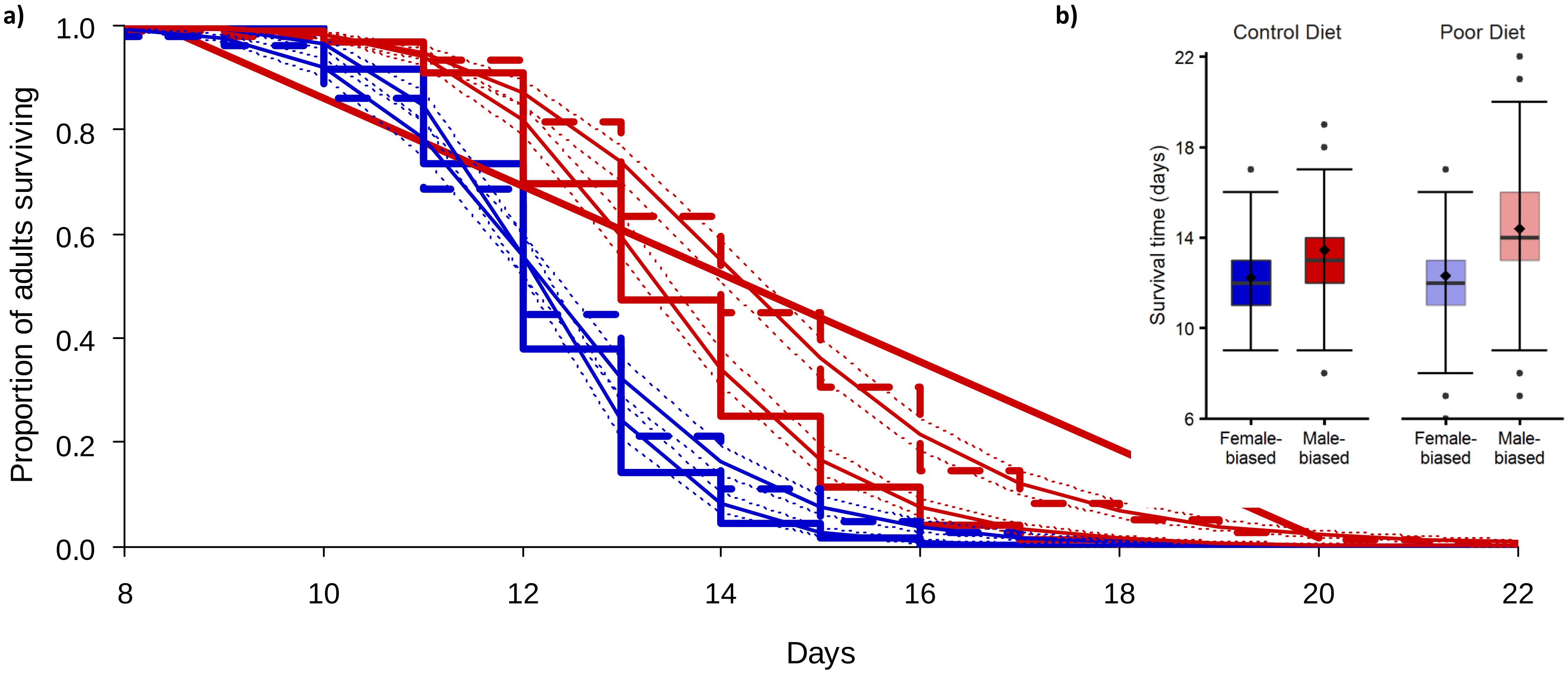


Figure 2.6 | Adult survivorship under starvation conditions for individuals from contrasting female-biased (blue) and male-biased (red) sexual selection treatments, under control (solid lines) and poor (dashed lines) diets. Data grouped by sexual selection treatment (n = 3 independent lines). a) Survivorship curves of raw data (thick lines) overlaid with AFT (log-logistic hazard distribution) model fit (mean (thin line) and 95% confidence intervals (thin dashed lines)). Significant effect of sexual selection treatment ($z = 7.13$, $p < 0.001$) and nutritional treatment ($z = 4.19$, $p < 0.001$) and a significant interaction ($z = 3.53$, $p < 0.001$). b) Boxplot to show distribution of survivorship.

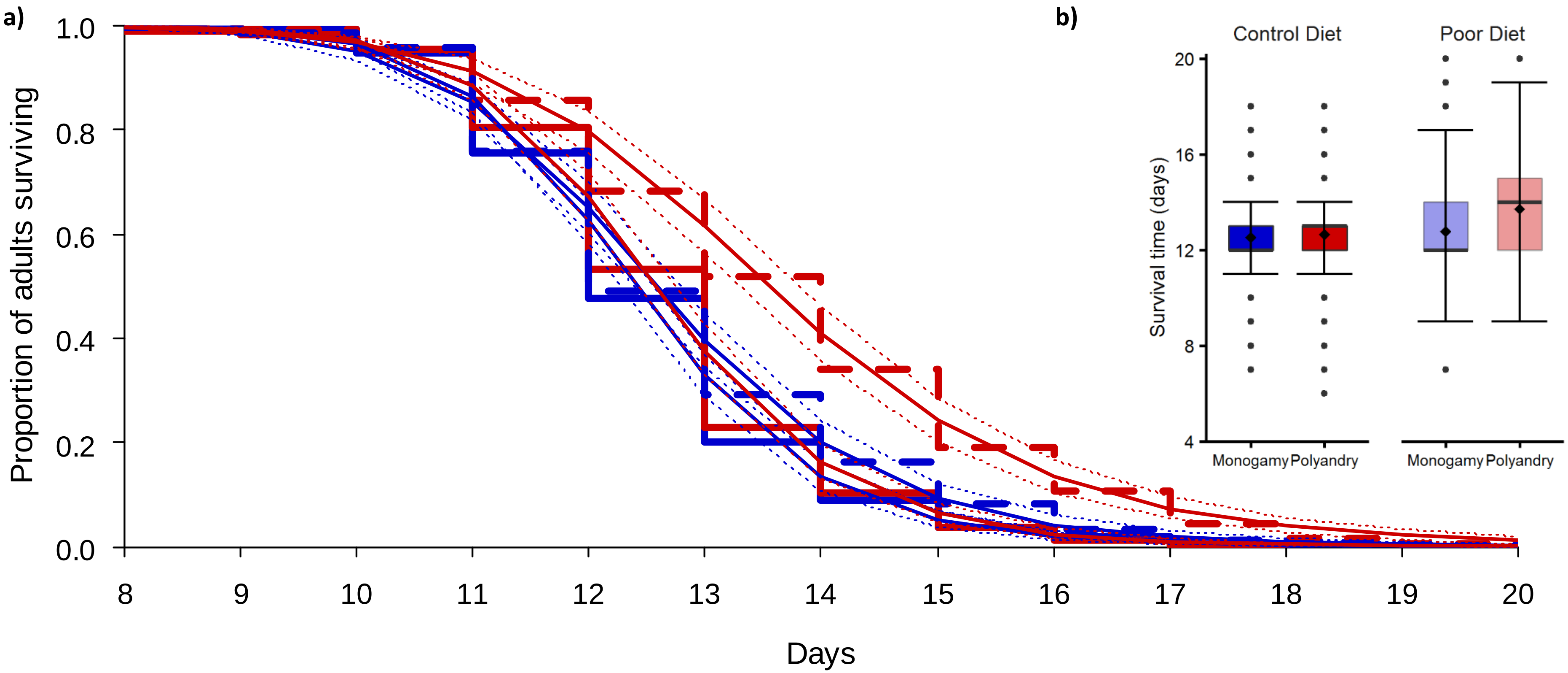


Figure 2.7 | Adult survivorship under starvation conditions for individuals from contrasting monogamous (blue) and polyandrous (red) sexual selection treatments, under control (solid lines) and poor (dashed lines) diets. Data grouped by sexual selection treatment (n = 3 independent lines). a) Survivorship curves of raw data (thick lines) overlaid with AFT (log-logistic hazard distribution) model fit (mean (thin line) and 95% confidence intervals (thin dashed lines)). No effect of sexual selection treatment (z = 0.88, p = 0.38) but a significant effect of nutritional treatment (z = 3.36, p < 0.001) and a significant interaction (z = 2.31, p < 0.05). b) Boxplot to show distribution of survivorship.

2.4 Discussion

Overall, this study provides no evidence for a consistently positive or negative influence of sexual selection history on population fitness under environmental stress or ageing. Previous work with the populations used here provided strong evidence that a long-term history of strong sexual selection, in the form of high male-male competition and female choice experienced under male-biased and polyandrous populations, resulted in a reduction in genetic load revealed through fitness variance under extreme inbreeding (Lumley *et al.* 2015). A reduction in the strength of natural selection with increasing age is predicted to allow deleterious mutations which act in later-life, to accumulate (Medawar 1952) or persist (Williams 1957). If strong sexual selection has decreased genetic load, this was expected to be reflected in an increase in intrinsic lifespan in those populations with histories of high sexual selection. However, no difference was found between male-biased and female-biased sexual selection treatments or polyandrous and monogamous treatments in intrinsic lifespan (Table 2.1, Figure 2.1 and 2.2). This finding suggests that those mutations under purifying selection from male-male competition and female choice in male-biased and polyandrous populations, are not associated with ageing later on in life. Increased longevity has not evolved indirectly as a 'by-product' of strong sexual selection purging deleterious mutations across the genome, therefore specific loci may have an important influence. The historical experimental evolution in these populations focuses sexual selection on 'young' adults and, as such, ageing was not under any direct selective pressure by the regimes. To determine whether ageing could be influenced directly by sexual selection, experimental evolution of sexual selection could be applied to an older cohort of individuals at each generation, therefore creating selection for both increased intrinsic lifespan and contrasting sexual selection. If sexual selection augments natural selection then this experimental regime might result in more rapid evolution of increased intrinsic mortality under high male-male competition and female choice compared to low competition/choice or enforced monogamy. The experimental evolution protocol for all populations in this study, regardless of sexual selection treatment, imposes an artificially high extrinsic mortality immediately post-reproduction because no adults older than 20 days post-eclosion contribute to the next generation. Both evolutionary theories of ageing predict that any deleterious mutations acting after this age are hidden from selection and will therefore accumulate/persist (Medwar 1952; Williams 1957). This is predicted to cause a sharp decline in survivorship after this age (Partridge & Barton 1993), although curiously this was not seen in this *Tribolium* system where survivorship did not begin to decline until 3-5 months (80-140 days) post-eclosion.

Consequently, to select for increased lifespan in this species it might be necessary to age individuals for a minimum of 6 months, which would create significant limits on the number of generations that could be run.

Alternatively, although low/no sexual selection may lead to a relatively higher genetic load, organisms may be able to mitigate this under benign environmental conditions, through physiological stress pathways and phenotypic plasticity, however, the combination of environmental and genetic stress may cause minor detrimental fitness effects to become significant (Agrawal & Whitlock 2010). Evidence from studies of inbreeding supports the idea that environmental and genetic stress interact, such that the effects of deleterious alleles are amplified in stressful environments (Armbruster & Reed 2005; Fox & Reed 2011). Although a recent study using mutation accumulation lines found a consistent reduction in fitness as a result of increased genetic load, no evidence was seen of an interaction with abiotic stress (Andrew *et al.* 2015). A further investigation of intrinsic lifespan in individuals also facing sub-optimal abiotic conditions may reveal a benefit to sexual selection. The interaction between genotype and environment is discussed further below in relation to the results of resistance to abiotic stress.

Although no differences in intrinsic lifespan between contrasting sexual selection treatments were seen, unexpected sex-specific ageing profiles were discovered with males from all sexual selection treatments living much longer than females. Additional work investigating these sexually dimorphic ageing profiles is presented and discussed in more detail in Chapter 5 of this thesis. However, it is of interest to note that the divergent intrinsic ageing profiles correspond to equally divergent sex-specific declines in fertility and reproductive ageing. Males maintain consistently high fertility for at least 12 months of their life, while female fertility declined significantly after just 1 month of adulthood (see Chapter 5). These divergent reproductive profiles suggest intralocus conflict between male and female reproductive and ageing optima. Therefore, if sexual conflict prevents either or both sexes obtaining their fitness optima, this negative impact may outweigh beneficial effects from sexual selection. High sexual conflict in male-biased and polyandrous lines could therefore outweigh beneficial purging of mutation load, limiting differences between contrasting sexual selection treatments.

Contrary to the results of intrinsic ageing, the response of contrasting sexual selection histories on the responds to nutritional stress, provides more persuasive evidence that strong sexual selection has acted to decrease genetic load within loci responsible for coping with the environmental stress of starvation. Where the effects of deleterious mutations are small, favourable environmental conditions may mask negative fitness effects, however, under additional environmental stress these may be uncovered (Agrawal & Whitlock 2010). Stressful environments are predicted to either increase the mean cost of deleterious mutations and/or make their effects more variable, or alternatively increase the numbers that are expressed (Martin & Lenormand 2006; Reed *et al.* 2012). Theoretical work suggests that stress most often increases the variance of the negative fitness effects of mutation load (Martin & Lenormand 2006), although a review of the impact of stress on new mutations found the response to be inconsistent and dependent on the type of stress (Agrawal & Whitlock 2010).

Here, in control nutritional conditions, no differences were seen in viability between contrasting sexual selection treatments. However, under poor nutritional conditions the proportion of eggs that developed from monogamous lines decreased significantly. Although not replicated in the female-biased lines, this might reflect the fact that sexual selection is still acting to some extent in these lines, which is not the case under enforced monogamy in Regime B. In female-biased populations there may be competition between females, although this is likely to be less intense as males will also be under selection to mate with as many females as possible. However, there will certainly be a strong form of non-competitive selection acting on males to be able to mate with and fertilise many females, as is the case in some polygynous systems. In addition, under poor nutritional conditions, although mean development time did not differ between pairs of contrasting sexual selection treatments, the variance in development time increased in female-biased and monogamous lines, but not in male-biased and polyandrous lines.

The resistance of adults to starvation was greater in male-biased than female-biased individuals, and an interaction between sexual selection treatment and nutritional treatment was seen, such that individuals from male-biased and polyandrous lines showed significantly greater survivorship under nutritional stress than individuals derived from histories of weak sexual selection. Although increased survivorship following nutritional stress initially seems counter-intuitive, calorie-restriction is well known to extend lifespan across taxa (reviewed in Mair & Dillin 2008; Colman 2009). My results, showing enhanced survivorship under nutritional stress for strong sexual selection histories, clearly indicate a fitness advantage for individuals coming from populations experiencing high male-male competition and female choice. Increased resistance to starvation of strong sexual selection individuals suggests a better ability to acquire and make efficient use of nutritional resources in the face of competition and choice, which is consistent with the definition of condition applied by the genic capture hypothesis (Rowe & Houle 1996; Tomkins *et al.* 2004). Furthermore, following eclosion, no opportunity was given for feeding, so adults relied on resources gained as larvae to survive and operate, which suggests that, although sexual selection regimes are imposed on the adult life stage, benefits are also seen during development. Significant and consistent patterns across two independent but equivalent experimental evolution regimes applying weak versus strong selection, indicate a biologically relevant change and point towards nutrition as an ecologically relevant stress in this system, which sexual selection has also acted upon.

The results from the nutritional stress assays appear to provide good evidence for the population genetic benefits of sexual selection. However, these patterns were not corroborated by the results for thermal stresses. No difference was seen between contrasting sexual selection treatments in resistance to heat stress, and a history of male-biased sexual selection actually appeared to be a disadvantage under cold stress. Repeating this assay to include a comparison with control conditions is needed to investigate possible differences in variance. An explanation for the inconsistency in response to nutritional versus thermal stress might be that the stress must be ecologically relevant and, consequently, populations have experienced it in their evolutionary history for selection to act. All experimental evolution populations are maintained at a constant moderate temperature, and are therefore under no direct selection to be resistant to extreme heat or cold. However, although protocols aim to ensure nutritional medium is '*ad libitum',* it is plausible that varying density creates local competition between individuals for nutritional resources within populations, thereby generating some selection to be more efficient at obtaining and using nutritional resources. Both juvenile and adult flour beetles live in the medium that is also their food resource, therefore it can gradually lose its nutritional quality and accumulate waste products (Sokoloff 1974), which will increase nutritional stress, particularly when population densities become high. Furthermore, 'wild' populations of *T. castaneum* are likely to experience cycles of nutritional stress as a result of the management of dried food stores. Therefore, *T. castaneum* will have come under selection to cope with nutritional stress throughout their evolutionary history, while regular exposures to extreme temperatures are less likely to have occurred.

A review of the impact of stress on selection against new mutations found that where stress was purely abiotic, and therefore impacted all individuals equally, selection against mutations was inconsistent. However, where stress was density dependent, causing some individuals to gain limited resources but not others, this created stronger selection against deleterious mutations (Agrawal & Whitlock 2010). This could further explain the inconsistent results between thermal and nutritional stress in this study. Strong sexual selection will drive the evolution of individuals that are good at winning competitions over mating, however, if mating success is condition dependent these individuals are also likely to be good at competing for limited resources. Therefore, a history of sexual selection may provide an advantage where the stress is density/competition-dependent. To investigate this further, the nutritional stress during development assay could be repeated at different larval densities. If a history of strong sexual selection increases ability to acquire resources under competitive conditions, the greatest resistance to nutritional stress might be predicted where density is highest. Conversely, individuals with a history of low sexual selection, and therefore low competitive ability, combined with high density would be expected to show the greatest decrease in viability and subsequent adult survivorship. Chapter 3 of this thesis also investigates whether sexual selection creates all round better competitors by introducing individuals with contrasting sexual selection histories into novel populations, where they must perform successfully at every biotic and abiotic level to successfully reproduce and establish.

Chapter 3

Sexual selection history predicts the rate and extent of new population invasion

Abstract

Sexual selection is theorised to act either positively or negatively on population fitness. If sexual selection focuses mainly on reproductive traits, and/or generates sexual conflict, then overall population fitness could be constrained by trade-offs, or reduced productivity. On the other hand, genic capture theory proposes that sexual selection acts on condition in a wide range of traits, therefore selecting upon a wide range of naturally selected loci across the genome. Sexual selection should therefore promote natural selection by enhancing condition through the creation of populations which carry reduced mutation load and show greater fitness across a wide range of life history traits, and not just traits closely linked to mating, fertilisation and reproduction. Here I test this idea by examining whether sexual selection influences ability to biotically invade a new population. Such a test is a fair measure of genic capture theory because the ability to invade a population of new competitors, across multiple generations, captures condition and fitness across a range of relevant biotic levels and traits. This experiment also has relevance for understanding the role of mating pattern and sexual selection in population invasion, which has become a more prevalent phenomenon as humans increasingly modify, move and fragment natural systems. Male *Tribolium castaneum* from experimentally evolved populations with histories of contrasting strengths of sexual selection were introduced into intraspecific competitor populations carrying an observable dominant phenotypic mutant, and invasion was measured over 8 generations as the sexual selection wild-type introgressed and diluted the mutant population. Measuring from the second generation following introduction, when the dominant visible mutation could be disrupted and reproduction and fitness by both sons and daughters could become evident, I found that invasion was more rapid when the original introduction was by males from polyandrous sexual selection backgrounds, compared to those with a history of enforced monogamy. As well as being more rapid, introductions from polyandrous backgrounds achieved more complete saturation in the new populations, occupying 94% of the phenotypic variation, as opposed to 78% for saturation following introductions from monogamous backgrounds. These results provide clear evidence that a history of stronger sexual selection improves population fitness in the face of biotic challenge, and supports genic capture and a positive influence of sexual selection upon overall population fitness.

3.1 Introduction

Sexual selection is a distinct form of evolutionary selection which favours the most attractive or competitive individuals that gain the greatest mating and fertilisation success (Andersson 1994). Secondary sexual signals are energetically costly to develop and maintain, and can have negative impacts on survival (reviewed in Andersson 1994). Within a limited energy budget, when resources are allocated to one trait they become unavailable for others, therefore, investment in mating and fertilisation success is predicted to trade-off with other components of fitness (Stearns 1992). As a result, sexual selection is often viewed as working in opposition to natural selection, as benefits gained by greater reproductive success will be balanced against survival costs (Lande 1980; Kirkpatrick & Ryan 1991; Houle & Kondrashov 2001). Furthermore, sexual selection may generate sexual conflict when the evolutionary interests of males and females differ, and can result in the evolution of traits which increase the relative fitness of one sex while imposing costs on the other (Parker 1979). Empirical evidence reveals that male adaptations to manipulate female reproductive rate reduce lifetime reproductive output of females (reviewed in Arnqvist & Rowe 2005), which could have important implications for population fitness (reviewed in Holman & Kokko 2013). Therefore, either due to trade-offs between reproductive and other fitness components, or as a result of loss of fitness caused by sexually antagonistic traits evolving under sexual conflict, sexual selection is predicted to have negative or negligible effects on population fitness.

Alternatively, genic capture theory proposes that this increased reproductive success is not specifically due to secondary sexual traits, but ultimately a result of condition, defined as ‘the pool of resources available for fitness-traits’ (Rowe & Houle 1996). This resource pool includes both the ability to acquire resources, as well as make efficient use of them, and is expected to be associated with a wide range of physiology and behaviour determined by genes at many loci (Rowe & Houle 1996; Tomkins *et al.* 2004). As a result, sexual selection may act across an extensive proportion of the genome involved in the expression of condition, and not just on a relatively small number of loci responsible for secondary sexual traits (Rowe & Houle 1996). A significant outcome of the genic capture hypothesis is, therefore, that sexual selection and natural selection should work in synergy, by adding an additional layer of competition for mates on top of that for resources and survival, to ensure that only the highest condition individuals contribute to the next generation (Rowe & Houle 1996; Whitlock & Agrawal 2009). As a consequence of amplified selection for condition, sexual selection is theorised to both increase the rate of adaptation to changes in environment (Lorch *et al.* 2003), as well as increasing the rate of fixation of beneficial alleles and removal of deleterious mutations, thus reducing the negative fitness effects of genetic load (Whitlock 2000; Agrawal 2001; Siller 2001; Whitlock and Agrawal 2009). If sexual selection via genic capture has the potential to create populations with high condition and low genetic load, this will have a positive impact on all aspects of fitness, and not simply on traits related to mating (Lorch *et al.* 2003; Whitlock & Agrawal 2009). Furthermore, this positive impact on mean fitness may be important for determining population persistence and extinction avoidance, particularly in challenging or changing environments where the relative fitness costs and benefits of traits are altered (Whitlock & Agrawal 2009; Long *et al.* 2012; Holman & Kokko 2013).

Faced with unfavourable environmental pressures from modern anthropogenic impacts, organisms can either adapt, or disperse to more favourable conditions (Gienapp *et al.* 2008). Statistically significant shifts in range boundaries and in the timing of biological events are well documented across taxa and geographical regions (Parmesan & Yohe, 2003; Root *et al.* 2003) and are predicted to result in spatial and temporal disruption to biotic interactions (Tylianakis *et al.* 2008; Berg, *et al.,* 2010; Van der Putten *et al.* 2010; Walther 2010). Within a community, species' abilities to disperse and therefore the extent of their range shifts, will vary, creating novel, 'no-analogue', communities whose interactions currently have unknown outcomes (Williams & Jackson 2007; Gilman *et al.* 2010). Early theoretical models predicted climatic variables to have the greatest impact on species distributions, however, inclusion of biotic interactions, in order to predict responses to global environmental change, is now seen as fundamental, and theorised to have a greater impact on species persistence than the direct effects of abiotic changes (Araujo & Luoto 2007; Van der Putten *et al.* 2010; Gilman *et al.* 2010; Wisz *et al.* 2013). A recent review of empirical studies examining the impact of climate change on species extinctions found that species interactions, rather than physiological intolerance, is the common proximate cause of extinction (Cahill *et al.* 2013). Therefore, the ability to predict the outcome of biotic interactions will be important for informing the composition of future community assemblages, and consequently their conservation, under global environmental change (Tylianakis *et al.* 2008; Van der Putten *et al.* 2010; Walther 2010; Wisz *et al.* 2013; Louthan *et al.* 2015). Here I examine the impact of sexual selection on population fitness, and the consequences for the outcome of biotic interactions.

