Pre- and post-copulatory sexual selection:

Their role in trait evolution in field crickets

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Pre- and post-copulatory sexual selection: Their role in trait evolution in field crickets

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Statutory declaration

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Ich versichere hiermit an Eides statt, dass die vorgelegte Dissertation von mir selbständig und ohne unerlaubte Hilfe angefertigt ist.

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Statement

Erklärung

Hiermit erkläre ich,

- dass die Dissertation weder ganz noch in wesentlichen Teilen einer anderen Prüfungskommission vorgelegt worden ist.
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Abbreviations

Abbreviation	Written-out	Meaning
Pre-M	Pre-copulatory monogamous	Mating treatment: animals were mated with intense pre-copulatory selection, monogamously
Post-P	Post-copulatory polyandrous	Mating treatment: animals were mated with intense post-copulatory selection, polyandrously
No-Sel	No Selection	Mating treatment: animals were mated with weakened sexual selection through enforced monogamy
LFP	Low frequency pulse	Courtship song element; multiple pulses of low frequency (4-5 kHz) within one chirp
HFP	High frequency pulse	Courtship song element; one single pulse of high frequency (11-16 kHz) within one chirp
SE	Standard error	Standard deviation divided by the sample size's square root
n	Number	Sample size measured

List of embedded publications

Matzke, M., Rossi, A., & Tuni, C. (2023). Pre-and post-copulatory sexual selection increase offspring quality but impose survival costs to female field crickets. *Journal of Evolutionary Biology*, *36*(1), 296-308.

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Summary

Sexual selection is responsible for shaping extravagant secondary sexual traits that enhance individual reproductive success. It operates both before and after mating (namely, pre- and post-copulatory sexual selection), giving rise to traits that not only secure access to mates, but also access to gametes (respectively, pre- and post-copulatory traits). In recent years, there has been increasing interest in addressing both episodes of selection, with studies revealing a complex interplay between the two forces that warrant further clarification on the net action of pre- and post-copulatory processes when addressing the evolutionary potential of sexual selection. Hence, the overall aim of my PhD project was