Theoretical and empirical evidence from invasion biology identifies competition within a trophic level, along with interactions with predators, parasites and pathogens across trophic levels, as key determinants of population establishment and growth following emigration (reviewed in Shea & Chesson 2002). Theoretical modelling predicts that competition for resources slows the ability of a species to track climate change by decreasing population abundance, preventing colonisation and/or causing extinction (Johansson 2008; Urban *et al.* 2012). Range overlap, or immigration, of ecologically similar species will create particularly high competition for resources (Gilman *et al.* 2010; Urban *et al.* 2012; Alexander *et al.* 2015). Competition for resources is costly for all participants, whether successful or not, as it will lead to increased resource use and decreased resource uptake. However, this negative impact is not equal for all participants as competition creates a situation where those better able to exploit a resource will decrease its availability for those already less proficient at gaining resources, and thus accentuate fitness differences between individuals (Begon *et al.* 2009). Therefore, the competitor that is most efficient at acquiring and using a resource, or if it has a smaller maintenance requirement, is predicted to dominate (Tilman 1982; Shea & Chesson 2002). Competition between members of the same sex for access to the opposite sex, intra-sexual competition, is a particular form of within-species competition, where mates are the limited resource (Andersson 1994). Genic capture theory assumes mating success is dependent on condition, the definition of which encompasses the ability to acquire resources (Rowe & Houle 1996; Tomkins *et al.* 2004). If sexual selection drives the evolution of individuals that are good at acquiring resources, then this might be reflected in competition for all resources and not just for mates. Conversely, if sexual selection benefits reproductive success at the expense of other fitness components, or leads to negative fitness effects of sexual conflict, individuals from backgrounds of high sexual selection could be inferior competitors across the full demands of life history. Therefore, a history of strong sexual might be an important indicator of inter-specific competition ability, and consequently biotic interaction/invasion success.

Reduction of genetic load, as a result of synergy between natural and sexual selection (Whitlock & Agrawal 2009), could also confer an advantage under competitive conditions. Negative biotic interactions, like an unfavourable abiotic environment, will reduce fitness of individuals relative to average conditions and are therefore considered to be a cause of stress (Bijlsma & Loeschcke 2005; Martin & Lenormand 2006). Organisms are often able to mitigate stress through physiological pathways and phenotypic plasticity, however, there is a common assumption that faced with additional reductions in fitness from deleterious mutations, the combined impact may become significant (Agrawal & Whitlock 2010). Evidence from studies of inbreeding support the theory that environmental and genetic stress interact, such that the effects of deleterious alleles are amplified in unfavourable environments (Armbruster & Reed 2005; Fox & Reed 2011). However, a review by Agrawal & Whitlock (2010), found that the only consistent empirical evidence of this was from studies that manipulated density and created intra-specific competition for limited resources. In these studies, the deleterious fitness effects of mutations were greater at higher density, which the review suggested was due to the asymmetry in fitness under competition (Agrawal & Whitlock 2010). Furthermore, where closely-related or ecologically analogous species (those with similar resources requirements and acquisition ability) compete for resources through inter-specific competition, modelling shows that a small increase in genetic load in one species can lead to extinction through competitive exclusion (Agrawal & Whitlock 2012). These studies suggest genetic load can have significant ecological consequences in competitive environments, and both individuals and populations with relative reductions in mutation load compared to competitors, may gain an advantage. Therefore, if sexual selection via genic capture improves the removal of deleterious mutations, this may provide a further benefit to populations facing competitive interactions.

Empirical studies of genic capture have investigated the reduction of genetic load by introducing mutations, then manipulating the level of sexual selection operating in populations to compare relative improvements in fitness. The results of these studies are inconsistent, with positive (Radwan 2004; Hollis *et al.* 2009; Almbro & Simmons 2014), negative (Hollis & Houle 2011) and no effect (Arbuthnott & Rundle 2012) of high male-male competition and female choice on the removal deleterious mutations being reported. An alternative approach is to consider the response of populations to inbreeding, which is expected to increase homozygosity and consequently expose deleterious mutation load (Charlesworth & Charlesworth 1987). Sexual selection was shown reduce the proportion of populations that went extinct as a result of inbreeding, suggesting a reduced rate of deleterious mutation accumulation (Jarzebowska & Radwan 2010; McGuigan *et al.* 2011). The problem with a number of these studies, however, is that population fitness was measured in tandem with sexual selection, which could, in turn, be confounded by simultaneous sexual conflict (Parker 1979; Whitlock & Agrawal 2009; Holman & Kokko 2013). To overcome this problem, Lumley *et al.* (2015) examined evidence for relative differences in genetic load and fitness in populations after they had been through long-term experimental evolution of high versus low sexual selection. By exposing load via inbreeding, populations with a history of strong male-male competition and opportunities for female choice showed subsequent resilience to extinction through inbreeding, and maintained fitness better than populations with histories of weak or no sexual selection. These results suggest a historic reduction in mutation load where populations had experienced strong inter-sexual competition and intra-sexual choice, and provide compelling evidence that sexual selection can work alongside natural selection to increase underlying fitness and potential for population viability in the face of genetic stress (Lumley *et al.* 2015). Here, I conduct a further empirical test by examining whether sexual selection leads to improved population fitness as measured by the ability to overcome biotic challenges and invade a novel intra-specific competitor population over 8 generations.

This study measures population fitness benefits of a history of sexual selection, with particular reference to a range of naturally selected traits that will be acting in addition to mating, and in both males and females. I use experimentally evolved lines of the promiscuous flour beetle *Tribolium castaneum*, which have been maintained for 77 generations under sole variation in contrasting levels of sexual selection. The common ancestry, and long-term experimental evolution in these lines make them an invaluable resource to investigate the impact of sexual selection history on population fitness. Moreover, the contrasting sexual selection regimes in the adult phase were applied while equalising effective population size, and measures of genome-wide heterozygosity demonstrate that there is no evidence for any differential inbreeding or genetic variation between the two regimes (Lumley *et al.* 2015). Manipulation of mating pattern and adult operational sex ratio was used to create populations evolving under enforced monogamy (1 male : 1 female) which removed the opportunity for sexual selection, and polyandry (5 males : 1 female) which allows male-male competition and female choice over mating and fertilisation. These populations were part of the study by Lumley *et al.* (2015), discussed above, which provided evidence of historic reduction in genetic load in populations with a history of stronger male-male competition and female choice. In addition, data presented in Chapter 2 of this thesis showed that individuals from polyandrous populations showed increased survivorship under starvation following larval development in a poor nutritional environment. These findings could indicate that individuals from polyandrous populations are more efficient at gaining and using nutritional resources, including during larval development, despite experimental evolution regimes only manipulating the strength of selection during the adult life stage. This new study will use invasion of an intra-specific competitor population as a holistic measure of the biotic fitness of individuals from populations with histories of contrasting sexual selection. Invasion success will be determined through the ability to compete across the entire life history and successfully reproduce, therefore indicating whether sexual selection can positively impact on a wider range of fitness traits. If a history of strong sexual selection increases condition and reduces genetic load, invasion of the competitor population is predicted to be more rapid and complete where introduced individuals have a history of polyandry, with higher male-male competition and opportunity for female choice, compared to a history of enforced monogamy. Conversely, if sexual selection leads to high investment in reproduction at the expense of survival, or generates sexual conflict, then benefits could be negated or even outweighed, which would be seen as slower and less extensive invasion and or even local extinction of the invader.

3.2 Methods

The invasion experiment was carried out using replicate lines which have been experimentally evolved under contrasting strengths of sexual selection using monogamous versus polyandrous mating patterns (Regime B) for 77 generations, and the 'Reindeer' (Rd) mutant stock population. For details of stock maintenance and experimental evolution regimes see General Introduction 1.5 and Figures 1.2.

The invasive ability of individuals from contrasting monogamous and polyandrous sexual selection treatments was investigated by introducing 10 males from these backgrounds into replicate populations of the Rd mutant, which is a standard phenotypic marker (Lewis *et al.* 2005; Tregenza *et al.* 2009) with distinctive swollen antennae, compared to wild-type which are filiform (Figure 1.1). The mutation is dominant and also homozygous within the Rd stock population, therefore, any offspring sired by introduced males from wild-type sexual selection treatment backgrounds were not visible in the F1 generation. For the wild-type phenotype to be expressed, individuals must be homozygous for the wild-type allele, therefore comparison of the extent of invasion began in the F2 generation, when wild-type sexual selection females may also have been produced. Only males were introduced at first to prevent against potential assortative mating in the parental generation, thereby allowing more focused tests of genetic introgression once they had mixed with the new population.

Population invasion protocol

All adults in the parental generation were sexed at pupation, and left to eclose and mature in single sex groups (20 individuals) for 10-12 days in *ad libitum* control food medium. Populations of Rd contained 50 females and 40 males. Ten sexual selection treatment males were then introduced such that populations had a 1:1 sex ratio, which creates levels of intra-sexual competition and inter-sexual choice consistent with natural populations. Four replicate populations (1 to 4) were created for each of the three independent lines (A, B, C) within both sexual selection treatments (monogamy versus polyandry) (n = 4 replicate populations x 3 independent lines = 12 total populations per sexual selection treatment). Parental populations were provided *ad libitum* control medium, and oats to aid locomotion, and left for 7 days to mate and oviposit. Adults were then removed from the medium and eggs/larvae were left to develop for 32-34 days allowing all to develop to full adult maturity.

For each subsequent generation ~450 (n = 167-586, mean = 461) of the adults were randomly sampled and placed in fresh medium for 7 days to mate and oviposit. Adults were then removed and frozen for counting, while eggs/larvae were left to develop. Due to high larval densities, additional control medium was provided 14 days into development to prevent population crashes as a result of extremely poor nutrition or disease. Frozen adults were divided into sexual selection treatment (wild type) phenotypes and reindeer phenotypes by examination of the antennae, and counted. The extent of population invasion at each generation was measured as the proportion of individuals in the population inheriting the wild type phenotype. See Figure 3.1 for protocol summary.

Statistical analyses

The invasive ability of groups from contrasting monogamous and polyandrous sexual selection treatments was compared by analysing the changes in proportions of wild-type phenotype in the replicate populations across generations. Proportion data are strictly bounded between 0 and 1, with non-normal errors and non-constant variance, making parametric analysis inappropriate therefore, the binomial distribution was used to describe the data. Proportions were compared by constructing a generalised linear mixed model (GLMM) in the 'glmmADMB' (Fournier *et al.* 2012) and 'R2admb' packages (Bolker *et al.* 2015). These packages allow models to be fitted using the negative binomial distribution which was necessary due to overdispersion in the data. GlmmADMB has two options for fitting a negative binomial model which both incorporate an additional parameter into the mean, variance relationship to account for overdispersion; i) nbinom: where variance = mean(1+mean/dispersion parameter) or ii) nbinom1: where variance = mean*scale parameter (Bolker *et al.* 2008; Fournier *et al.* 2012). Each option was tried and the model with the lowest AIC was selected. The response variable was entered as a paired variable containing the number of 'successes' and the number 'failures' to retain information on sample size within the model (Crawley 2013) and a maximal model was fitted with treatment (monogamy or polyandry) and generation (2 to 8) entered as fixed effects. Generation was also entered into the random effects to account for temporal autocorrelation, and population (1 to 4) was nested within independent line (A, B, C) to account for the hierarchical design.

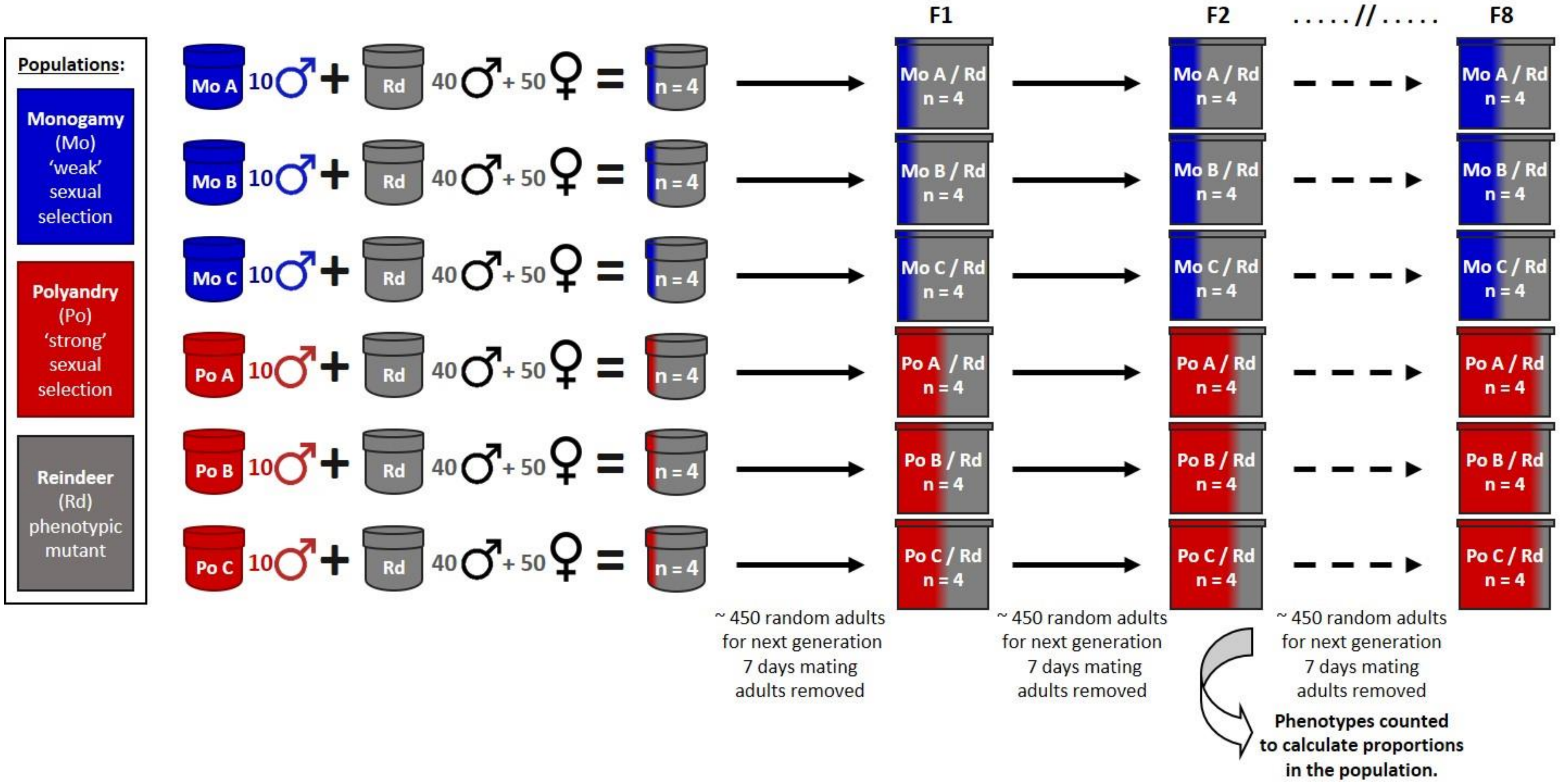


Figure 3.1 | Protocol – Introgression as a measure of condition and competitive ability. Males from contrasting monogamous (blue) and polyandrous (red) sexual selection treatments with a wild-type phenotype, were introduced into competitor populations of the 'Reindeer' phenotypic mutant. Proportion of the population expressing the wild-type phenotype was used as a measure of the extent of introgression (n = 4 replicates x 3 independent lines per sexual selection treatment = 24 total populations).

3.3 Results

Invasion of an intra-specific competitor population was more rapid when the initial introduction contained individuals with a history of polyandry, compared to a history of monogamy (Table 3.1, Figure 3.2). In generation 2 the mean proportion of individuals expressing the wild-type phenotype was 2% higher in populations where introduced males had a history of polyandry compared to monogamy. This difference increased to 9% in generation 3, and 27% in generation 4. After generation 4 the rate of invasion in populations where introductions came from a polyandrous background began to decelerate as the wild-type allele moved closer to fixation and the reindeer mutant locus was diluted. Where introductions had a history of monogamy, invasion experienced a greater lag phase before exponential population growth commenced (Figure 3.2).

Table 3.1 | Summary of invasion of an intra-specific competitor population by individuals with contrasting histories of sexual selection (SS) (* p < 0.05, *** p < 0.001).

Generation	**Mean proportion of sexual selection phenotype in the population (± SE)**		**Outcome of negative binomial GLMM (z)**	
	Monogamy	**Polyandry**		
2	0.04 (± 0.01)	0.06 (± 0.01)		
3	0.11 (± 0.01)	0.20 (± 0.02)		
4	0.22 (± 0.02)	0.49 (± 0.02)	SS treatment	**2.17 ***
5	0.44 (± 0.02)	0.71 (± 0.02)	Generation	**8.90 *****
6	0.63 (± 0.02)	0.84 (± 0.02)	SS treatment * Gen	-0.28
7	0.75 (± 0.03)	0.90 (± 0.01)		
8	0.78 (± 0.03)	0.94 (± 0.01)		

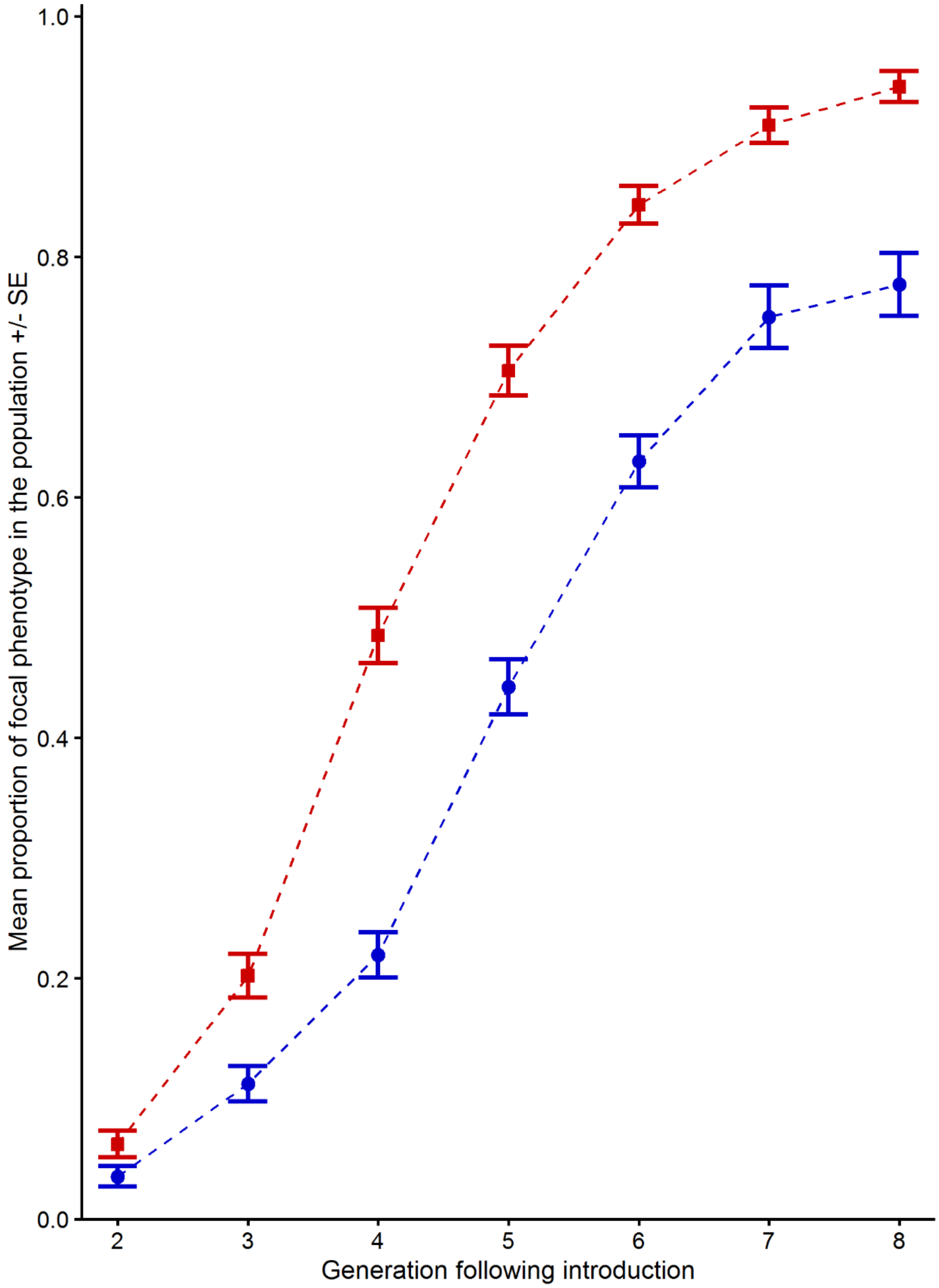


Figure 3.2 | Extent of invasion of intra-specific competitor populations by individuals with a history of monogamy (blue circles) versus polyandrous (red squares) across 8 generations. Data grouped by sexual selection treatment (n = 4 replicate populations x 3 independent lines per treatment). Extent of invasion differs significant between sexual selection treatments (z = 2.17, p < 0.05).

3.4 Discussion

In general, my findings provide support for genic capture theory within sexual selection, and no evidence that sexual selection or sexual conflict have negative influences on population fitness across the full life history and 8 generations. Introduced individuals from populations experiencing stronger sexual selection under a history of polyandry were more rapidly and effectively able to invade a new population, resulting in introgression of the wild-type genotype and dilution of the resident Reindeer phenotype. This improved ability to invade and penetrate the new population indicates that overall superior biotic fitness for individuals from populations with a history of stronger sexual selection.

It is established that sexual selection drives the evolution of traits that increase mating and fertilisation success (Darwin 1859, 1871; Parker 1970), and experimental evolution of contrasting strengths of sexual selection has been shown to lead to increased paternity share where males evolve under high male-male competition (Tilszer *et al.* 2006; Simmons & Garcia-Gonzalez 2008; Michalczyk 2011; Nandy *et al.* 2013; also see Chapter 4). Therefore, the higher rate of invasion where introduced individuals have a history of strong sexual selection could be driven by increased male-male and sperm competition success. However, invasion success is a holistic measure of fitness as it requires improved performance in both males and females across all life history traits from the egg, through larval development, pupation to adulthood and reproduction. Moreover, if the improved mating and fertilisation success of males alone explained superior invasion ability, we would expect to see a significant difference between polyandrous and monogamous introductions in the F2 generation, as a result of superior mating success in the Parental and F1 generations. Instead, we see a non-significant difference at the F2 generation of 2% versus 4% invasion success; as time proceeds, and the wild-type SS genetic background spreads, there is an accelerating rate of invasion, which eventually starts to saturate by generation 7 (Fig. 3.2). In addition, since fathers produce daughters as well as sons, we would expect female-derived fitness improvements to also contribute to this superior invasion for the high sexual selection background introductions. These patterns, together with the fact that lifetime fitness depends upon a wide range of traits, do not indicate that male mating success alone explains invasion.

High investment in energetically costly traits to increase mating and fertilisation success is predicted to trade-off with other components of fitness, therefore sexual selection is often viewed as working in opposition to natural selection (Lande 1980; Kirk patrick & Ryan 1991; Houle & Kondrashov 2001). However, genic capture theory states that mating and fertilisation success are ultimately determined by condition, which is primarily shaped by naturally-selected genes, consequently leading to synergy between sexual and natural selection, rather than antagonism (Rowe & Houle 1996; Lorch *et al.* 2003). High condition individuals will be able to sequester a greater pool of resources to allocate to fitness components, therefore alignment of natural and sexual selection for condition can increase resources available for non-mating fitness components (Lorch *et al* 2003). Higher fitness where there is a history of male-male competition and opportunity for female choice in polyandrous populations, suggests that natural and sexual selection are working in synergy in this system, creating populations where mutation load is reduced, condition is improved, and any trade-offs between lifetime and reproductive fitness components, in either males or females, are reduced.

An important goal of invasion biology has been to establish life history characteristics of organisms which predict successful invasion (Mack *et al.* 2000; Blackburn *et al.* 2011; Sol *et al.* 2012). Traits which enable rapid population growth, including high fecundity and growth rate, are considered to be crucial for establishment following movement to a new locality (Sakai *et al.* 2001; Estrada *et al.* 2016). If a history of strong sexual selection creates individuals that can achieve high condition, additional resources are expected to be allocated to viability and fecundity, which are additional key component of fitness (Rowe & Houle 1996). Mean offspring production of 'within-sexual-selection' treatment pairs was previously shown to be lower where there was a history of monogamy compared to polyandry (Lumley *et al.* 2015). It is not yet known whether this is driven by males or by females. In order to ascertain the extent to which this difference in fecundity contributes to invasion success, measures of mean offspring production of sexually selected males and females crossed with reindeer individuals, under both competitive and non-competitive mating scenarios, are needed and currently planned.

A recent within-species study of harlequin ladybirds showed that invasive populations not only produced more offspring, but also matured earlier, thus increasing their reproductive lifespan and producing a 'bet-hedging' strategy which could account for their invasion success (Tayeh *et al.* 2015). Data presented in chapter 2 of this thesis showed that individuals from strong sexual selection histories were more resistant to adult starvation. Furthermore, this result was augmented following larval development under nutritionally stressful conditions. These results indicate greater resource acquisition ability in juveniles from strong sexual selection backgrounds, which could increase the rate of development and provide an advantage in terms of earlier reproductive success. In addition, more rapid development could increase competition success across life history stages, not simply for adult reproduction, by allowing those that develop first to gain the highest quality resources. Depletion of resources is expected to exacerbate differences between competitors as those already less able to gain resources are also faced with fewer available (Begon *et al.*2009). Furthermore, in *T. castaneum* the active life history stages, larvae and adults, are known to sometimes cannibalise sessile eggs and pupae, particularly where density is high (reviewed in Sokoloff 1974), and nutritional resources are scarce (Via 1999). If the rate of development differs between competitors, then this may lead to cannibalism of slower developing rivals and aid invasion success even further. Finally, rather than faster development, improved juvenile resource acquisition could be invested in increased size, which is beneficial both for males in competition with rivals and for female fecundity (Stearns 1992; Andersson 1994) and could also contribute to invasion success. Comparison of the pupal mass of individuals from contrasting sexual selection treatments would provide a simple estimate of differences in resource acquisition during development, which is also planned. In order to link higher invasion rate by individuals with a history of strong sexual selection to heightened selection for condition, an important future study will be to repeat this invasion protocol under both control and nutritionally stressful conditions. If sexual selection creates individuals that are efficient at gaining and making use of resources, this will further increase their advantage where resources are limited, thus the divergence in invasion ability revealed by this study would be predicted to be exaggerated under nutritionally stressful conditions.

Polyandry is predicted to increase the opportunity for sexual conflict, which negatively impacts female fitness (reviewed in Arnqvist & Rowe 2005). Original work by Holland & Rice (1999), revealed greater reproductive rate of populations in which conflict was removed via experimentally enforced monogamy compared to promiscuous control populations, and suggested that promiscuous populations experience a ‘conflict load’ in which fitness benefits of sexual selection are outweighed by costs of conflict. Although, Holman & Kokko (2013) suggest that neither positive nor negative impacts of polyandry are likely to be visible where favourable environmental conditions allow females to produce higher numbers of offspring than are able to survive at density-dependent equilibrium. If sexual conflict is acting to dampen the benefits of sexual selection, then the result presented here is a conservative estimate of those benefits.

Furthermore, this experiment provides clear empirical evidence that sexual selection can have visible consequences for population demography.

A further theoretical outcome of genic capture is that greater reproductive success of high condition males can act as a filter that removes deleterious alleles, while promoting the fixation of beneficial ones, thus reducing genetic load (Whitlock & Agrawal 2009). Previous work with the experimentally evolved populations used in this study showed greater resistance to extinction under inbreeding in polyandrous populations, compared to monogamous ones, which suggests a reduced genetic load where there is a history of sexual selection (Lumley *et al.* 2015). Theoretical work predicts that deleterious mutations accumulate in individuals at the leading edge of a range shift, referred to as 'expansion load' (Peischl *et al.* 2013). If a history of sexual selection reduces genetic load, this should increase resilience of populations to expansion load, and aid range shifts and colonisation. Furthermore, an important aspect of invasion and colonisation is the increased opportunity for hybridisation as a result of the breakdown of physical, temporal and behavioural reproductive barriers (Anderson 1948; Allendorf e*t al.* 2001; Chunco 2014). Although recognised as an important aspect of historical evolution (Barton 2001; Seehausen 2004), hybridisation due to anthropogenic activities has been linked to the extinction of rare species and therefore is a concern for conservation (Rhymer & Simberloff 1996; Allendorf e*t al.* 2001). However, increases in genetic variance as a result of gene flow may be beneficial for colonisation and adaptation due to the increased possibility of advantageous allele combinations and phenotypic variants, as well as increases in heterozygosity and consequently reductions in the effects of deleterious mutations when populations face new environments (Hedrick 2013; Rius & Darling 2014; Colautti & Lau 2015; Dlugosch *et al.* 2015). Introgressive hybridisation (introgression) is the movement of genes from one population or species into another by the backcrossing of a hybrid with one of the parents (Anderson & Hubricht 1938; Anderson 1948). Theory predicts that advantageous alleles will introgress more rapidly than others, known as 'adaptive introgression' (Barton 2001), which could provide a significant advantage over new mutations or standing genetic variation in the rate of adaptation to changing environments and interactions (reviewed in Hedrick 2013 and Hamilton & Miller 2016). Therefore, if strong sexual selection aids the fixation of beneficial alleles, introgression might be expected to be more rapid where there is a history of male-male competition and opportunity for female choice. Exaggeration of male secondary sexual traits and associated mating success have been shown to increase the rate of introgression in wall lizards (While *et. al* 2015). In this *Tribolium* study, the wild-type antennae phenotype/allele has been used as a marker of the sexual selection history, however, it is likely that this association will break down with ongoing genetic mixing, although it is unclear how rapidly this may occur. While the wild-type allele appears to be moving to complete fixation in populations where introductions had a history of polyandry, the proportion of wild-type phenotype in monogamous populations appears to be levelling off at approximately 0.8 of the population. If adaptive alleles introgress more rapidly and individuals with a history of polyandry hold those adaptive alleles, this could explain how the marker allele could rapidly move to fixation where there is a history of polyandry, but become broken away from its genetic background where there is a history of monogamy and therefore persist at a lower level in the population.

Evidence presented here provides clear support for genic capture by showing that a history of strong sexual selection, with high male-male competition and opportunity for female choice, is beneficial to population fitness and viability and outweighs costs of sexually selected signals and/or negative impacts of sexual conflict. This effect was determined over a year-long population invasion study across 8 generations, using independent lines and replicated populations. A history of strong sexual selection conferred a significant advantage during intra-specific competition and invasion. Sexual selection drives the evolution of mating success, however, invasion and colonisation requires superior fecundity, viability and success in competition for all resources by both sexes, not just competition by males for mates, so these results provides evidence that sexual selection can provide wider fitness benefits to both sexes through all life histories. Finally, this evidence suggests that the strength of sexual selection acting in a population, which is determined by mating system, could be used to predict the outcome of competitive interactions leading to biological invasions.

Chapter 4

Experimental evolution reveals that sperm competition selects for directional, not stabilising, selection on sperm length

Abstract

Sexual selection continues after copulation in polyandrous mating systems through sperm competition and cryptic female choice. Sperm competition increases between-male variation in fertilisation success and consequently creates intense selection on traits which improve sperm competitive ability. Although number of sperm is known to be a vital component of fertilisation success in competition, the adaptive significance of sperm quality traits, including length, remain unclear. Sperm are fundamentally small, but size varies profoundly between species. If longer sperm are more competitive, sperm competition may drive directional selection for increasing sperm length. On the other hand, a reduction in sperm length may allow greater numbers of sperm for competition. Sperm competition is also predicted to stabilise variation in length, both between and within males, as sperm are driven towards a competitive optimum. So far, most studies into the evolution of sperm length under sperm competition are restricted to observational analyses across species, where other uncontrollable confounds may have influence. Here, I use experimental evolution with replicate populations of the promiscuous flour beetle *Tribolium castaneum* to measure how sperm competitiveness, and sperm length and variance, evolve when exposed to divergent intensities of sexual selection through female-biased versus male-biased adult sex ratios over 75+ generations. First, I confirmed that divergent sexual selection had led to differences in sperm competitiveness between males: in competition with a single rival, males from male-biased sexual selection backgrounds gained a 20% higher paternity share than those from a female-biased sexual selection background. In association with increased sperm competitiveness, male-biased sexual selection males also had significantly longer sperm. However, contrary to comparative studies, inter-male or intra-male variance in sperm length did not differ between treatments. Having used experimental evolution to create differences in sperm competitive ability between populations, I then demonstrate within a species that sperm competition causes divergence in total sperm length, but does not reduce sperm length variance.

4.1 Introduction

Sexual dimorphisms in reproductive physiology, morphology and behaviour, 'Darwinian' sexual selection, ultimately arise as a result of anisogamy (Darwin 1874; Bateman 1948). Production of numerous, small gametes increases the chances of fusion success for fertilisation. However, a zygote formed of two tiny gametes, with little or no provisioning, will be at a disadvantage in terms of offspring fitness, so parallel selection will also favour large, well provisioned gametes. Theory proposed that disruptive selection as a result of these alternate and opposing selective forces, will act simultaneously on isogamous gametes leading to small competitive gametes (sperm), and large nutritive gametes (ova) (Parker 1972). These contrasting approaches to increasing fitness mean that (in general), females are reproductively limited by the number of offspring they can produce, while males are limited by access to mates and fertilisation success. Males are therefore under greater selection to maximise number of mates, while selection on females will act more upon ability to choose mates of the highest quality (Darwin 1871; Bateman 1948). It is these fundamental principles of potential reproductive rate and between-adult variance in reproductive success which underpin the force, and diverse outcomes, of sexual selection (Andersson 1994; Dewsbury 2005).

Along with these fundamental differences between males and females, we now know that polyandry, when females mate with multiple males, is a widespread mating pattern across animal taxa (Taylor *et al.* 2014). Parker (1970) first highlighted the important consequence of polyandry for creating an opportunity for sexual selection to continue beyond mating for fertilisations. The overlap of sperm from different males in the female reproductive tract creates competition between the sperm of rivals, sperm competition (Parker 1970), and also opportunities for cryptic female choice (Eberhard 1996). These two processes drive the evolution of peri- and post-copulatory courtship behaviours, genitalic and spermatozoal morphology, and a diverse set of non-sperm ejaculate components (reviewed in Eberhard 2009; Simmons & Fitzpatrick 2012; Rowe *et al.* 2015), some of which are the fastest-evolving traits known (Swanson & Vacquier 2002; Haerty *et al.* 2007). However, post-copulatory competition and choice are now thought to not only result in rapid divergence of reproductive traits, but critically to maintain anisogamy (Parker 1982), and also drive the subsequent 'sexual cascade' resulting in distinct sex roles and pre-copulatory 'Darwinian' sexual selection (Parker 2014; Parker & Pizzari 2015).

Sperm competition creates between-male variation in fertilisation success, and consequently intense selection on traits that either increase sperm competitive ability or reduce rival sperm success (Parker 1970). Sperm traits that contribute to fertilisation success are often partitioned into: i) the number of gametes and ii) their 'quality' or fertilising efficiency and performance (Snook 2005; Pizzari & Parker 2009). Theoretical models (Parker 1982, 1990; reviewed in Parker & Pizzari 2010) and empirical evidence (reviewed in Wedell *et al.* 2002; Gage and Morrow 2003) show number of sperm to be a vital component of fertilisation success in the face of competition, when fertilisations are won through a 'raffle principle'. However, although sperm contribute less to the zygote than ova, they are no longer considered to be a cheap, unlimited resource (Dewsbury 1982), and it is now recognised that male reproduction is also under the universal constraint of a fixed energy budget (Parker 1982; Parker 1998). Thus, investment in high numbers of sperm is predicted to trade-off against investment in provisioning individual sperm cells, as well as quality, non-sperm ejaculate components or post-mating behaviours such as mate guarding. Because of these, selection is expected to act so that males invest in ejaculates strategically (Parker & Pizzari 2010; Simmons & Fitzpatrick 2012; Perry *et al.* 2013). Parker (1982) theoretically showed that even a small risk of sperm competition is enough to ensure that the benefit from zygote provisioning is heavily outweighed by the cost in terms of producing numbers of sperm to compete for fertilisations. Sperm competition is therefore predicted to maintain small sperm size to ensure maximum numbers, and is a favoured mechanism for the maintenance of anisogamy (Parker 1982; Pizzari & Parker 2009).

Sperm cells are the most diverse eukaryotic cells known, with profound variation in morphology, behaviour and longevity (Gage 2012; Birkhead, Hosken & Pitnick 2009). This vast diversity, along with their fundamental role in reproduction, suggests intense selection on individual sperm cell form and function (Pitnick 2009). However, unlike sperm number, the adaptive significance of variation in sperm quality traits, including the simple morphometric variant of size, remains unclear (Snook 2005; Pizzari & Parker 2009). Despite the expected constraints due to anisogamy (Parker 1982), sperm size (usually measured as length) across taxa is hugely variable (Pitnick *et al.* 2009) and convergent evolution of species producing relatively few giant sperm is well known (e.g. Pitnick *et al.* 1995). As increasing sperm size will be costly due to the trade-off with number of sperm produced (Parker 1982), this must be outweighed by benefits from increased fertilisation success under competitive conditions (Simmons 2001; Pitnick 2009). Moreover, any relationships between sperm size and sperm competition can provide us with an insight into the mechanisms of sperm competition and how post-copulatory sexual selection acts on gamete evolution. Comparative analyses of associations between sperm length and sperm competition risk are inconclusive and have found positive, negative and no association between sperm competition and sperm length, as well as conflicting results within taxa (summarised in Snook 2005; Simmons and Fitzpatrick 2012). It is probable that additional differences between taxa, such as spatial and temporal influences surrounding the female fertilisation environment, act on the evolution of sperm form and function (Snook 2005; Pitnick *et al.* 2009). Sperm competition is theorised to be at its most intense where sperm from different males are stored together (Parker 1970), therefore internal fertilisation and sperm storage might be expected to generate quite different selective pressures to external fertilisation. Although some early studies suggested that the sperm of internally fertilising species are longer (and more diverse) than external fertilisers, this has yet to be rigorously analysed (Pitnick *et al.* 2009). Likewise, the extent and predictability of the fertilisation window will act on sperm longevity, behaviour and possibly structure, confounding relationships with sperm competition (Pizzari & Parker 2009).

Increasing sperm size is likely to result in qualitative influences on the male gamete. Longer sperm increased velocity, providing a competitive advantage in the race for fertilisation. Increasing flagella length may deliver additional thrust, or a longer mid-piece with more mitochondria may allow greater energetic capabilities. Despite this seemingly intuitive relationship between length and speed, evidence linking the two is ambiguous (reviewed in Snook 2005 and Simmons & Fitzpatrick 2012), and a critique by Humphries *et al.* (2008) suggests the biomechanics and physics of this assumption are flawed as the hydrodynamics experience by a sperm cell are very different to those on which these assumptions are based. In species that store sperm, it may be that competition is for storage, rather than a race for fertilisation. Evidence suggests longer sperm are better at displacing rivals or defending their position in female storage (Pattarini *et al.* 2006). Alternatively, sperm length may influence longevity. A longer mid-piece and better energy reserves may increase longevity and ensures greater male representation over time. However, longer flagella will have higher energy demands which could decrease longevity (Parker 1998). Mode of fertilisation (Parker 1993, Ball & Parker 1996; Snook 2005) and mechanism of sperm competition (Immler *et al.* 2011) are likely to be crucial in determining the relative importance of velocity and longevity, and therefore sperm size. Alternate benefits of increasing sperm length and conflicting empirical evidence shows our understanding of the impact of sperm length on fertilisation success and therefore the consequences of sexual selection on sperm length, are far from clear, and a more controlled experimental approach that is free from cross-species confounds may shed light on the true relationship between sperm competition and sperm size.

As well as directional effects on sperm length, sperm competition may act on the variation in sperm length. If sperm competition risk is high, then heightened selection could stabilise variation by driving sperm length more effectively towards the optimum, improving chances of fertilisation success under competition (Parker & Begon 1993). Such stabilisation could act at the population level, driving down between-male variation in sperm size, and at the level of individual males through selection for greater quality control within spermatogenesis, where vast numbers of cells are produced at very high rates in reproductively active males, to reduce production errors and ensure that every sperm is viable in competition (Cohen 1973; Hunter & Birkhead 2002). Conversely, relaxed post-copulatory sexual selection could reduce selection on the sperm size optimisation. Evidence for this comes from species with exceptionally degenerative sperm morphology for their taxa, which are also associated with very low or absent selection from sperm competition (van der Horst & Maree 2013). Alternatively, sperm competition may create variation between individual males (but not within) by promoting contrasting optima where alternative male roles have evolved. Parker (1990b) theoretically showed that alternative male mating strategies, ‘guarding’ and ‘sneak’, should invest differently in sperm due to the inherently different sperm competition risks posed by their respective mating roles. Tests of this possibility in Atlantic salmon anadromous *versus* sneaker parr male morphs with alternative mating strategies yielded no evidence for differences in sperm morphology between male roles (Gage *et al.* 1995). Conflict between diploid and haploid expression might also increase variation within an ejaculate, but this intra-ejaculate competition is expected to be non-significant when there is sperm competition between rival males (Parker & Begon 1993).

A number of comparative studies in passerine birds (Calhim *et al.* 2007; Immler *et al.* 2008; Kleven *et al.* 2008; Lifjeld *et al.* 2010), murine rodents (Šandera *et al.* 2013; Varea-Sanchez *et al.* 2014) and social insects (Fitzpatrick & Baer 2011), and an intraspecific study in barn swallows (Laskemoen *et al.* 2013), have found evidence for decreased sperm length variance, both between males and within males, in species with predicted high levels of sperm competition. In the promiscuous superb fairy wren (Calhim *et al.* 2011), some variation was maintained, but this was interpreted due to selection for alternative sperm length optima for either defending position in storage, or outcompeting already-stored sperm. Therefore, an increasing body of evidence indicates decreased variance (stabilising selection) is seen in species with high sperm competition levels, and it has been suggested that sperm length variance itself could be used as a measure of a species' level of sperm competition (Lifjeld *et al.* 2010). However, two problems face these studies: (i) all make use of indirect indicators of sperm competition level, such as extra pair paternity (EPP) or relative testis size, and (ii) the majority examine relationships between taxa where other confounds could be acting on sperm length variance. Currently, no investigation has used an experimental approach, coupled with direct measures of sperm competition, to measure the impact on sperm length divergence, or the variance in sperm length between and within males. Here, I use independently replicated lines of the promiscuous flour beetle *Tribolium castaneum,* which have been experimentally evolved under sole variation in the strength of sexual selection, to determine how sperm total length and variance evolve as a consequence of sperm competition.

Although often requiring longer-term approaches, experimental evolution has proven to be a powerful tool for studying evolved responses to sexual selection (Garcia-Gonzalez 2011). Experimental evolution of the opportunity or intensity of sexual selection enables the traits which respond to selection to be identified and measured, while all other aspects of the biotic and abiotic environment are kept constant. Sperm competitive ability has been shown to decrease following experimental removal of sexual selection through enforced monogamy (Hosken *et al.* 2001; Tilszer *et al.* 2006; Simmons & Garcia-Gonzalez 2008) and increase in males evolving in a high male-male competition background (Nandy *et al.* 2013). The studies of enforced monogamy by Hosken *et al.* (2001) and Pitnick *et al.* (2001) also measured sperm length. This was predicted to decrease following removal of sexual selection as males no longer needed to maintain a costly trait, but no significant difference in length was found between treatments. Only a single study in *C. elegans* has found an effect of sperm competition on sperm size (LaMunyon & Ward 2002), however this nematode has amoeboid sperm which makes comparisons with the majority of flagellate sperm more difficult to draw.

To allow a more controlled approach to the question of how sperm competition interacts with sperm length and sperm length variance, this study makes use of experimentally evolved populations of the model insect *Tribolium castaneum*, which vary only in the contrasting levels of sexual selection adults have faced over 75+ generations. *T. castaneum* are highly promiscuous (Lumley *et al.* 2015), females store sperm from multiple males (Arnaud *et al.* 2001), and show little evidence for pre-copulatory mate discrimination, while evidence for post-copulatory sperm competition and cryptic female choice is more plentiful (reviewed in Fedina & Lewis, 2008). This makes *T. castaneum* ideal for investigations into sperm competition and its impact on sperm trait evolution. Manipulation of the operational sex ratio was used to create male-biased (90 ♂ : 10 ♀) and female-biased (10 ♂ : 90 ♀) populations with contrasting levels of male-male competition and female choice. Contrasting male-male competition is expected to be particularly significant in these populations as, on average, nine males are in competition for access to every female and her ova in the male-biased treatment, while in the female-biased treatment competition between males is unlikely. Previous work with these populations, following just 20 generations of selection, showed males from the male-biased sexual selection treatment were 27% more successful in competition with a rival for both mating and fertilisation, than males from the female-biased sexual selection treatment. However, assays focusing on sperm competition alone showed no significant differences between the sexual selection treatments, suggesting that divergence in male competitive ability was a result of mating behaviour rather than sperm competitive ability (Michalczyk *et al.* 2011). The aim of this new study is to determine whether, following further generations of experimental evolution, sperm competitive ability has diverged significantly. Having established evidence for any divergence, I then investigate associated evolutionary changes in sperm length and sperm length variance. Although the impact of sperm competition on directional changes in sperm length is uncertain, if investing in sperm length allows qualitative improvements in sperm function that make these cells more competitive, I predict a directional shift in mean sperm length to be greater under male-biased sexual selection, and/or a reduction in sperm length under female-biased histories of sexual selection. In addition, sperm length variance is predicted to decrease, both within and between males, if male-biased sexual selection and sperm competition intensity drives sperm length closer to an optimum, or (conversely) female-biased sexual selection allows sperm size to drift away from the optimum, and/or relax the efficiency of spermatogenesis.

4.2 Methods

The following assays were carried out using replicate lines which have been experimentally evolved under differing intensities of sexual selection using female-biased versus male-biased adult population structures (Regime A). For details of stock maintenance and experimental evolution regimes see General Introduction 1.5 and Figure 1.2.

4.2.1 Sperm competitive ability

Sperm competitive ability of individual males from male-biased versus female-biased selection treatments was assessed following 77 generations of experimental evolution. Sexual selection treatment males (experimental focal males) were competed against ‘Reindeer’ (Rd) males for fertilisation of a balanced mix of females from the respective sexual selection treatment. The Rd mutation is dominant and homozygous within the Rd population, therefore all offspring sired by a Rd male have distinctive swollen antennae, while offspring of the focal males have ‘wild-type’ (WT), filiform antennae (Figure 1.1). The Rd marker is a standard mutant used in sperm competition experiments (Lewis *et al.* 2005; Tregenza *et al.* 2009). Treatment females were used to maintain any coevolved effects of cryptic female choice within treatments, and to avoid the possibility for differential effects of control females upon treatment male competitiveness. Within sexual selection treatment, both within- and between-line crosses were carried out to allow investigation of any differences in fertilisation compatibility between populations (Figure 4.1). A total of 139 sperm competition trials were run between Reindeer and female-biased males, and 143 trials with male-biased males.

All adults were virgin and 10-12 days post-eclosion, and were isolated at the pupal stage to equalise any developmental effects. Females were paired with a Rd male for 24 hours, allowing mating and full storage of Rd sperm. Rd males were then removed and females were presented with an experimental focal male from either the male- or female-biased sexual selection treatment, pairing male-biased males with male-biased females, and vice versa. Pairs were left to mate for 1 hour, then males were removed. After the second mating, females were transferred to a petri dish of fresh medium and left to oviposit for 10 days, and then transferred to another petri dish of fresh medium for a further 10 days, before finally being removed. Thus, I collected offspring to determine the outcome of sperm competition over 20 days of oviposition. Previous work in this lab group has shown that offspring production from the first

20 days of oviposition following a single mate predicts total offspring production for that mating (R^2= 0.38) and accounts for ~50% of the potential total offspring production from such a mating period (Gage & Dickinson unpubl. data). The petri dishes containing eggs/larvae were left to develop into adults and the number of each phenotype (Rd and wild-type) was counted. Sperm competitive ability between sexual selection treatments was assessed by comparing the proportion of offspring showing the wild-type phenotype.

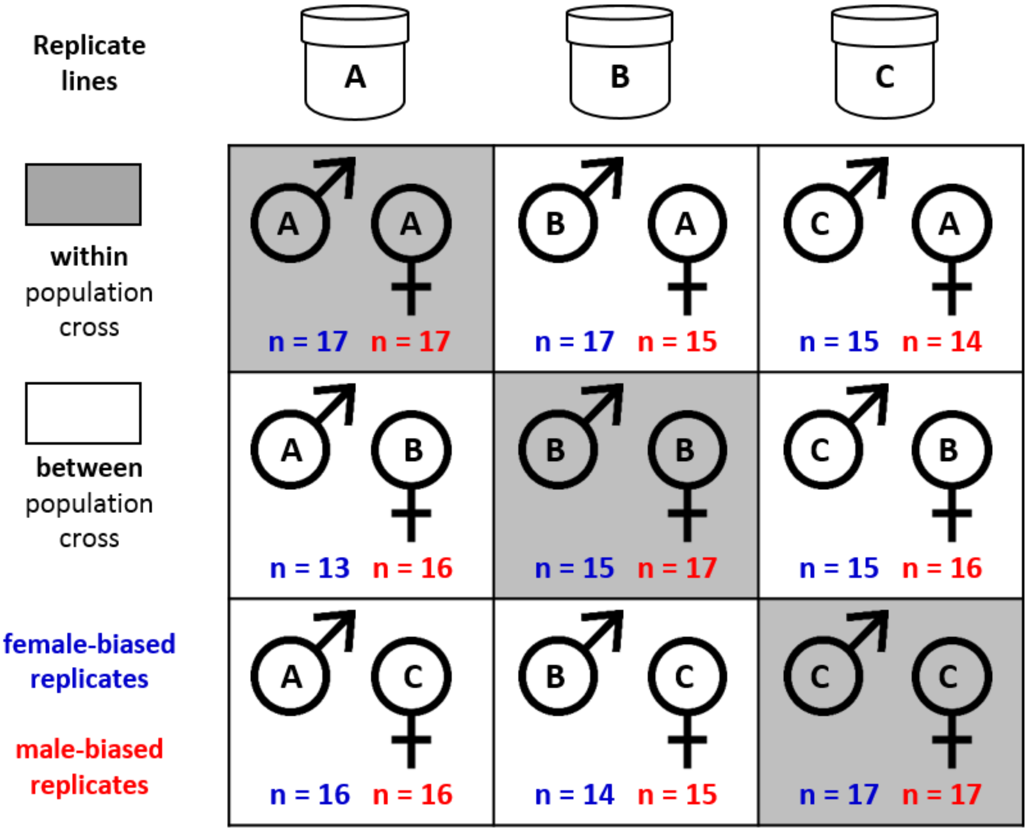


Figure 4.1 | Within-treatment mating crosses. All combinations of within (grey blocks) and between (white blocks) crosses were carried out to allow additional investigation of any differential reproductive isolation between treatment replicate lines (n = 3 lines per treatment).

Statistical analyses

Offspring production (20 days of oviposition) for each female was checked before analysis. Normal oviposition rate in these populations is 10+ eggs per day for the first few weeks after mating (Lumley *et al.* 2015). Females that died or produced <20 offspring over 20 days were considered aberrant and therefore discarded from the dataset (deaths: n = 3/290, low offspring production: n = 5/290). Total offspring production (wild type + Rd) was compared between sexual selection treatments to gather information regarding divergence in reproductive traits. Data met the assumptions of parametric analysis therefore a linear mixed effects model (LMM) was fitted using the 'lme4' package (Bates *et al.* 2015) with sexual selection treatment (female-biased or male-biased) entered as a fixed effect and female line (A, B, C), nested within male line (A, B, C), entered as random effects to account for the hierarchical design.

Sperm competitive ability

Sperm competitive ability was measured as the proportion of offspring sired by experimental focal males. Proportion data are strictly bounded between 0 and 1, with non-normal errors and non-constant variance, making parametric analysis inappropriate therefore, the binomial distribution was used to describe the data. Proportions were compared by constructing generalised linear mixed models (GLMMs) in 'lme4' (Bates *et al.* 2015) with a binomial error structure which incorporates a 'logit' link function to apply an inverse logistic transformation (Crawley 2013; Bates *et al.* 2015). The response variable was entered as a paired variable containing the number of 'successes' and 'failures' to retain information on sample size within the model (Crawley 2013). Models were assessed for overdispersion, using 'overdisp_fun()' (Bolker *et al.* 2008) and where necessary, this was accounted for using a negative binomial error distribution in the 'glmmADMB' (Fournier *et al.* 2012) and 'R2admb' packages (Bolker *et al.* 2015). GlmmADMB has two options for fitting a negative binomial model which both incorporate an additional parameter into the mean, variance relationship to account for overdispersion; i) nbinom: where variance = mean*(1+mean/dispersion parameter) or ii) nbinom1: where variance = mean*scale parameter (Bolker *et al.* 2008; Fournier *et al.* 2012). Each option was tried and the model with the lowest AIC was selected.

To investigate differences in the proportion offspring sired by focal males in contrasting sexual selection treatments, a maximal model was fitted with treatment (female-biased or male-biased) entered as the fixed effect and female line (A, B, C), nested within male line (A, B, C), entered as random effects to account for the hierarchical design. Where the proportion of offspring sired by the sexual selection treatment male was equal to 0 or 1 it was unknown whether this showed failure to mate by one of the males or total dominance of a single male's sperm in competition, therefore analyses were repeated without these data points to assess their influence.

Sperm competitive ability within treatments

Within each treatment GLMMs were fitted to investigate differences in the proportion of offspring sired by focal males in within and between replicate crosses. Higher values in within line crosses would suggest a degree of reproductive isolation between independent replicates; this might be expected to be more exaggerated in the male-biased sexual selection lines. A maximal model was fitted with cross (within and between) entered as the fixed effect and female line (A, B, C), nested within male line (A, B, C), entered as random effects to account for the hierarchical design. Within treatment analyses were also repeated following the removal of proportions equal to 0 and 1.

4.2.2 Sperm length

Having established sperm competition differences, sperm length was measured in the same lines after 83 generations of experimental evolution, using similar 10-12 day post-eclosion unmated males which had been isolated at the pupal stage to equalise any developmental effects. Sperm were recovered from females using microdissection, after spermatophores had been deposited during mating, thereby ensuring that sperm were functional and mature.

Pairs of beetles (n = 10 males x 3 lines = 30 total males per treatment) were placed in 1 x 1cm mating arenas at standard conditions for 30 minutes, which normally allows a successful mating. Females were then removed and decapitated to prevent movement of sperm to long-term storage. The female reproductive tract was isolated by extruding the ovipositor and gently pulling to detach it from the abdomen. This tract was placed in a drop of buffer (0.9% NaCl) and the spermatophore isolated. Finally, the spermatophore was transferred to a drop of fresh buffer on a clean microscope slide, teased open, and the slide flooded with further buffer to disperse the sperm. Slides were left to dry then dipped in distilled water to remove buffer residue, and re-dried.

Slides were viewed using an Olympus BX41 system microscope with phase contrast, at 60x magnification. Images were captured using a JVC KYF70B digital camera and Auto-Montage 4.00.0413 imaging software (Syncroscopy 2015). Sperm were measured using the 'ImageJ' image analysis package (Schneider *et al*. 2012). Total length was measured by creating a segmented line which traced the length of the cell (Figure 4.2). Length was measured in pixels then transformed into micrometers (um) by applying a conversion factor which was established by counting the pixels in one 10um division of a 1mm graticule. 30 sperm per male were measured which provides an accurate estimate of variation (Sokal & Rohlf 1981; Immler et al. 2008). All sperm from a single male from a male-biased replicate were eliminated due to obvious breakages as sperm were viewed and measured (n = 30 sperm x 9-10 males x 3 lines = 870-900 total sperm in either treatment).

Repeatability of the measuring technique

Sperm were measured by 2 investigators. To assess repeatability, 20 sperm were measured by both investigators, and the intra-class correlation coefficient (ICC) calculated. Repeatability was found to be very high, ICC = 0.99 (± 0.005) (Lessells & Boag 1987; Nakagawa & Schielzeth 2010). Pairs of measurements were also correlated against each other (r = 0.99) and a Bland-Altman plot created to visualise 'agreement' between investigators (Figure 4.3) (Bland & Altman 1986; Bartlett & Frost 2008).

Statistical analyses

Sperm length divergence

Sperm length data met the assumptions of parametric analysis, therefore a linear mixed effects model was fitted to investigate differences between treatments. Sexual selection treatment (female-biased or male-biased) was entered as a fixed effect and replicate male (a-j), nested within replicate line (A, B, C) were entered as random effects.

Figure 4.2 | Sperm measuring technique. Total sperm length was measured by creating a segmented line (yellow line) tracing the length of the cell using the 'ImageJ' image analysis package (Schneider *et al*. 2012).

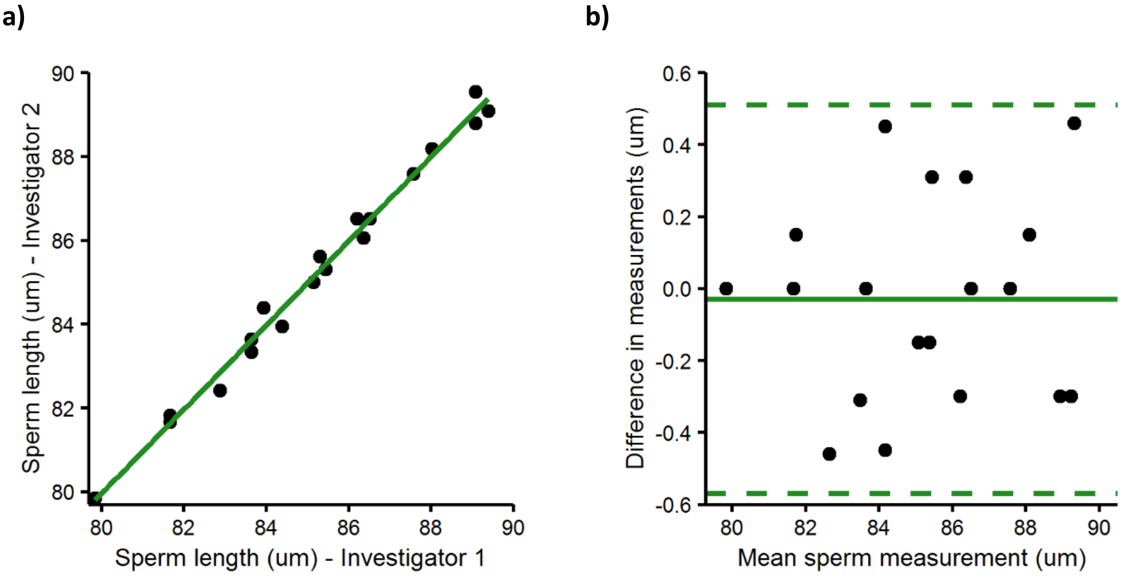


Figure 4.3 | Agreement of sperm measuring technique between 2 investigators. a) Correlation of measurements by 2 investigators (r = 0.99). b) Bland-Altman plot showing agreement between investigators, mean (solid line) ± 95% CI (dashed lines).

Sperm length variance

Sperm length variance was calculated as a standardised coefficient of variation (CV). This was calculated both within males (CV_{wm}) and between males (CV_{bm}) for each treatment replicate. The within male CV for a population is a mean of 10 individual male CVs. The between male CV for a population is calculated using the mean sperm length of each male, therefore this value has no confidence intervals. A linear mixed effects model was fitted to compare within male CV (n = 9-10 x 3 lines = 29-30 total CV_{wm} per treatment) between treatments. A maximal model was fitted with treatment (female-biased or male-biased) entered as the fixed effect and line (A, B, C) entered as a random effect to account for the hierarchical design. A Wilcoxon rank-sum test was used to compare between male CVs (n = 1 x 3 populations per treatment).

4.3 Results

4.3.1 Sperm competitive ability

Sperm competitive ability (mean ± SE) differed significantly between the males from female-biased and male-biased sexual selection treatments ($z = 3.5$, $p < 0.01$; Figure 4.5). Males from the male-biased sexual selection treatment sired 20% more offspring (0.65 ± 0.04) in sperm competition with a single rival, than males from the female-biased treatment (0.45 ± 0.04). Removal of individuals that appeared to sire all or none of the offspring (proportions equal to 0 and 1) (female biased males n = 13/139, male biased males n = 12/131) decreased the difference between treatments to 17% although this remained highly significant ($z = 2.85$, $p < 0.01$). Males from male-biased sexual selection lines sired 0.65 (± 0.04) of offspring, while the proportion for female-biased selection males increased slightly to 0.48 (± 0.04). Total offspring production of male-biased matings (151 ± 8) was slightly lower than in female-biased matings (166 ± 6) however this difference was not significant ($\chi^2_{(1)} = 2.95$, $p = 0.09$).

Within treatments

No evidence was found for any differential compatibility and/or reproductive isolation between lines following male-biased versus female-biased sexual selection treatments (Table 4.1 and Figure 4.4). Small differences in the proportion of offspring sired by experimental focal males in within and between line crosses were non-significant and also did not necessarily follow the expectation that greater paternity would be gained in within line crosses. Analyses were repeated following exclusion of proportions equal to 0 and 1 however this made little difference to the proportion of offspring sired by experimental focal males (Table 4.1).

Table 4.1 | Summary of within treatment sperm competitive ability analysis.

Sexual selection treatment	**Mean proportion of offspring sired by experimental focal males (± SE)**		**Outcome of GLMM**
	within line crosses	between line crosses	
Female-biased	**0.44** (± 0.04)	**0.46** (± 0.04)	z = -0.68, p = 0.50
0, 1 excluded	0.44 (± 0.04)	0.50 (± 0.04)	z = -2.36, p = 0.02
Male-biased	**0.68** (± 0.04)	**0.63** (± 0.04)	z = 1.13, p = 0.26
0, 1 excluded	0.67 (± 0.04)	0.64 (± 0.04)	z = 0.17, p = 0.87

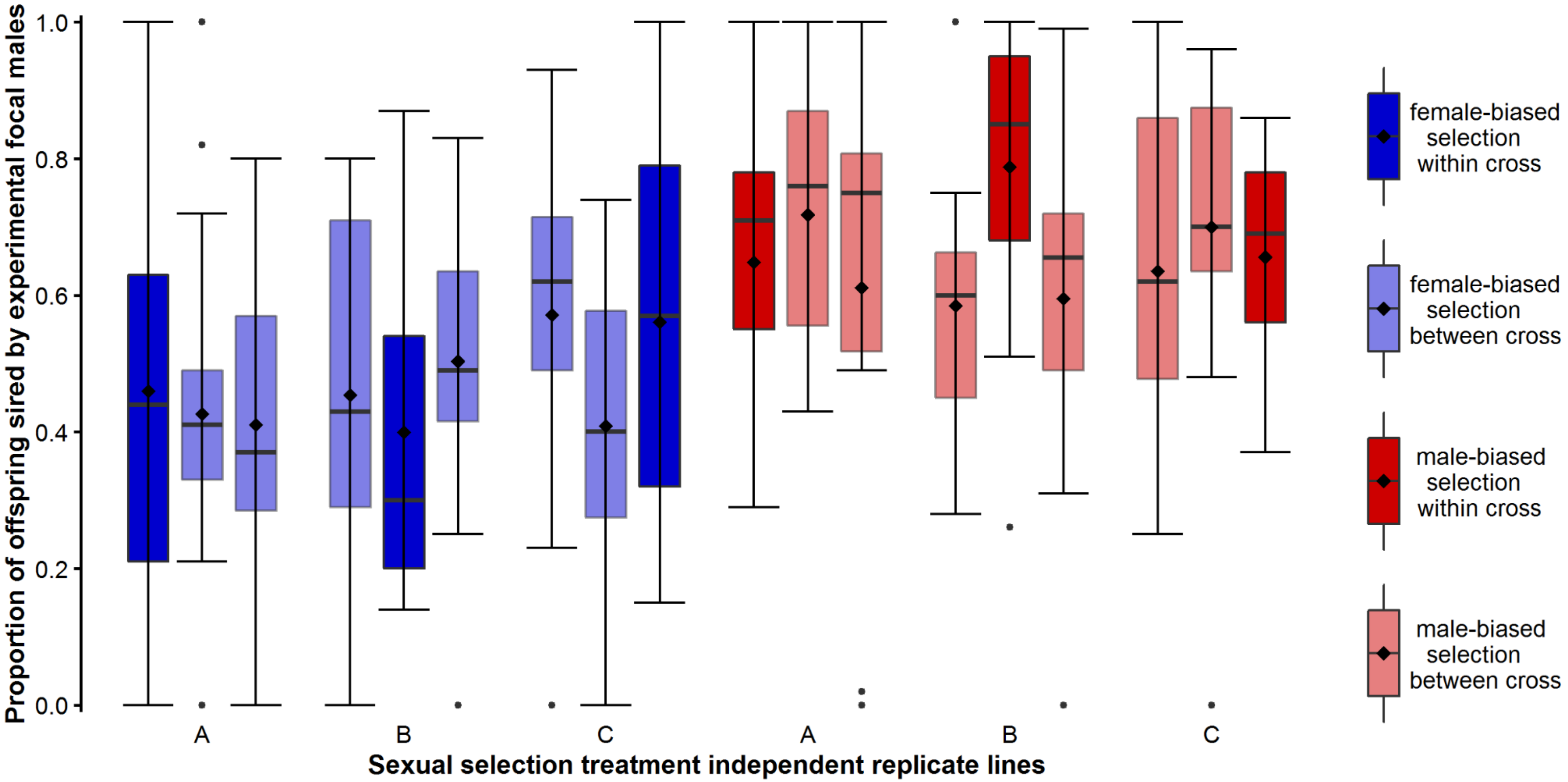


Figure 4.4 | Proportion of offspring (P2) sired by focal males from female-biased versus male-biased sexual selection treatments in sperm competition with single rivals. Data grouped by within treatment cross (n = 13-17). Within treatments the difference in within and between population cross were not significant (female-biased selection z = -0.68, p = 0.50; male-biased selection z = 1.13, p = 0.26).

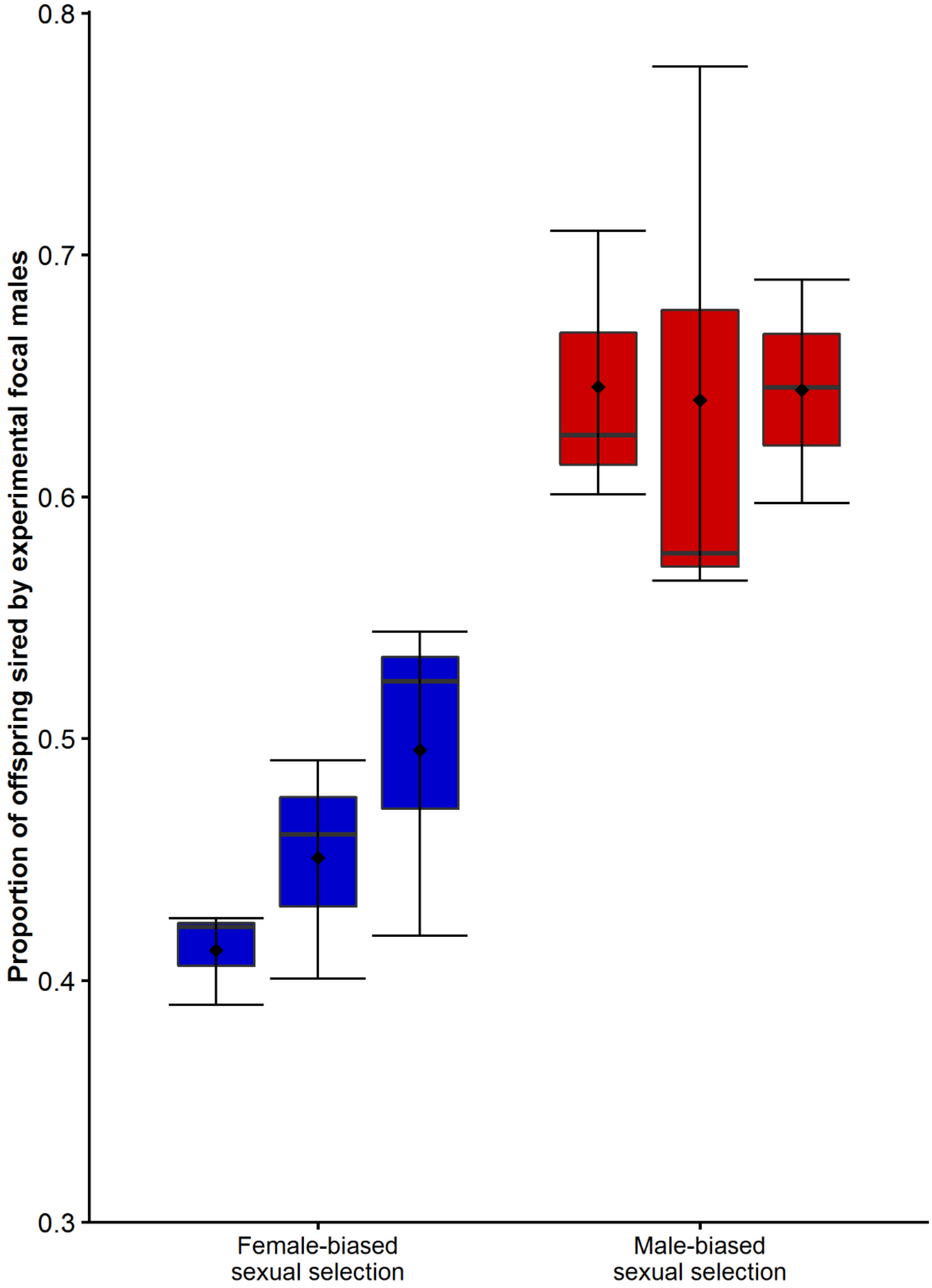


Figure 4.5 | Proportion of offspring (P2) sired by experimental focal males from female-biased versus male-biased sexual selection treatments in sperm competition with single rivals. Data grouped by independent line (n = 13-17 replicates nested in each of 3 crosses per line). Treatments means (n = 3 independent lines per treatment) are 0.65 (± 0.04 SE) for male-biased sexual selection males and 0.45 (± 0.04 SE) for female-biased sexual selection males. This difference is highly significant ($z = 3.5$, $p < 0.01$).

4.3.2 Sperm length divergence and sperm length variance

Sperm produced by males from male-biased sexual selection treatments were significantly longer than those from female-biased sexual selection males ($\chi^2_{(5)} = 9.88$, $p < 0.01$) (Figure 4.6 and 4.7). Mean sperm length in male-biased sexual selection males was 88.99um (± 0. 57 SE) compared to 86.11um (± 0.40 SE) in female-biased sexual selection males (see Table 4.2 for population means).

The variances in sperm length did not differ significantly between treatments, for either within male CVs ($\chi^2_{(4)} = 0.79$, $p = 0.37$), or between male CVs (Wilcoxon $_{(3, 3)}$ = 7, $p = 0.4$). For both treatments, variation in sperm length was greater within males than between males in a population line (Figure 4.8 and Table 4.2).

Table 4.2 | Mean sperm length (um) and coefficients of variation in sperm length, within males and between males for sexual selection treatment independent lines.

	Independent lines	**Mean sperm length (um) (± SE)**	**Within male CV (± SE)**	**Between male CV**
Female-biased sexual selection	A	85.47 (± 0.45)	3.06 (± 0.13)	1.66
	B	85.84 (± 0.61)	3.38 (± 0.23)	2.24
	C	87.02 (± 0.45)	2.85 (± 0.19)	1.65
Male-biased sexual selection	A	90.01 (± 0.35)	3.01 (± 0.10)	1.23
	B	88.32 (± 0.43)	3.20 (± 0.18)	1.55
	C	88.62 (± 0.51)	2.50 (± 0.12)	1.72

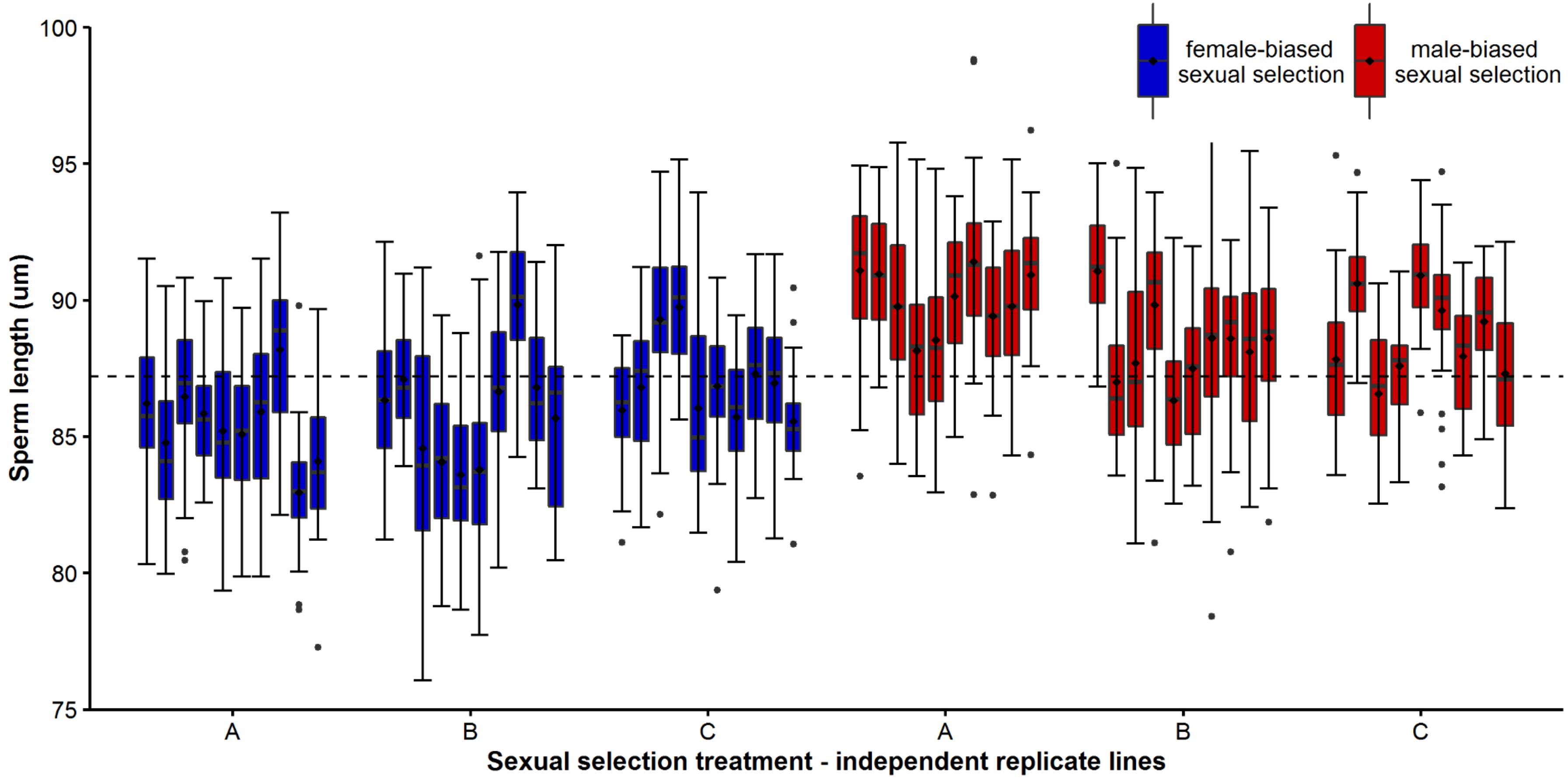


Figure 4.6 | Sperm length (um) of individual males from female-biased and male-biased sexual selection treatments (n = 30 sperm x 9-10 males x 3 lines = 870-900 total sperm in either treatment). Mean sperm length of wild type GA1 males (86.8 um) shown as a dashed line to compare increases and decreases to the ancestral population average.

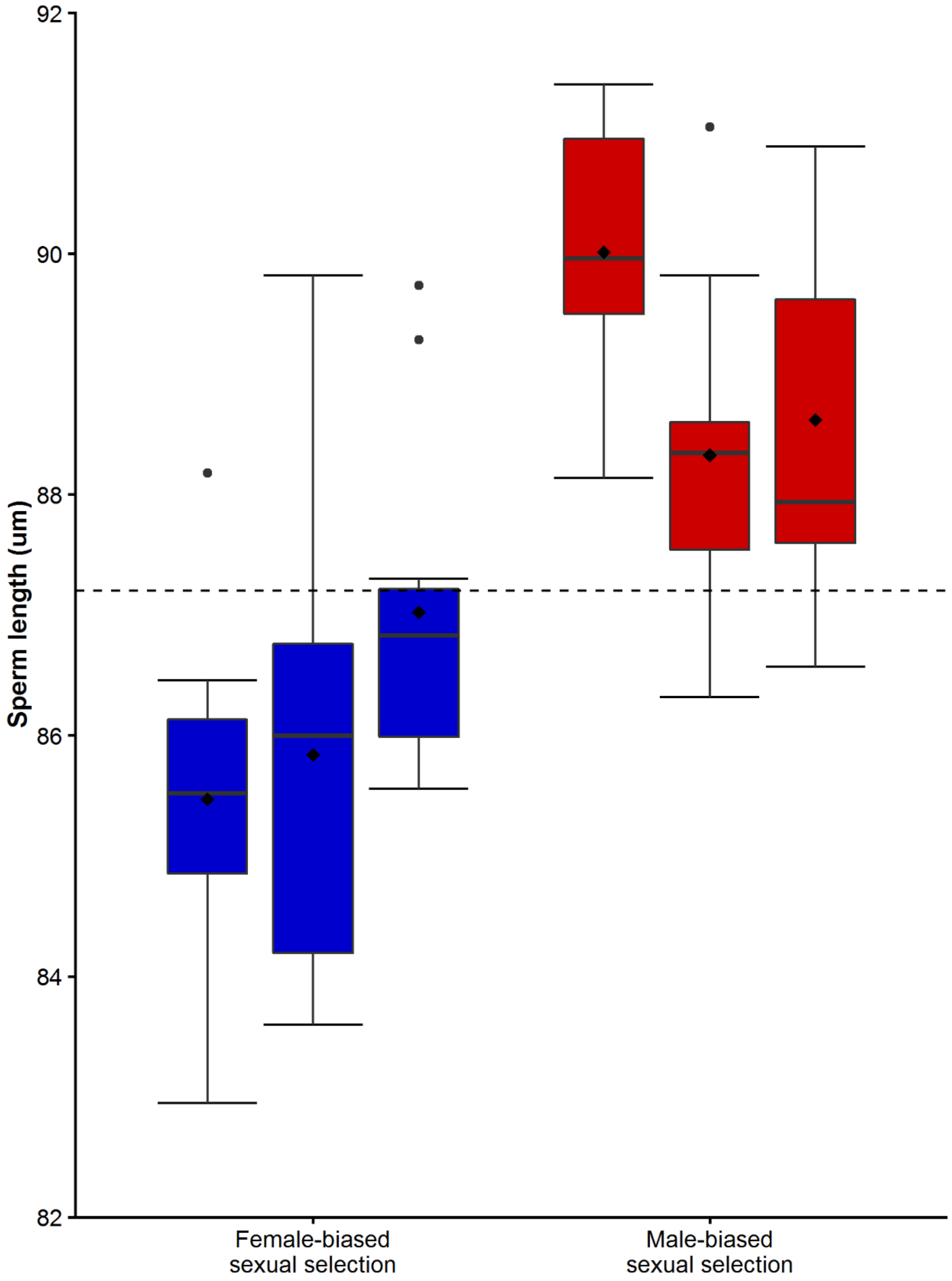


Figure 4.7 | Sperm length of males from female-biased and male-biased sexual selection treatments. Data grouped by independent line (n = 30 sperm x 9-10 males per line). Mean sperm length of wild type GA1 males (86.8um) shown (dashed line) to allow comparison with the ancestral average. Treatments means (± SE) are 86.09um (± 0.41) for female-biased sexual selection males and 89.01um (± 0.58) for male-biased sexual selection males (n = 3 independent lines per treatment). This difference is highly significant ($\chi^2_{(5)} = 9.88$, $p < 0.01$).

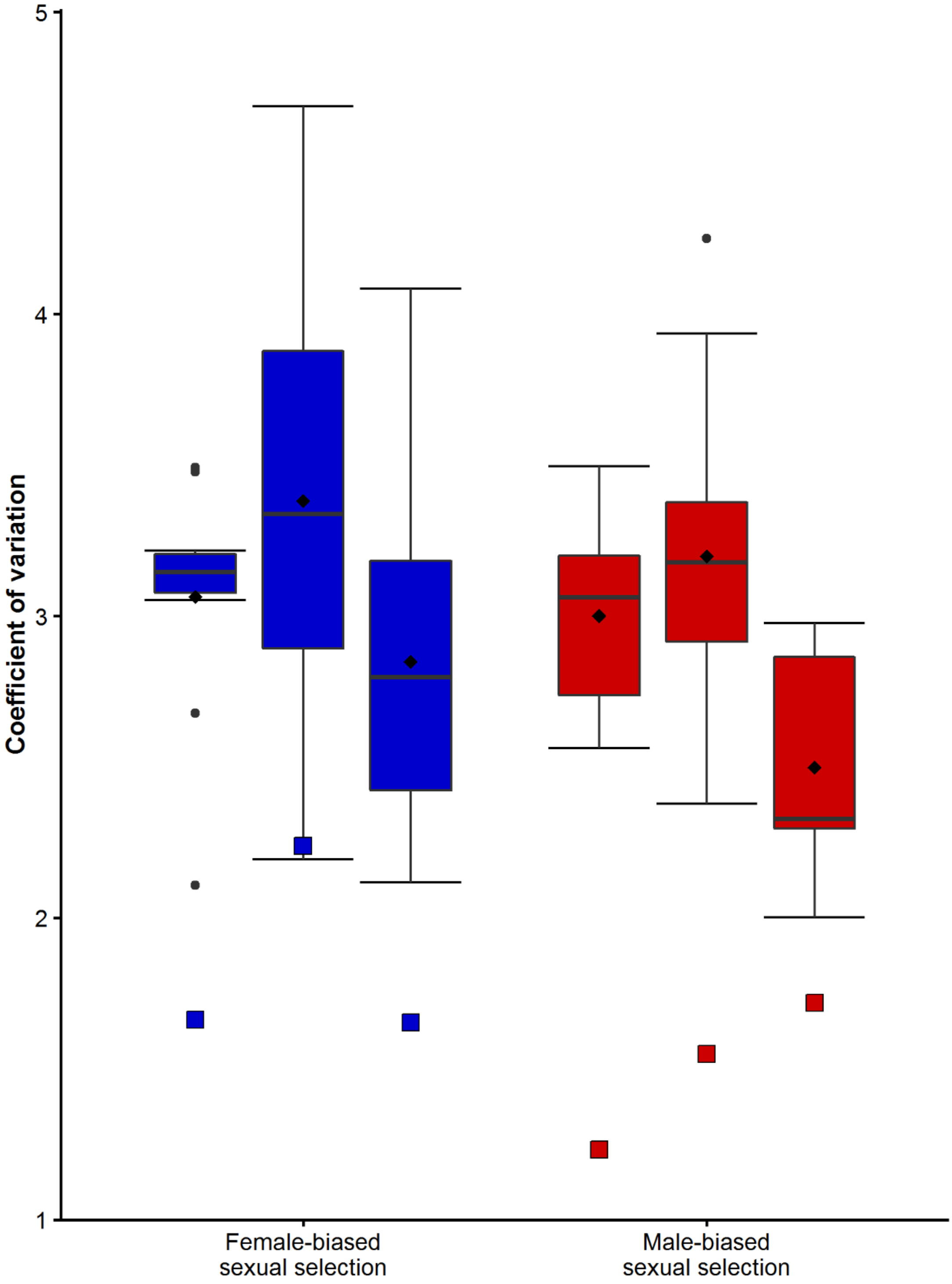


Figure 4.8 | Within and between male coefficient of variation (CV) in sperm length for replicate populations of female-biased and male-biased sexual selection treatments. Within male CVs (boxplot) (n = 30 sperm x 9-10 males per population). Between male CV (square points) calculated using mean sperm length of each male (n = 9-10 males per population).

4.4 Discussion

Experimental evolution through contrasting female-biased versus male-biased sexual selection treatments led to a significant divergence in sperm competitive ability between treatments. Males from a male-biased sexual selection history gained 20% higher fertilisation success through competition than males from a female-biased background. In this study, the focal males were the second males to mate, therefore their share of paternity is termed P2: the proportion of offspring sired by the second male to mate (Boorman & Parker 1976). In insects, the second or last male to mate usually gains the greatest share of paternity, and this non-random utilization of sperm is known as last-male sperm precedence (Simmons & Siva-Jothy 1998). Previous work using these experimentally evolved populations found high last male sperm precedence, and no significant difference between sexual selection treatments following 20 generations of selection (Michalczyk *et al.* 2011). Following a further 45 generations of experimental evolution, P2 for the male-biased sexual selection males has remained high, therefore the significantly lower P2 value for the female-biased selection males in this study suggests a relaxation in their sperm competitive ability, rather than a gain for the male-biased males. Under the female-biased regime, the risk of sperm competition is greatly reduced, while the male mating opportunity is greatly increased, therefore selection on sperm competitive ability is relaxed, but selection on male mating ability and frequency will be high. Since high investment in sperm competitive ability is likely to be a costly strategy to maintain for males (perhaps at the expense of inseminating multiple females), within a fixed energy budget for reproduction, resources should be allocated strategically (Dewsbury 1982; Parker 1990a). Males from the female-biased sexual selection treatment are likely to be under selection to be competent re-maters as a result of the high number of available females and mating opportunities, providing a different route to increasing individual fitness. These males should therefore allocate resources to produce the maximum number of ejaculates across multiple females, rather than increasing the sperm competitive ability of fewer ejaculates (Dewsbury 1982; Parker 1990a). Altering the number of sperm per ejaculate is a possible mechanism to achieve this, as sperm number is known to be a vital component of sperm competition success (Parker 1982; 1990a; reviewed in Wedell *et al.* 2002) and strategic ejaculation has been shown across taxa (delBarco-Trillo 2011; Kelly & Jennions 2011). Males experiencing female-biased selection might therefore be predicted to allocate the minimum number of sperm to the maximum number of ejaculates as their populations experience a consistently low sperm competition risk, and mating opportunities are very high (90 females shared among 10 males). In contrast, male-biased sexual selection males could gain greater fitness by maximising sperm numbers over the fewer, more competitive mating opportunities. Comparing the number of sperm within a single and successive ejaculates in males from both treatments is an important future investigation.

Differences in sperm competitive ability were also examined between populations within treatments, in order to determine whether differential fertilisation compatibility and/or reproductive isolation were evolving between independent replicate lines. Over time, the physical separation of two populations will lead to inevitable reproductive incompatibilities and consequently allopatric speciation (Turelli *et al.* 2001). If this is the case, males might be expected to gain a greater share of paternity in within population crosses, when mating with females with whom they are co-evolving, than in between population crosses where reproductive incompatibilities might decrease sperm competition success. Sexual selection (Lande 1981) and sexual conflict (Rice 1998; Gavrilets 2000) are proposed to be important drivers of reproductive isolation due to the intense selection on reproductive traits and the rapid nature of antagonistic coevolution between the sexes. Any differences in within- versus between-line crosses were therefore predicted to be exaggerated in the male-biased sexual selection treatment. Martin & Hosken (2003) provided empirical evidence for greater divergence between populations under high sexual conflict, measured as a significant decrease in copulation success and increased female reluctance to copulate in between population mating trials. *Tribolium sp.* show little pre-copulatory discrimination and females multiply-mate, therefore this was a good system to investigate post-copulatory reproductive isolation. However, no evidence was found for any differential compatibility and/or reproductive isolation between lines following male-biased versus female-biased sexual selection treatments. Although experimental evolution has become a crucial approach within sexual selection research, it has been suggested that it may be of limited use in the study of reproductive isolation and speciation due to the extreme lengths of time needed for these processes (Kawecki *et al.* 2012).

Having established divergence in sperm competitive ability between treatments, a key objective of this study was to investigate the pattern of selection on sperm length. In addition to greater sperm competitive ability, males from a male-biased sexual selection background had evolved significantly longer sperm than males from a female-biased sexual selection history. Comparison of sperm length data from this experiment with ancestral GA1 stock population sperm length (see Chapter 5 for GA1 data) suggests that male-biased sperm has increased in length, while female-biased has also decreased. Although neither are significantly different to the ancestral stock, the amount of change appears to be greater in the increase by male-biased sperm (Wilcoxon rank sum tests: male-biased vs GA1; W=7, p=0.08 and female-biased vs GA1; W=35, p=0.16), suggesting more intense selection on sperm length under this regime. Experimental evolution through sexual selection has therefore had a directional effect on sperm length. Now, the priority is to understand the functional benefit of increasing sperm length for sperm competition. Additional evidence may be gained by partitioning influences of the flagella, which aids motility, and the mid-piece, which stores mitochondria for metabolic energy conversion (Humphries *et al.* 2008), although this may be a challenge in insect models where the midpiece is not distinct. Alternatively, longer sperm may have no functional benefit, but act simply as a signal of male quality or condition, much like elaborate and costly pre-copulatory traits that were Darwin's original inspiration for the theory of sexual selection (Miller & Pitnick 2002). Unlike the many contradictory studies regarding evolution of sperm length under sperm competition, comparative studies of the relationship between sperm length and the female storage organs are unanimous in finding positive associations between female tracts and male gametes (reviewed in Pitnick *et al.* 2009b). Experimental evolution of female sperm-storage organ length drove the evolution of sperm length in *D. melanogaster* (Miller & Pitnick 2002), and longer sperm were more likely to be stored in diverged female storage, irrespective of mating order (Pattarini *et al.* 2006). This evidence suggests an interaction between sperm and variation in the environment in which they must function and compete, so cryptic female choice may be a significant evolutionary force acting on sperm length. Measuring the length and number of tubules in the *Tribolium* spermathecae is a priority for future work to investigate female responses and potential effects in these different sexual selection treatments. Also, following the pioneering work of Manier *et al.* (2010), it should be possible to investigate differences between females in each treatment in sperm storage and utilisation using males with sperm tagged with either green or red fluorescent proteins. Coevolution between males and females is often antagonistic. If optimum sperm length differs for males and females, sexual conflict will prevent either sex gaining that optimum (Arnqvist & Rowe 2005). Antagonistic coevolution has been implicated in the rapid evolution of ejaculate proteins (reviewed in Pitnick *et al.* 2009b), therefore sexual conflict is also expected to play a role in the evolution of sperm form and function (Snook 2005; Pitnick *et al.* 2009b).

Contrary to a growing number of comparative studies suggesting sperm competition has a stabilising effect on sperm length variance (e.g. Immler *et al.* 2008; Lifjeld *et al.* 2010; Varea-Sanchez *et al.* 2014; Fitzpatrick & Baer 2011), I found no difference between female-biased and male-biased sexual selection treatments in either within- or between-male sperm length variance. These previous comparative studies all used indirect measures of sperm competition level in their populations, therefore my study has been the first to rigorously control, within a species, the evolution of sperm length variance as a consequence of direct selection from, and impacts upon, sperm competition. Testis size has frequently been used as a predictor of sperm competition level due to a wealth of comparative data supporting the evolution of increased investment in sperm production in species with high sperm competition (reviewed in Birkhead & Moller 1998). More recently, however, it has been questioned whether post-copulatory sexual selection may also act on the composition and organisation of the testes (Ramm & Schärer 2014). Firman *et al.* (2015) showed that polyandrous males had a higher proportion of sperm producing tissue within the testes and produced correspondingly higher numbers of sperm than monogamous males, although testis size did not vary between the treatments (Firman & Simmons 2010). This raises doubts about the use of testis size alone as a measure of mating pattern (Ramm & Schärer 2014; Firman *et al.* 2015). Preliminary investigation of testis size in the female-biased and male-biased sexual selection lines used in this study have found no difference in size between treatments (Vasudeva & Gage unpubl. data). This is unsurprising if, as already speculated, males in these treatments are under selection to either produce many ejaculates (female-biased treatment) or fewer highly competitive (high sperm number) ejaculates (male-biased treatment) as alternative routes to increased fitness. In other words, the female-biased treatment has reduced selection from competitiveness, but increased selection from mating competence. In addition, investment in the testes has been suggested as a mechanism to allow sperm number and size to vary independently in species where there is no evidence of a trade-off between these traits as in *T. castaneum* (Arnaud *et al.* 2001). This may also be seen as more complex testicular function and organisation, rather than simply differences in size (Ramm & Schärer 2014).

The absence of stabilising selection under high male-male competition and sperm competition, might also be due to the unexpectedly low coefficient of variation found between males in both sexual selection treatments, which consequently provides an already-narrow range of variation for selection to act upon. Morrow & Gage (2001) found consistent, significant between-male variation in total sperm length across a wide range of species. However, in this study I found variation between males to be lower than that within males (Figure 4.8, Table 4.2) which is unusual. Regular genetic bottlenecks from pest control and founder effects may have resulted in low genetic variation for sperm length *T. castaneum* (Sokoloff 1972). Although low within-male and high between-male variation in sperm length was found in dairy and beef cattle, known for their low genetic variation (Morrow & Gage 2001). A previous study estimated heterozygosity in the *Tribolium* populations used in these experiments and showed it to be relatively low, although no difference was found between contrasting sexual selection treatments (male-biased $H_{est} = 0.312$, female-biased $H_{est} = 0.318$) (Lumley *et al.* 2015). Previous studies of variation in sperm length make use of wild bird populations which are not only likely to have greater genetic variability than captive/laboratory populations but also be subject to environmental selection pressures which are likely to increase trait variation (Frankham *et al.* 2010).

Finally, experimental evolution studies often compare polyandry with enforced monogamy, which completely removes sexual selection and conflict (Hosken & Ward 2001; Pitnick *et al.* 2001; Martin & Hosken 2003; Simmons & Garcia-Gonzalez 2008). However, this is unlikely to be a normal case in natural populations where levels of post-copulatory sexual selection are more likely distributed along a continuum rather than either being present or absent (Snook 2005). Here, I find that experimental evolution of contrasting strengths of sexual selection has led to significant divergence in both sperm competitive ability and sperm length between treatments. Although males in the male-biased sexual selection treatment had both more competitive and longer sperm, it is not possible from the results here to say that sperm length increases are the specific cause of increased sperm competitiveness. Further investigation into sperm number, testis size and female reproductive tract dimensions and the distribution of sperm therein are needed to build a certain understanding of the mechanism of sperm competition, and the relative level of selection from sperm competition and cryptic female choice on sperm length in *T. castaneum.*

Chapter 5

Sex-specific reproductive ageing is associated with intrinsic lifespan in a promiscuous insect

Abstract

Evolutionary theories of ageing agree that greater reproductive fitness, and therefore stronger selection, in early life compared to later life, causes decreases in fecundity/fertility and increased intrinsic mortality with age. As a result of anisogamy, males and females maximise reproductive fitness via contrasting strategies. Selection acts more on males to gain fitness by increasing mating opportunities and fertilisations, while selection on females favours greater direct investment in gametes and offspring. Sex-specific differences in reproductive investment, and in trade-offs between reproduction and survival, could therefore explain sexual dimorphism in ageing and longevity. The aim of this study was to investigate whether sex-specific reproductive ageing in the promiscuous flour beetle, *Tribolium castaneum*, is associated with sex-specific intrinsic mortality, as would be predicted by ageing theories. In this species, males invest in pre- and post-copulatory competitions to maximise reproductive success, while females invest in nutrient rich ova to maximise fecundity. Reproductive fitness was measured in both sexes up to 12 months of age and, in parallel, adult longevity was assessed by measuring intrinsic lifespan in stress-free, food-rich conditions. Sex-specific assays measured declines in female reproductive fitness over a finer timescale, while deteriorations in male reproductive fitness were examined over a wider timescale. In addition, variation in sperm length as males aged was measured, and effects of paternal age on reproductive success of sons were explored, in order to look for evidence of trans-generational germ-line mutation accumulation. Intrinsic lifespan of males was significantly greater than that of females, with males living 35% longer. In association, male reproductive fitness showed no decline in ageing individuals up to 12 months old, while female reproductive fitness decreased significantly by 50% within 1 month of adulthood, in comparison with control young females. No evidence of ageing in males up to 12 months old was found in sperm length variance or reproductive fitness of sons. Therefore, a clear sexual dimorphism in reproductive longevity exists in *Tribolium,* which could explain the sexual dimorphism in intrinsic ageing profiles. These findings indicate either, a significant sexual dimorphism in costs of early reproduction, or that the probability of future reproduction across a longer timescale is much greater for males, or both.

5.1 Introduction

Ageing or senescence is the rise in intrinsic mortality and decrease in fecundity with increasing age which, despite the negative impact on fitness and absence of adaptive benefit, is pervasive across living organisms (Hamilton 1966; Rose 1991; Partridge & Barton 1993). Mortality from extrinsic sources, such as predators, disease and the abiotic environment, results in fewer representatives of older age groups, and a reduction in future contributions to fitness with increasing age, the outcome of which is a decrease in the strength of selection acting on older individuals (Haldane 1941; Medawar 1952; Williams 1957; Hamilton 1966; Charlesworth 1994). The consequence of this unidirectional reduction in the strength of selection is that deleterious mutations with effects in later life can accumulate and act ('mutation accumulation' theory, Medawar 1952), and lead to the increased intrinsic mortality and decreased fecundity associated with ageing. Alternatively, high risks of extrinsic mortality will favour biased investment in early reproduction, and mutations with pleiotropic early life benefits but inadvertent later life costs could persist within a population and cause ageing ('antagonistic pleiotropy' theory, Williams 1957). The life history strategy of an organism is shaped by evolution to maximise lifetime fitness by optimising the allocation of resources to the various aspects of survival and reproduction (Stearns 1992). Acquired resources will most often be limited, therefore, trade-offs take place such that one process will benefit, but another will experience a cost. Major life history trade-offs occur between early/current reproduction and future reproduction/survival (Stearns 1992). An increase in intrinsic mortality and decrease in fecundity with age will reduce future contributions to fitness, and increase natural selection for investment in early/current reproduction at the expense of somatic maintenance and consequently future reproduction/survival ('disposable-soma theory' Kirkwood 1977; Kirkwood & Rose 1991). Which of these specific models, which are unlikely to be mutually exclusive, best explains the evolution of ageing remains controversial. However, the general theory that ageing evolves as a by-product of higher fitness (survival and reproduction), and therefore stronger selection, in early life compared to later life, is widely accepted (reviewed in Partridge & Gems 2002 and Williams *et al.* 2006).

Differences in the longevity of males and females are documented across taxa (Promislow 1992; Promislow & Haselkorn 2002; Liker & Szekely 2005; Clutton-Brock & Isvaran 2007). Despite this, understanding of the causes of sexual dimorphism in ageing and longevity remains uncertain, and is a central question in current evolutionary biology (Bonduriansky *et al.* 2008; Maklakov & Lumma 2013; Regan & Partridge 2013; Lemaitre *et al.* 2015). If ageing and longevity evolve as a result of a reduction in fitness, and therefore selection, of older individuals, sex-specific ageing must be driven by sex-specific fitness differences in later life. Males and females optimise their fitness via contrasting life history strategies, therefore sex-specific trade-offs between reproduction and survival can drive the evolution of sexual dimorphism in ageing and longevity (Bonduriansky *et al.* 2008; Maklakov & Lumma 2013). The fundamental difference between males and females is in gamete size, anisogamy, with females producing relatively low numbers of large, nutrient rich ova, while males produce high numbers of tiny sperm which do not contribute to zygote provisioning (Darwin 1871; Bateman 1948). The consequence of this ultimate difference in gamete size, is that male potential reproductive rate is high, but their actual reproductive success is limited by female reproductive rate, which is itself governed by energetically costly egg/offspring production (Darwin 1871; Bateman 1948; Clutton-Brock & Vincent 1991). Males are therefore under greater selection to maximise fitness via increased numbers of fertilisations, while selection on females will act more upon fecundity and mate quality (Darwin 1871; Bateman 1948).

Males maximise fertilisations via traits and behaviours which improve success during pre-copulatory competition for mating opportunities, and post-copulatory sperm competition for fertilisations (Darwin 1871; Parker 1970). However, these are often associated with increased risk of extrinsic mortality (Williams 1957) from predation and combat (reviewed in Andersson 1994), or energetic costs associated with increased size dimorphism, mate searching, combat and displays, including calls and song (reviewed in Andersson 1994), which reduce resources available for somatic maintenance and repair, leaving males more vulnerable to extrinsic and intrinsic mortality (Williams 1957; Kirkwood 1977; Bonduriansky *et al.* 2008). Male investment in competition success could therefore lead to reduced contributions to fitness of older males, and consequently male-biased ageing and decreased longevity. Comparative evidence from polygynous birds and mammals supports this (Promislow 1992; Clutton-Brock & Isvaran 2007), with early senescence and reduced average male longevity clearly evident in species where males are territorial (Tidiere *et al.* 2015), although associations may depend on how male competition is measured (Lemaitre & Galliard 2013). A recent review of early-late trade-offs in wild vertebrates found that these were more often found in males than in females (Lemaitre *et al.* 2015). Within-species studies also find more rapid ageing in males that experience high levels of competition for mating (Lemaitre *et al.* 2014; Beirne *et al.* 2015).

Theoretically, male reproductive success can also increase with age as an outcome of increasing size, weapons or social rank, which could reduce rates of ageing even where extrinsic mortality is high (Graves 2007). High reproductive success of older high-ranking human males in early/traditional societies, could explain the maintenance of high fertility with age of modern human males, despite their lower life expectancy (Tuljapurkar *et al.* 2007). Alternatively, Wiklund *et al.* (2003) showed that multiple re-mating by females, polyandry, led to increased male longevity and reduced the gender gap in butterflies, compared to monogamy. It was proposed that this could be explained by increased mating opportunities, and consequently reproductive success over time for males, through polyandry (Wiklund *et al.* 2003). Finally, male mating and fertilisation success is predicted to be dependent on condition, the ability to acquire and make efficient use of resources (Fisher 1915; Zahavi 1975; Rowe & Houle 1996). Theoretical (Williams & Day 2003; Shokhirev & Johnson 2014) and empirical evidence (Reznick *et al.* 2004; Chen & Maklakov 2012) suggests that, where extrinsic mortality risk is condition-dependent, this could lead to the evolution of increases in lifespan as high condition individuals have more resources to allocate to maintenance and repair, and therefore a lower rate of extrinsic mortality. Most recently, Chen & Maklakov (2014) have shown that condition-dependent extrinsic mortality in males not only raises male longevity, but does not appear to benefit females, and therefore also influences the extent of the gender gap in lifespan.

Females maximise fitness via investment in nutrient-rich ova which are energetically costly compared to sperm (Bateman 1948; Parker 1970; Trivers 1972; Maynard Smith 1978). Although the cost of ejaculate production is no longer considered to be trivial (Dewsbury 1982), comparison of gamete biomass production rates found this to be two to four times higher in females compared to males, across a broad range of taxa (Hayward & Gillooly 2011). High investment in ova explains the increased prevalence of parental care in females, as the gains from deserting and producing future offspring are small compared to the potential costs (Trivers 1972; Clutton-Brock 1991). Females are consequently expected to adopt a lower risk strategy compared to males, in order to minimise extrinsic mortality and maximise the opportunity for reproduction which is often time-, as well as resource-limited (Stearns 1992; Bonduriansky *et al.* 2008). However, the significant energetic costs of gamete production and parental care for females may constrain investment in their survival by decreasing available resources for allocation to maintenance and repair (Kirkwood 1977; Kirkwood & Rose 1991; Stearns 1992). Despite these theories, empirical evidence for early-late trade-offs in females is inconclusive, with negative, positive and no relationships reported (reviewed in Lemaitre *et al.* 2015). Decreased longevity of females compared to males is described in monogamous mammals (Promislow 1992), invertebrates (Prosmislow & Haselkorn 2002; McCulloch & Gems 2003), and is predominant in birds (Promislow *et al.* 1992; Liker & Szekely 2005). Female-biased mortality in birds is not explained by investment in parental care (Liker & Szekely 2005), and is theorised to be a result of reduced male competition intensity in both monogamous mammals and birds, which allows males to be spared the cost of competition, and consequently to outlive females (Promislow 1992; Liker & Szekely 2005). However, empirical studies have not investigated whether differences in the cost of gametes in females, and the consequent trade-off with survival, can explain interspecific and within-species variation in the extent of female-biased mortality.

A limited number of within-species studies have measured ageing and longevity in both sexes. Some have estimated ageing from mortality rates (Fox *et al.* 2003; Kawasaki *et al.* 2008), while others have measured changes in metabolic rate (Promislow & Haselkorn 2002) and somatic senescence (Tafani *et al.* 2012; Beirne *et al.* 2015). Those that have investigated reproductive ageing have often done so indirectly in males by measuring courtship effort (Zajitschek *et al.* 2009; Archer *et al.* 2012), or sexual ornamentation and gamete production (Cornwallis *et al.* 2014). Only two studies have directly measured sex-specific reproductive declines and longevity; in red deer, a polygynous species where males compete for dominance via displays and combat, males have more rapid decreases in offspring production and corresponding reduced longevity (Nussey *et al.* 2009), and guillemots, long-lived, monogamous sea birds in which reproductive ageing is more rapid in females and also shows a corresponding decrease in longevity (Reed *et al.* 2008). Therefore, empirical evidence for within-species sex-specific associations between reproduction and survival and associated longevity is limited. Further within-species studies, across a wider range of taxa, with differing mating systems and consequently routes to fitness in males and females, will add important empirical evidence to the question of how tightly reproduction is linked to intrinsic ageing.

To gather empirical evidence regarding sex-specific trade-offs and ageing, this study compares intrinsic lifespan in the absence of stress, selection and reproduction, with changes in reproductive fitness in increasing age cohorts in the promiscuous red flour beetle, *Tribolium castaneum*. *T. castaneum* is a relatively long-lived, iteroparous insect species, with mating and reproduction occurring repeatedly throughout the adult lifespan (Sokoloff 1974). These characteristics make *Tribolium* an effective model for investigations of ageing. In addition, *T. castaneum* is regularly used in empirical studies of sexual selection and conflict (Fedina & Lewis 2008, Zuk *et al.* 2014), therefore much is known about sex-specific reproductive investment and behaviour that could lead to sexual dimorphism in ageing and longevity. Both males and females in *T. castaneum* are highly promiscuous (Lumley *et al.* 2015), showing little evidence for pre-copulatory mate discrimination, while evidence for post-copulatory sperm competition and cryptic female choice is plentiful (reviewed in Fedina & Lewis, 2008). Following fertilisation and oviposition, neither sex invests further in offspring through parental care, therefore sex-specific ageing and longevity in *T. castaneum* are predicted to evolve as a consequence of (a) the relative cost of male ejaculate and female ova production, (b) sex-specific trade-offs with physiological maintenance and repair processes, and/or (c) future reproductive potential.

Here, I measure intrinsic lifespan under controlled, benign conditions, and investigate changes in reproductive fitness up to the ages of 12 months in male and female adults, as indicators of ageing. Data regarding longevity of *T. castaneum* provide a wide range of estimates of mean lifespan (16 to 80 weeks for males and 15 to 41 weeks for females), although all sources agree that males are longer lived than females (reviewed in Sokoloff 1974 and Spratt 1980). Much of the variation in these estimates is likely to be a result of differences in standard rearing conditions including nutritional quality, temperature and humidity therefore, a measure of intrinsic lifespan under standard conditions for our laboratory was required. Oviposition rate of mated females is reported to be on average 10 eggs per day for 3-6 months (Sokoloff 1974; Fedina & Lewis 2008), however nothing is known about changes in male fertility. Offspring production following a single mating was used as a measure of the reproductive fitness of males and females from cohorts of increasing age. Early results indicated more rapid loss of reproductive fitness in females compared to males, therefore additional investigations focused on male and female reproductive fitness in isolation. To establish how rapidly female reproductive fitness decreases with age, changes in offspring production were measured over a finer timescale. Sperm are produced by spermatogenesis continuously throughout adult life and, as such, enforced sexual deprivation while males age may allow sperm reserves to build up and cause fertility to appear to be maintained (Pizzari *et al.* 2008). Increasing male age is also predicted to increase errors during sperm production, resulting in a decrease in fertilising efficiency and/or genetic quality of sperm (Pizzari *et al.* 2008) therefore, sperm quality was used as an additional measure of male ageing rate. A simple measure of the quality of sperm production was predicted to be the variation in sperm length within males. Older males were predicted to show greater variation in sperm length as a result of increased sperm production errors. Finally, a reduction in the genetic quality of sperm could have important implications for offspring fitness. Increased paternal age has been associated with negative effects on offspring development, fecundity and longevity (reviewed in Johnson & Gemmell 2012). Therefore, male ageing was also assessed via the effect of paternal age on the reproductive ability of sons of young and old males.

5.2 Methods

The following assays were carried out using individuals from the GA1 stock population. For details of stock maintenance and experimental evolution regimes see General Introduction 1.5.

5.2.1 Intrinsic ageing and lifespan

All adults (n = 147 males and 149 females) were virgin, and 10-12 days post-eclosion. Extrinsic impacts on mortality were equalised and minimised by isolation of individuals in vials containing *ad libitum* control food medium and oats to aid locomotion. Individuals were checked weekly for deaths as evidenced by complete immobility, and food medium/oats were refreshed every 4 weeks.

Statistical analyses

Intrinsic survivorship of males and females was compared using the ‘survival’ package (Therneau 2015). A parametric accelerated failure time (AFT) model was used which allows an appropriate error distribution to be specified (Crawley 2013). Event times are positively skewed such that a normal distribution is inappropriate for survival data therefore, a number of distributions were compared using AIC values, along with visual assessment of log-log plots and raw data overlaid with model fits (Crawley 2013). A logistic error distribution provided the best model fit and accounted for non-proportional hazards shown in the log-log plot. Censoring was not required as time of death of all experimental individuals was known. A Kaplan-Meier object was created and sex (male versus female) was included as a categorical explanatory variable.

5.2.2 Reproductive ageing

Control reproductive fitness measure

All adults were virgin and 10-12 days post-eclosion (control). Males and females were randomly allocated to pairs (control: n = 34 pairs) for 48 hours to mate, and the male was then removed. Females were transferred to a petri dish of *ad libitum* control medium/oats and left to oviposit for 10 days, and then transferred to another petri dish for a further 10 days. This was repeated every 10 days for 100 days after mating. The petri dishes containing eggs/larvae were left to develop into adults and counted. Total offspring production over 100 days following a 48 mating period was thus used as a measure of reproductive fitness.

Changes in reproductive fitness with age

At the start of the control reproductive fitness protocol, additional males and females were isolated in vials, containing *ad libitum* control medium and oats, to age. Individuals were checked weekly for deaths, and food medium/oats were refreshed every 4 weeks.

Following 4 months and 12 months of senescence, surviving males and females were paired with control adults of the opposite sex from stock populations, and the fertility protocol was repeated (4 month males: n = 21, 12 month males: n = 25, 4 month females: n = 23, 12 month females: n = 22). In addition, 4 month males were paired with 4 months females (4 month pairs: n = 25) and 12 month males were paired with 12 month females (12 month pairs: n = 21) to investigate how the fertility of males and females interact.

Statistical analyses

Reproductive fitness, total offspring production over 100 days following a 48 mating period, of 10-12 days post-eclosion (control) individuals and males, females and pairs of each age group (4 months and 12 months) were compared. Count data have non-normal errors and the variance increases with the mean, making parametric analyses inappropriate, therefore, the Poisson distribution was used to describe the data (Crawley 2013). Counts were compared by constructing generalised linear models (GLMs) in ‘lme4’ (Bates *et al.* 2015) with a poisson error structure which incorporates a ‘log’ link function to apply a log transformation (Crawley 2013; Bates *et al.* 2015)**.** Models were assessed for overdispersion using the ratio between the residual deviance and the residual degrees of freedom. Where dispersion was >2 this was accounted for using a quasi-poisson error structure which rescales the variance (Crawley 2013). A maximal model was fitted with total offspring as the response variable and a single treatment variable incorporating all treatments (7 levels: control, 4 month males, 4 month females, 4 month pairs, 12 month males, 12 month females, 12 month pairs) as a fixed effect. Contrasts were set to compare each level of the treatment factor to the control and also to investigate within age group pairs.

Data checks before analysis showed that female deaths occurred during the 100 days of oviposition/offspring production, particularly where females were 12 months old. Where a female died after she had finished producing offspring (offspring production = 0 in 10 days preceding death) these were left in the analysis, however where a female died before she had finished producing offspring (offspring production >0 in 10 days preceding death) these data points were discarded from the analysis as incomplete replicate data (n = 4/175). Many 12-month-old females also failed to produce offspring (12 month females n = 11/22, 12 month pairs n = 10/21); however, it was unknown whether this showed a failure to mate by either member of the pair, or a complete loss of fertility. Therefore, analyses were repeated also without these data points to assess their influence.

Finer scale changes in female reproductive fitness

The control reproductive fitness protocol and the changes in reproductive fitness with age protocol (above) were repeated, but only females were aged for 1 month, 2 months and 3 months, to investigate finer scale changes in female fertility with age (control n = 25, 1 month n = 18, 2 months n = 18, and 3 months n = 20).

Statistical analyses

Reproductive fitness of control, 1 month old, 2 months old and 3 months old females were compared. Count data were treated as for the reproductive ageing analysis (above). A GLM was fitted with total offspring as the response variable and age group (control, 1 month, 2 months and 3 months) entered as a fixed effect. Contrasts were set to compare each age group to the control and also to compare age groups with older age groups (control versus 1, 2 and 3 months old, 1 month old versus 2 and 3 months old and 2 months old versus 3 months old).

Sperm length variance in old males

Sperm length was measured in 12 month old males (n = 15) from the reproductive ageing protocol (above) and control 10-12 days post-eclosion males (n = 15), which had been isolated at the pupal stage to equalise any developmental effects. Sperm were recovered from females using microdissection, after spermatophores had been deposited during mating, thereby ensuring that sperm were functional and mature. For complete micro-dissection and sperm measurement protocols, including repeatability analysis of the measuring technique, see Chapter 4.2. 30 sperm per male were measured which provides an accurate estimate of variation (Sokal & Rohlf 1981; Immler et al. 2008) (n = 30 sperm x 15 males = 450 total sperm in either treatment).

Statistical analyses

Sperm length variance was calculated as a standardised coefficient of variation (CV). This was calculated both within males (CV_{wm}) (n = 15) and between males (CV_{bm}) in each age group. The within male CV for an age group is a mean of 15 individual male CVs. The between male CV is calculated using the mean sperm length of each male therefore this value has no confidence intervals. An independent t-test was used to compare within male CVs for each treatment.

Transgenerational effects of old males

12 month old males (n = 16) from the changes in reproductive fitness with age protocol (above) and control males (n = 16) were paired with control females for 48 hours to mate, then the male was removed. Females were transferred to a petri dish of *ad libitum* control medium/oats and left to oviposit for 10 days before being removed. The petri dishes containing eggs/larvae were left to develop then pupae were sexed and 2 sons from each male were isolated and allowed to reach full adult maturity (n = 2 sons x 21 control males, and n = 2 sons x 23 12 month old males).

The reproductive potential of sons of control and 12 month old males was assessed by stretching the mating and fertilisation ability of both groups across multiple mating opportunities. Sons were presented with a control virgin female for 30 minutes, then females were removed and replaced with another control female. This was repeated every 30 minutes for 5 hours such that sons were presented with 10 females consecutively. Following removal from the mating arena, each female was transferred to a petri dish of *ad libitum* control medium/oats and left to oviposit for 10 days, then transferred to another petri dish for a further 10 days, before finally being removed. Thus, each male had the opportunity to mate with and fertilise 10 different females and I collected offspring over 20 days of oviposition. Previous work in this lab group has shown that offspring production from the first 20 days of oviposition following a single mating significantly predicts total offspring production for that mating (R^2= 0.38), and accounts for ~50% of the potential total offspring production from such a mating period (Dickinson & Gage unpubl.

data); thus, 20 days of offspring production is a fair measure of total reproductive fitness for a mating. The petri dishes containing eggs/larvae were left to develop into adults and then counted. Offspring production when reproductively stretched was thus used as a measure of the reproductive potential and fitness of males.

Statistical analyses

Reproductive potential of sons of control male and sons of 12 month old males were compared. Count data were treated as for the reproductive ageing analysis (above). Number of successful mates was compared by scoring the presence or absence of offspring. A GLMM was fitted with age of the father (control versus 12 months) as a fixed effect and son (a, b) nested within father (n = 21 and 23) entered as random effects to accounted for the hierarchical structure of the data. Total offspring production over 10 mating opportunities (10 females) was also compared by fitting a similarly structured GLMM, with age of the father (control versus 12 months) as a fixed effect and son (a or b) nested within father (n = 21 and 23) entered as random effects. To investigate changes in offspring production over consecutive mating opportunities, the mean number of offspring per father (mean of 2 sons) was calculated to remove nesting and a GLM was fitted with age of the father (control versus 12 months old) and female (1 to 10) entered as fixed effects. A polynomial contrast was set to investigate the trend across consecutive females (Field *et al.* 2013).

5.3 Results

5.3.1 Intrinsic ageing and lifespan

Intrinsic lifespan differs significantly ($z = 9.52$, $p < 0.001$, Figure 5.1) between males and females in *T. castaneum*. Mean male lifespan (66.03 ± 1.59 weeks) is 35% longer than mean female lifespan (49.18 ± 1.12 weeks). In females, mortality began to increase rapidly around 20 weeks post-eclosion, however, in males this increase did not begin until around 38 weeks (Figure 5.1).

5.2.2 Reproductive ageing

Changes in fertility with age

Mean (± SE) reproductive fitness, measured as offspring production over 100 days following a 48hr mating period, for 10-12 day post-eclosion (control) pairs of *T. castaneum* was 512 offspring (± 42). Ageing had no significant effect on male fertility after either 4 months (583 ± 54 offspring, $t_{6,\,164} = 1.12$, $p = 0.26$) or 12 months (438 ± 41 offspring, $t_{6,\,164} = -1.31$, $p = 0.19$). However, female reproductive output decreased significantly to 43% of the young control output after 4 months of senescence (221 ± 26 offspring, $t_{6,\,164} = -5.22$, $p < 0.001$), and to just 7% after 12 months (34 ±13 offspring, $t_{6,\,164} = -7.22$, $p < 0.001$). Removal of 12 month old females that produced no offspring from the dataset increased this value to 14% ($t_{6,\,142} = -5.65$, $p < 0.001$). For pairs where both adults were 4 months old, reproductive output decreased to 34% of the control level, which was not significantly different to the value for young control males paired with 4 month old females ($t_{6,\,164} = -1.23$, $p = 0.22$). For 12 month old pairs, fertility was 4% of the control level, which also did not differ significantly from the fertility of 12 month old females paired with young control males ($t_{6,\,164} = -0.75$, $p = 0.46$). Removal of 12 month old pairs that produced no offspring from the dataset increased this value to 8%, however this remains a 92% reduction in fertility compared with young controls ($t_{6,\,142} = -5.55$, $p < 0.001$) (Figure 5.2).

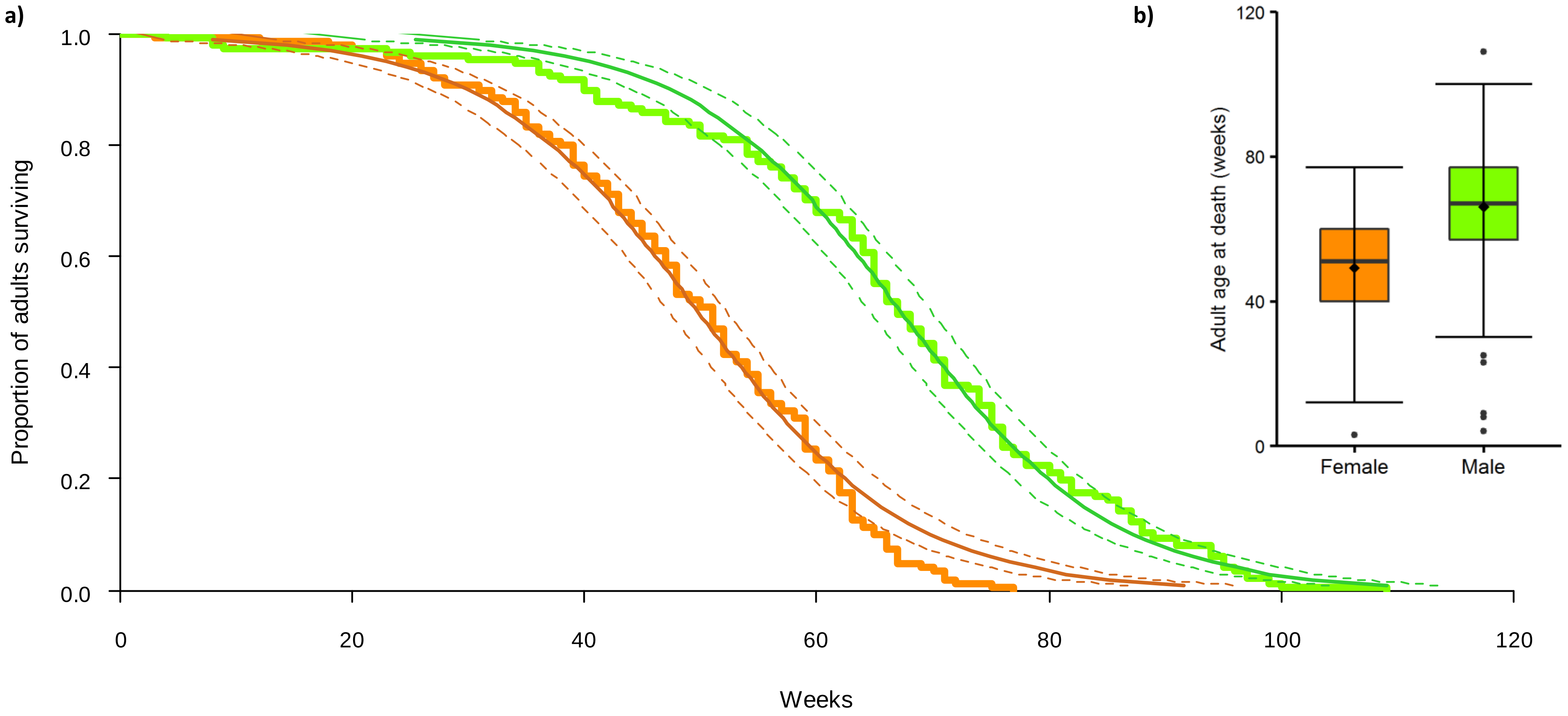


Figure 5.1 | Intrinsic survivorship of females (orange, n = 149) and males (green, n = 147). a) Survivorship curves of raw data (thick lines) overlaid with AFT (logistic hazard distribution) model fit (mean (thin line) and 95% confidence intervals (dashed lines)). The difference between females and males is highly significant ($z = 9.52$, $p < 0.001$). b) Boxplot to show distribution of adult age at death. Mean male lifespan (66.03 ± 1.59 weeks) is 35% longer than mean female lifespan (49.18 ± 1.12 weeks).

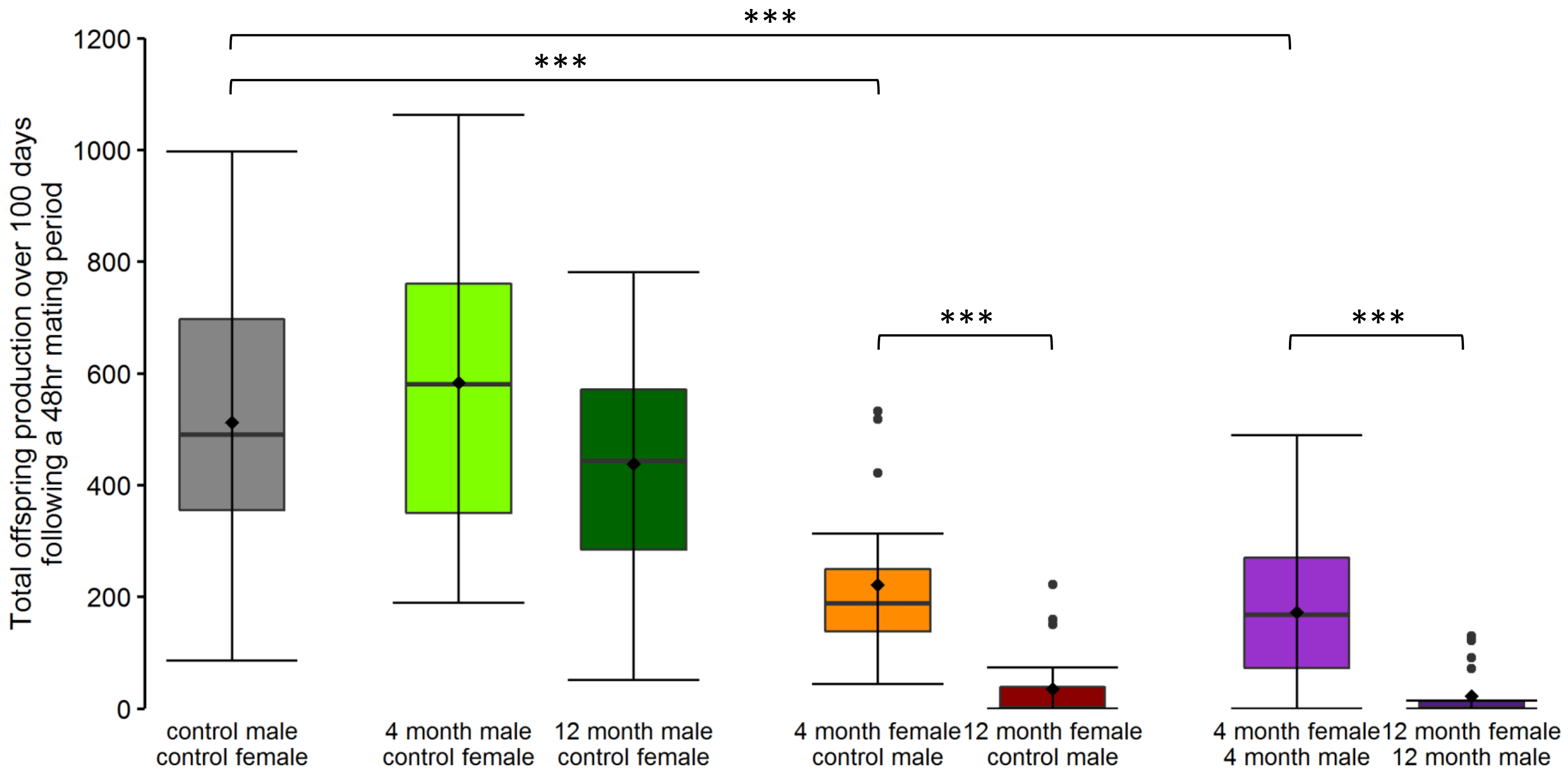


Figure 5.2 | Reproductive fitness of control young pairs (grey), aged males (green), aged females (orange) and aged pairs (purple) from 4 month (light) and 12 month (dark) age groups (n = 21-34 per treatment, brackets show significant pairwise comparisons, ***p < 0.001).

Finer scale changes in female reproductive fitness

Mean (± SE) female reproductive fitness, measured as offspring production over 100 days following a 48hr mating period, declined rapidly with increasing age of females. Reproductive fitness of 10-12 day post-eclosion (control) female *T. castaneum* was on average 554 offspring (± 44), but this declined significantly to 51% after just 1 month of female ageing (280 ± 40 offspring, $t = -8.27$, $p < 0.001$), further to 36% after 2 months (202 ± 31) and finally to 29% after 3 months (158 ± 25 offspring). Reproductive fitness of control females was significantly higher than older female age groups ($t_{3, 77} = -8.27$, $p < 0.001$), as was fertility of 1 month old females compared to older females ($t = _{3,77} -2.56$, $p < 0.05$) (Figure 5.3).

Sperm length variance in old males

No evidence was found for differences in sperm length variation between control versus 12 month old males. The mean (± SE) coefficient of variation (CV_{wm}) within males was 2.80 (± 0.10) for control males and 2.66 (0.10) for 12 month old males. Between males there was slightly more variation between control males ($CV_{bm} = 1.96$) than between 12 month old males ($CV_{bm} = 1.63$) although this difference is slight (Figure 5.4).

Transgenerational effects of old males

No evidence was found of differences in reproductive potential of sons of control males and sons of 12 month old males. The mean number of successful mates was 7 for both sons of control males and sons of 12 month old males ($z = 1.2$, $p = 0.23$). Mean (± SE) total offspring production also did not differ between sons of young control males (1036 ± 59) versus sons of 12 month old males (1042 ± 50 offspring) ($z = 0.02$, $p = 0.98$) (Figure 5.5a). Offspring production declined over consecutive mating opportunities ($t = -8.24$, $p < 0.001$), however, the rate of decline did not differ between sons of control and old males ($t = -0.40$, $p < 0.69$) (Figure 5.5b).

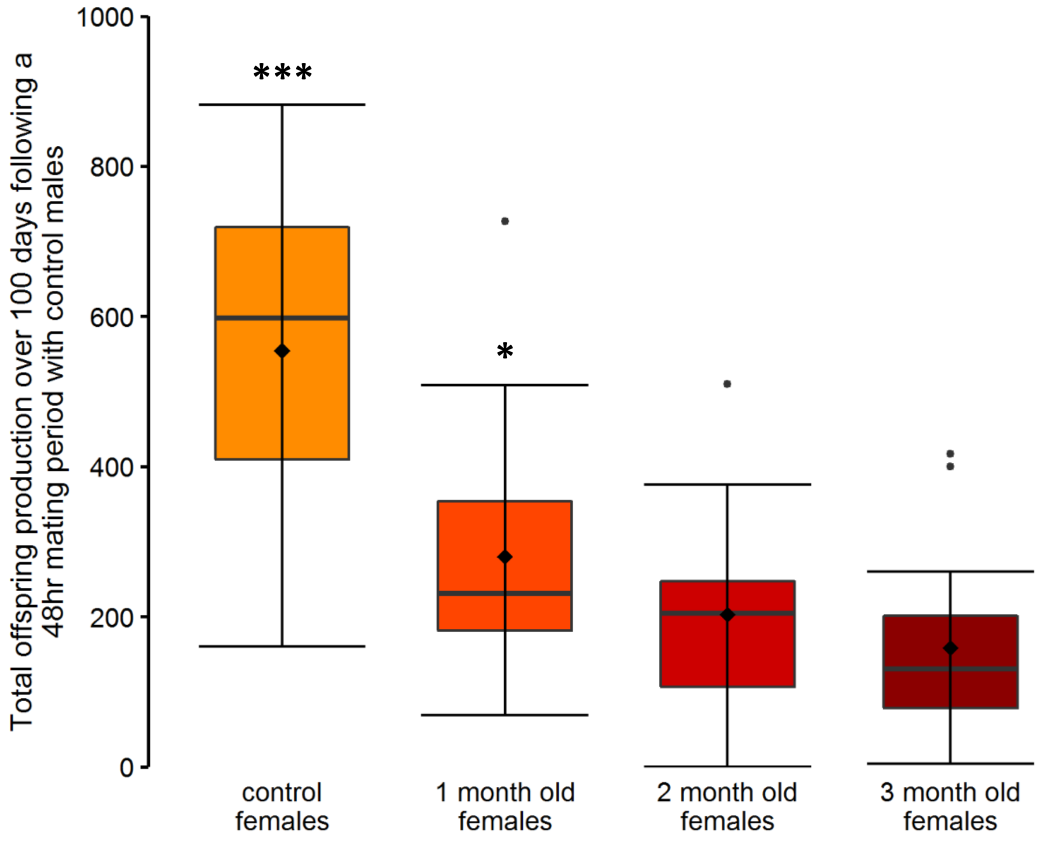


Figure 5.3 | Reproductive fitness of females from increasing age groups (n = 18-25 per age group, asterisks show age groups in which fertility was significantly different to older age groups, *p <0.05 ***p < 0.001).

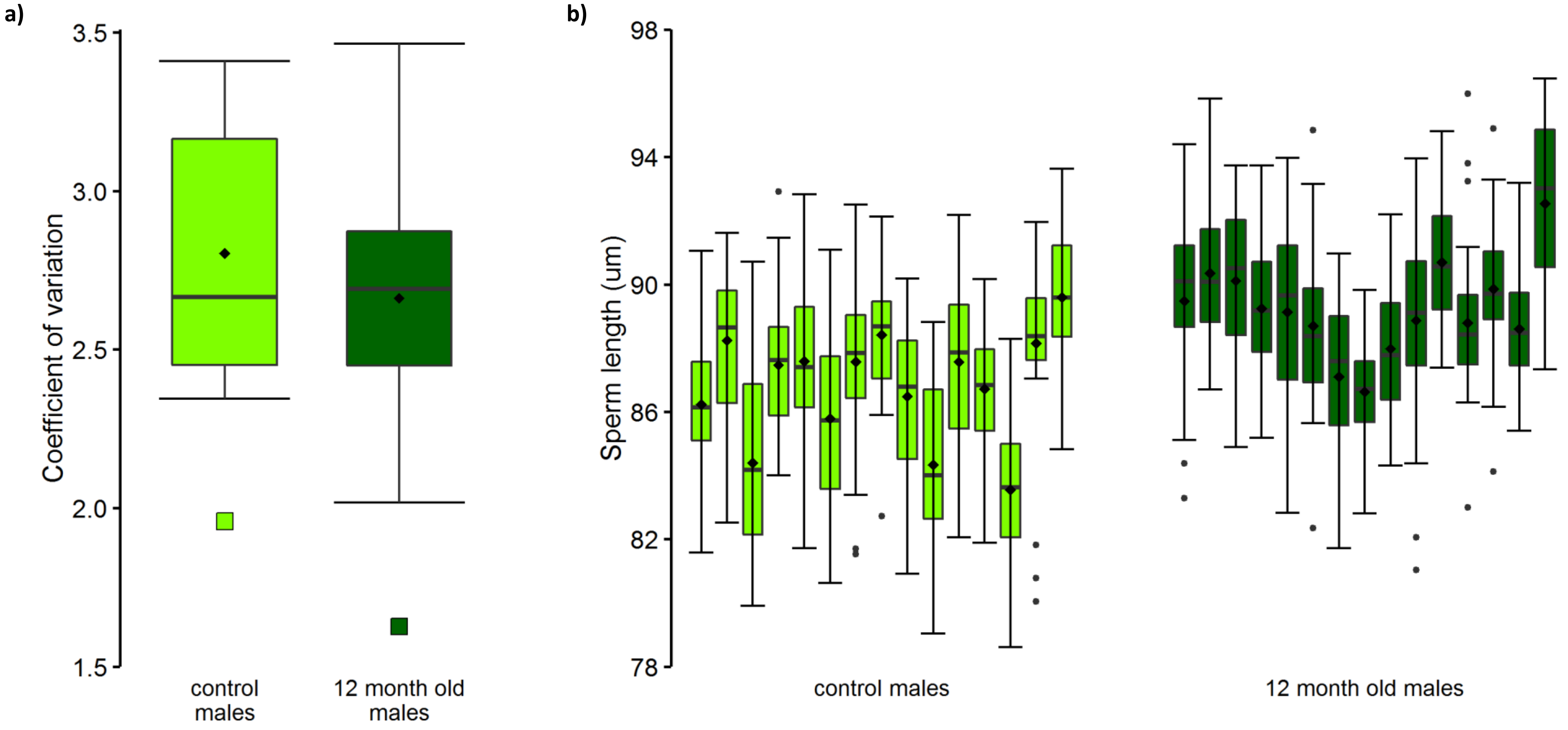


Figure 5.4 | Variation in sperm length of control (light green) and 12 month old (dark green) males (n = 30 sperm x 15 males per age group). a) Within male coefficient of variation (boxplot) and between male coefficient of variation (square points) in sperm length. b) Boxplots of the sperm length of individual males highlights the greater variation between control males than 12 month old males.

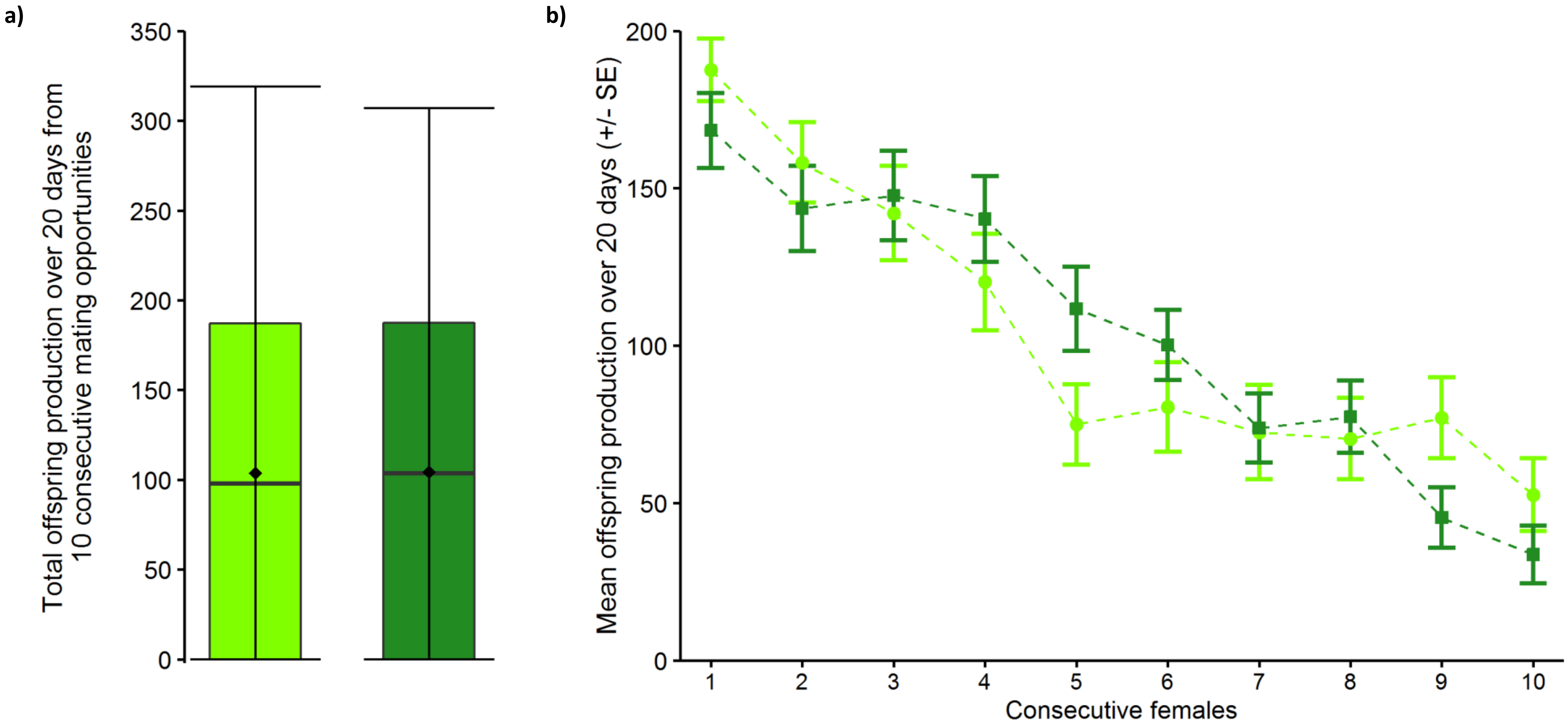


Figure 5.5 | Reproductive capacity of sons of control males (light green), and sons of 12 month old males (dark green) (n = 2 sons x 21 control males, n = 2 sons x 23 12 month old males)**.** a) Total offspring production. b) Offspring production per mating opportunity declined linearly (t = -12.73, p < 0.001) however this did not differ between sons of control and 12 month old males.

5.4 Discussion

Investigations into ageing in *Tribolium castaneum* have revealed significant sexual dimorphisms in intrinsic lifespan and age-related reproductive fitness. Females experience far more rapid reproductive ageing, and a much shorter lifespan compared to males. Evolutionary theories of ageing agree that reductions in the strength of natural selection with increasing age allows mutations with late deleterious effects to accumulate/persist, favouring investment in early life reproduction (Medwar 1952; Williams 1957; Kirkwood 1977). Sex-specific routes to increasing fitness will lead to sex-specific trade-offs in early/current reproduction versus future reproduction/survival (Stearns 1992), therefore sex-specific optimal allocation of resources could underlie sexual dimorphism in ageing and longevity (Bonduriansky *et al.* 2008; Maklakov & Lumma 2013). The results presented here indicate that males maintain more prolonged reproduction and survival, suggesting either a lower cost of reproduction, or a greater potential reproductive fitness through time, or both. By contrast, rapid declines in female fecundity and/or fertility with age indicates that investment in early reproduction is costly, and either the capacity of females to invest in future reproduction, maintenance and repair is reduced, or that future reproduction has a low fitness value, or both.

Rapid ageing and reduced longevity in females compared to males, suggests strong selection on females for investment in early reproduction and a corresponding substantial trade-off with survival. The significant decline in fertility, following just 1 month of ageing, could be consistent with expectations of extrinsic mortality in this species, where laboratory stock maintenance regimes impose regular mortality after a few weeks of adult life. Selection for individuals to invest in maintenance for older age is eliminated by removal of adults, following mating and oviposition, at ~20 days post-eclosion within every generation. Terminal investment theory predicts that reproductive effort should increase as the prospect of reproduction and survival decline (Williams 1966; Clutton-Brock 1984). This total removal of natural selection against late acting mutations, and increased selection for early reproduction, theoretically leads to the evolution of rapid senescence and reduced lifespan, as seen in semelparous species that die after a single reproductive event because no future fitness will be gained (Stearns 1992). This situation logically applies to *Tribolium* females in the light of high levels of extrinsic mortality after ~20 days of reproduction, and high costs of egg production. The question arises, however, as to why this situation is not seen in males?

Mounting empirical evidence recognises that allocation of resources to pre-copulatory male-male competition to increase mating success constrains resources available for survival, and is responsible for the reduced longevity of males, compared to females, observed in the majority of mammals (Clutton-Brock & Isvaran 2007; Lemaitre *et al.* 2014; Beirne *et al.* 2015; Tidiere *et al.* 2015). High intra-sexual competition is also associated with increased reproductive senescence in females of a cooperative breeding species where females compete for dominance (Sharp & Clutton-Brock 2011). The promiscuous mating system of *T. castaneum* decreases selection for pre-copulatory male-male competition over mating, and places the emphasis on mate searching and post-copulatory sperm competition to increase fertilisation success. Sperm are no longer considered to be a cheap, unlimited resource (Dewsbury 1982) and males are predicted to allocate resources strategically, such that sperm competition risk will increase investment in sperm number, quality and other ejaculate components (reviewed in Parker & Pizzari 2010 and Perry *et al.* 2013). Therefore, post-copulatory sperm competition, like pre-copulatory male-male competition, could select for males to bias investment in gaining fertilisations at the cost of resources available for survival. However, there is no evidence that this is the case in *T. castaneum*. Greater longevity and maintenance of high fertility in individuals up to 12 months old indicates that males are able to invest in maintenance and repair, therefore, the cost of investment in ejaculates, even where risk of sperm competition is high, appears to be at a level which does not impact on longevity or long-term fertility. Increasing male age was also predicted to decrease the efficiency of sperm production (Pizzari *et al.* 2008). However, no difference was found between control and 12 month old males in sperm length variance, and there was no evidence of transgenerational effects of paternal age on sons. The fact that neither of these traits change across 1 year of an adult male's lifespan, points towards relatively low costs for production of high quality ejaculates and investment in physiological maintenance and repair. Evidence from the meta-analysis by Hayward & Gillooly (2011) found rates of gamete production were two to four orders of magnitude higher in females across animal taxa. Therefore, although males experience costs of gamete/ejaculate production which require consideration with regards to male investment in alternative routes to mating and fertilisation success (Parker & Pizzari 2010), gamete costs for males are usually smaller than those experienced by females (Hayward & Gillooly 2011). Reduced cost of investment in reproductive fitness for males compared to females in this system could therefore explain sex-specific reproductive declines and ageing.

Alternatively, male reproductive fitness could be maintained if future opportunities for reproductive success are sufficiently beneficial to fitness for selection to favour investment in increased lifespan and fertility maintenance. Dispersal of individuals can create opportunities for increased reproductive success and evidence suggests males disperse more readily than females in *T. castaneum* (Ritte & Lavie 1977). Therefore, dispersal could create opportunities for future reproduction in males and generate stronger selection for maintenance and repair (survival) compared to females. This is the first within-species study to investigate sex-specific ageing and longevity in a promiscuous mating system, and the findings suggest that either males bear a lower cost of investing in reproductive fitness than females or that the probability of future reproduction across a longer timescale is much greater for males, or both. Further studies of sex-specific ageing and longevity in species where male reproductive investment is limited to ejaculate production and post-copulatory sperm competition are needed to establish the relative contribution of these.

In order to gather further evidence regarding the cost of reproduction, an important future assay will be to measure lifespan of both males and females as they reproduce. If investment in reproduction is at the expense of survival the lifespan of both sexes will be decreased, however the extent of the decrease in both sexes will provide valuable additional information regarding the cost of reproduction. It is likely, that reproductive investment is switched off to different degrees between males and females when isolated in flour. Virgin females are known to oviposit unfertilised eggs (Sokoloff 1974), therefore, if females maintain reproductive activity in the presence of suitable oviposition conditions, but males reduce activity in the absence of mates, then this differential investment could explain some of the dimorphism in longevity. Therefore, it will be important to measure the number of unfertilised eggs laid by virgin females in benign conditions, to help inform whether the sex difference in longevity shown in this study is due to investment in eggs and consequently reduced female investment in maintenance and repair. If this is the case, given such high costs for females, there is a question as to why females do not adjust reproductive investment on the basis of matings rather than oviposition conditions. Likewise, it would be informative to measure longevity of males when facing proximate conditions of high male-male competition and mating opportunities.

High male-male competition within a population is predicted to augment selection for male condition that improves the ability to acquire and use resources (Rowe & Houle 1996). Data presented in Chapter 2 of this thesis using experimentally-evolved populations with histories of contrasting strengths of sexual selection and conflict, showed no difference in intrinsic lifespan of individuals from contrasting sexual selection histories. However, following the results of this study where the reduction in female fertility with increasing age compared to males is much more significant than the reduction in lifespan, an important next study will be to measure sex-specific changes in fertility with increasing age. If individuals from populations with a history of high male-male competition are better at acquiring and making efficient use of resources, the trade-off for females between reproduction and survival would be expected to be reduced as more resources are available for survival. Furthermore, data presented in Chapter 4 shows divergence in sperm competitive ability of males from contrasting sexual selection backgrounds. Therefore, further investigation of changes in fertility with increasing age my reveal differences in the cost of reproduction for males evolving under contrasting sperm competition risk. Another interesting question would be whether male competitive ability changes with age as reproductive success must usually be achieved in the face of post-copulatory competition and choice in promiscuous species. Therefore, a more relevant and informative measure of male reproductive fitness with increasing age may be fertility within the demands of sperm competition.

Recently, the importance of sexual conflict within sex-specific patterns of ageing and longevity has become an important topic for discussion and investigation (Bonduriansky *et al.* 2008; Malakov & Lumma 2013; Adler & Bonduriansky 2014). Sexual conflict arises when there are differences in the evolutionary interests of the sexes (Parker 1979). Sex-specific optima for trait expression levels (intra-locus conflict), or conflict regarding the outcome of a male-female interaction (inter-locus conflict), will result in the outcome being more beneficial to one sex whilst being potentially costly for the other, shifting one or both sexes away from their optimum (Parker 1979; Chapman *et al.* 2003). Conflict between males and females may arise over courtship, mating, fertilisation and/or parental investment (Chapman 2006). The outcome will be disruption of optimal reproductive strategies and consequently allocation of resources to survival (Bonduriansky *et al.* 2008 and Malakov & Lumma 2013 and Adler & Bonduriansky 2014). Experimental reduction of sexual conflict in *T. castaneum,* by decreasing the number of males and therefore male-male competition, results in the evolution of females that suffer significant fitness costs when conflict is re-imposed. In contrast, females evolving in the face of conflict show no cost of high numbers of males (Michalczyk *et al.* 2011). These findings indicate that sexual conflict exists within this mating pattern, although it is difficult to reveal because females are adapted to it. However, when it is removed, females rapidly become susceptible which indicates that the coping mechanisms are costly to maintain and therefore adds to the cost of reproduction for females. In addition, faster oviposition in mated compared to virgin females

(Sokoloff 1974) and the identification of potential male accessory gland seminal fluid proteins in *Tribolium* (Fedina & Lewis 2008; South *et al.* 2011), suggests sexual conflict could follow patterns revealed in *Drosophila* in which seminal proteins manipulate female reproductive strategy, and both increase the cost of reproduction, and reduce survival of females (Chapman *et al.* 1995; Wigby & Chapman 2005). Female harm as a direct or indirect result of male-male competition, may also select for females to shift their reproductive strategy towards one of terminal investment (Stearns 1992; Wedell *et al.* 2006). Sexual conflict may therefore play a part in sex-specific ageing and longevity in *T. castaneum*, however, the extreme extent of the sexual dimorphism in reproductive ageing suggests that this is not the only driver as females would be predicted to evolve counter-adaptations to mitigate costs imposed by males (Parker 1979; Chapman *et al.* 2003). Investigations of changes in fertility with increasing age in populations with a history of enforced monogamy will improve understanding of the impact of sexual conflict on ageing and longevity in this species, which we have the opportunity to explore.

Evidence presented in this study shows a remarkable sexual dimorphism in reproduction and ageing in *T. castaneum*, which most likely suggests that there are significant differences in the energetic costs of reproduction between males and females. Male costs of ejaculate production appear to be low, despite sperm competition risk, and consequently allow for investment in longer-term survival and fertility, despite very high levels of male-male competition. However, investment by females in early/current reproduction appears to incur a significant trade-off against future reproduction/survival, resulting in rapid reproductive ageing and a 35% shorter intrinsic lifespan compared to males. Although sexual conflict may increase reproduction costs for females, it is unlikely to explain the extreme dimorphism in reproductive ageing in this species due to predicted counter-adaptation by females. Investigation of the rate of reproductive ageing of males and females from experimentally evolved populations with contrasting histories of sexual selection and sexual conflict will increase our understanding of the cost of reproduction, sex-specific allocation of resources to reproduction and survival, and the impact of sexual conflict. Assays of longevity where males and females are actively engaged in reproduction will also shed light on mechanisms within this sexual dimorphism in adult lifespan.

Chapter 6

General Discussion

The objective of this thesis was to address notable gaps in our understanding of the direct and indirect influences of sexual selection on three important levels of phenotypic variation. First, I aimed to measure the impact of sexual selection on a wider range of life history traits, beyond those closely linked to mating and fertilisation success, and their direct consequences for population fitness. This addressed important questions about how sexual selection interacts with natural selection and sexual conflict, as detailed in chapters 2 and 3. Second, I aimed to increase our understanding of how gamete-level traits are shaped by sexual selection. An incredible amount of biological diversity exists at the gamete level, but we understand very little of the adaptive significance of these traits. Using an intra-specific, experimental approach, I examined how sexual selection shapes sperm competitiveness, and how that is associated with divergence in sperm length and sperm variance (chapter 4). Thirdly, I measured sex-specific differences in reproductive ageing in the *Tribolium* model system, which showed profound male-female dimorphism, and mapped these differences onto sex-specific dimorphism in intrinsic ageing and longevity, in order to explore theories of how reproduction and ageing are linked (chapter 5). This general discussion draws the experimental chapters together, and provides an integrated summary of the main findings, along with a discussion of implications for current applied questions and future directions.

6.1 Main findings

No evidence of a consistent cost or benefit from a history of strong sexual selection for individuals facing environmental stress or ageing.

Individuals with histories of contrasting strengths of sexual selection did not differ in the length of their intrinsic lifespan, and survivorship of thermal (heat and cold) stress was inconsistent between sexual selection treatments. However, adults from populations with a history of strong sexual selection, survived significantly longer under starvation than those from weak sexual selection histories, and this result was augmented where juveniles developed in nutritionally stressful environment (chapter 2). Sexual selection is often viewed as working in opposition to natural selection, due to the high cost of development and maintenance of sexually selected signals (Lande 1980; Kirkpatrick & Ryan 1991; Houle & Kondrashov 2001). Alternatively, if sexually selected traits are dependent on condition, which is shaped by a large number of naturally selected genes, sexual and natural selection should be aligned and reinforce one another (Rowe & Houle 1996; Lorch *et al.* 2003). Heightened selection for condition under strong sexual selection could consequently aid the purging of deleterious mutations, increase fixation of beneficial alleles, and therefore provide significant fitness benefits for populations (reviewed in Whitlock & Agrawal 2009). The lack of a consistent negative influence of sexual selection history on fitness of individuals suggests that investment in reproductive success does not universally trade against the ability to cope with environmental stress, or resist ageing. However, the lack of a consistent positive effect also suggests sexual selection does not have a universal benefit on fitness, but may augment the evolution of more specific naturally selected traits (discussed further below).

Consistent evidence of a specific benefit of a history of strong sexual selection for individuals under nutritional stress.

The pattern of longer adult survival under starvation by adults with a history of strong sexual selection, which was augmented following nutritional stress during development, was consistent across both complementary experimental evolution regimes applying contrasting weak and strong sexual selection (chapter 2). This consistency provides strong evidence that sexual selection history influences ability to acquire, and make efficient use of, nutritional resources and therefore, provides evidence in support of genic capture theory, which predicts heightened selection for resource acquisition and efficiency of resource use as a result of the condition-dependence of sexually selected traits, and the alignment of natural and sexual selection (Rowe & Houle 1996). As *Tribolium* beetles live in their food medium and depend upon it (and mates) for reproductive fitness, nutritional stress is likely to be an ecologically relevant selection pressure which has acted on individuals and populations throughout the *Tribolium* evolutionary history. Therefore, rather than conferring a general fitness benefit, this evidence suggests sexual selection may provide a positive fitness benefit in the face of specific, ecologically relevant traits, already favoured by natural selection.

The extent of nutritional stress experienced by an individual will be dependent on local density/competition, as consumption of nutritional resources by some individuals will make them unavailable for others (Begon *et al.* 2009); this phenomenon is likely to apply as laboratory populations change in size and structure, and competition for the best food resources occur. Sexual selection favours traits which increase success in competition for mating and fertilisation opportunities (Darwin 1859, 1871), however this result suggest that there may also be a benefit of a history of strong sexual selection in the competition for nutritional resources. Therefore, this evidence points towards an interesting competition-dependent advantage for individuals with a history of strong sexual selection, across a wider range of non-sexual traits, and not just those directly involved in reproduction. Further evidence of this was seen in investigations of population invasion ability across multiple generations (chapter 3, discussed below).

Sexual selection history predicts the rate and extent of new population invasion.

A history of polyandry significantly increased the rate, and extent, of invasion of a novel intra-specific competitor population, compared to a history of monogamy (chapter 3). Sexual selection drives the evolution of mating and fertilisation success. However, colonisation and invasion within a competitor population require superior fecundity, viability and success in competition for all resources across all life stages by both sexes, not just competition by males for mates. Thus, the evidence for superior colonisation and invasion shows that sexual selection can provide wider fitness benefits to both sexes through all life history traits, not just those involved in mating and fertilisation success, translating into a net positive fitness effect of sexual selection in this system, and evidence for a wider impact of sexual selection on biotic fitness. Consequently, these findings also strongly support genic capture theory within sexual selection, providing the mechanism for these wider fitness benefits via shared condition-dependent pathways (Rowe & Houle 1996; Lorch *et al.* 2003). This evidence also endorses the idea of a competition-dependent advantage for individuals with a history of strong sexual selection as suggested by the advantage seen in the face of nutritional stress. Further studies are currently under way to investigate reproductive fitness of crosses between sexually selected males and intra-specific competitor females, in order to ascertain how much of the divergence in invasion ability is initially driven by any differences in male fertility between monogamous and polyandrous backgrounds.

Divergence in sperm competitive ability under contrasting sexual selection.

I found that experimental evolution of the strength of sexual selection, via manipulation of the operational sex ratio (OSR), has led to divergence in the paternity share gained by males experiencing sperm competition (chapter 4). Males with a history of strong sexual selection gained a significantly higher share of paternity than males from weak sexual selection backgrounds. A previous comparison of sperm competition success also found high second male precedence (P2) following contrasting sexual selection treatments (Michalczyk *et al.* 2011). However, this result was explained in terms of overall differences in mating activity, and not ejaculate-specific effects. Here, following a further 45 generations of experimental evolution, and executing a sperm competition experiment which measured ejaculate-specific effects by the competitor male's single mating, I found that males from a weak sexual selection background produce ejaculates that then result in a reduced P2. The relaxation in selection on sperm competitive ability that is expected in the female-biased OSR, appears to have resulted in a reduction in ejaculate investment per mating, perhaps to share investment across a greater number of potential mates under a female-biased OSR.

Sperm competition selects for directional not stabilising selection on sperm length.

Males from populations with a history of strong sexual selection have significantly longer sperm than males with a history of weak sexual selection. Despite the divergence in sperm size, the variance in sperm length did not differ between contrasting sexual selection treatments, either within or between males. These findings indicate that selection on sperm morphology is more directional than stabilising in *T. castaneum* (chapter 4). Theoretically, selection is predicted to disfavour increasing sperm size, as the energetic cost is expected to trade against sperm number, which is a key predictor of sperm competition success (Parker 1982). However, I found a clear association between increased sexual selection and increased sperm competitiveness and increased sperm length, despite a potential decrease in resources available for increasing sperm number. Further work is required to confirm whether a direct link exists between sperm length and increased sperm competitiveness, and to improve understanding of the mechanisms by which this could occur at the level of the gamete. Interestingly, I found no evidence of any differences in the extent of stabilising selection between strong and weak sexual selection, which might predictably result in reduced within- or between-male variation in sperm size under strong sexual selection (Parker & Begon 1993, Kleven *et al.* 2008). Perhaps the extremely promiscuous mating pattern of *Tribolium castaneum*, even under female-biased adult OSRs, still creates a sufficiently high level of sexual selection on gamete optima to stabilise canalised sperm length optima.

Sex-specific intrinsic lifespan corresponds with reproductive ageing profiles.

In the *Tribolium* system, males are able to maintain remarkably high reproductive success with increasing age to beyond a year, and show a correspondingly longer lifespan compared to females, whose reproductive output decreases by 50% after 1 month of adult life. Intrinsic female lifespan is 35% shorter than males (chapter 5). These findings provide an informative intra-specific test of the links between reproductive ageing and intrinsic lifespan where such obvious dimorphisms exist between males and females within one system. The contrasting reproductive strategies of males and females, which are rooted in fundamental differences in gamete size and number, are predicted to lead to sex-specific trade-offs between investment in early/current reproduction and future reproduction/survival (Stearns 1992), which could explain sex-specific ageing and longevity profiles (Bonduriansky *et al.* 2008). The evidence presented here indicates a significant sexual dimorphism in the costs of early reproduction, and/or that the probability of future reproduction is higher for males. Male costs of ejaculate production appear to be low, despite sperm competition risk, and consequently allow for investment in longer-term survival and fertility, despite very high levels of male-male competition. However, females experience a significant cost of early reproduction, which clearly reduces ability to invest in future reproduction/survival, and/or that future reproductive potential is very low. Planned future work will investigate intrinsic lifespan of both males and females when they are reproductively active, in order to gain further insight into the divergence in the cost of reproduction between the sexes.

6.2 Implications for current applied questions

Although the main emphasis of this thesis was to improve understanding of fundamental evolutionary questions, these findings have important implications for applied questions regarding population viability in the face of environmental change. Rapid, global environmental change as a result of modern anthropogenic activity is a major threat to biodiversity (Thomas *et al.* 2004; Parmesan 2006), therefore, understanding and predicting species responses to rapid change is a key aim of evolution, ecology and conservation (Williams *et al.* 2008; Dawson *et al.* 2011; Huey *et al.* 2012).

Is sexual selection beneficial during adaptation to environmental change?

Faced with unfavourable environmental pressures from modern anthropogenic impacts, organisms can either disperse to more favourable conditions, or adapt to cope (Gienapp *et al.* 2008). Within generations, phenotypic plasticity enables individuals to deal with unfavourable environmental pressures (West-Eberhard 2003; Ghlambor *et al.* 2007; Charmantier *et al.* 2008). However, genetic/evolutionary adaptation is likely to be most important for long-term solutions to changing environmental pressures (Gienapp *et al.* 2008; reviewed in Hoffman & Sgro 2011).

The costs versus benefits of sexual selection for adaptation under environmental change are not obvious (reviewed in Candolin & Heuschele 2008). Experimental evolution studies in which populations were allowed to adapt to a novel abiotic environment have proved inconsistent, with some reporting that sexual selection increased rate of adaptation (Fricke & Arnqvist 2007) and prevented extinction (Plesnar-Bielak *et al.* 2012), but others finding no benefit (Holland 2002; Rundle *et al.* 2006), or even antagonism (Chenoweth *et al.* 2015). However, an important confound in these types of study, as already discussed in this thesis, is unresolved sexual conflict which can concurrently decrease short-term fitness (Arnqvist & Rowe 2005), which I have aimed to overcome by testing experimentally-evolved populations after they have experienced a history of sexual selection, and when placed in standardised conditions where sexual conflict is equalised between treatments (Lumley *et al.* 2015). Evidence presented by Lumley *et al.* (2015) that strong sexual selection reduces extinction risk in the face of the genetic stress of inbreeding, is congruent with my findings that individuals from histories of high sexual selection are superior colonisers and invaders of competitor populations across multiple generations, where phenotypic quality across a range of traits must exist to achieve biotic superiority (chapter 3).

Together, these multi-generational studies, under rigorous experimental evolution and control, point to genetic benefits for populations from sexual selection, and therefore for adaptive evolution. Investigating the rate of evolutionary adaptation to abiotic stress under varying sexual selection in these populations would therefore be an informative next step. However, these various forms of stress are unlikely to occur in isolation and are often predicted to interact and amplify negative impacts on fitness (Armbruster & Reed 2005; Fox & Reed 2011). A more relevant investigation therefore, is the response of populations when faced with a combination of stresses (discussed below).

Can sexual selection protect populations from the extinction vortex?

Many populations are under genetic pressure as a result of anthropogenic-driven degradation of the natural environment, causing fragmentation, isolation and reductions in effective population size (reviewed in Keller & Waller 2002). Adaptation to environmental change is expected to be hindered where environmental stress leads to negative effects of demography and leads to population bottlenecks in which genetic variation is depleted (Plesnar-Bielak *et al.* 2012). The interaction of negative environmental, genetic and demographic factors has been theorised to lead to a downward spiral in fitness, eventually reaching depletion in population size and genetic diversity to such an extent that an extinction vortex exists (Gilpin *et al.* 1986; Fagan & Holmes 2006). Some evidence for this phenomenon comes from natural populations facing such pressures (McGinnity *et al.* 2003; Rowe & Beebee 2003; Blomqvist *et al.* 2010). An obvious question is how important sexual selection might be for resisting such extinction vortices, and an ongoing investigation using the *Tribolium* lines is in progress to answer such a question. We are testing fitness and resilience in populations from contrasting sexual selection histories following cycles of environmental (nutrition and heat) or genetic (inbreeding) stress over consecutive generations. Divergence in the rate of decline in fitness, or rate of extinction of replicate populations, will provide an important insight regarding whether sexual selection history can protect populations facing a potential extinction vortex (Gage *et al.* unpubl).

6.3 Summary

Does sexual selection act positively or negatively on population fitness?

Overall, no evidence was found of a trade-off between investment in sexually selected reproductive success and other naturally selected fitness components, or of negative impacts of sexual conflict. However, an improved biotic ability to colonise and invade new competitor populations suggests that a history of strong sexual selection, where male-male competition is high and there is ample opportunity for female choice, is beneficial to mean population fitness in *Tribolium castaneum*. However, this benefit does not appear to arise as a result of a universal improvement across fitness traits, but is more likely a result of an advantage when facing specific, competition-dependent and/or ecologically relevant challenges.

Does sexual selection act mainly on reproduction, or also on survival?

Evidence presented here suggests that sexual selection may benefit traits, and therefore fitness components, other than those directly involved in mating and fertilisation success. Longer adult survival under starvation suggests improved resource acquisition ability. Furthermore, invasion success requires superior fecundity, viability and success in competition for all resources by both sexes across all life stages, not just competition by males for mates.

The most parsimonious explanation for both of the above findings comes from the theory of genic capture, which predicts that natural and sexual selection will be aligned and reinforced as a result of the condition-dependence of sexually selected traits. Therefore, in summary this thesis provides **empirical evidence in support of genic capture theory.**

How does sperm competition risk influence sperm morphology/quality traits?

High sperm competition risk is predicted to increase investment in sperm number which is predicted to prevent/trade against increased sperm size. However, evidence presented here reveals that high sperm competition risk creates more competitive ejaculates, which are associated with directional selection on sperm length. This finding suggests the outcome of sperm competition could depend on sperm qualitative traits, and not just number of sperm.

Is sexual dimorphism in ageing explained by sex-specific investment in reproduction?

Notable sexual dimorphism in reproductive and intrinsic ageing suggests significant differences in the energetic cost of reproduction for males and females, and/or major differences in future reproductive potential. These differences map onto a clear dimorphism in ageing profile between males and females. In the absence of pre-copulatory male-male competition, the cost for males appears to be low, despite high sperm competition as a result of promiscuity. For females, investment in early reproduction is favoured at the expense of future reproduction/survival.

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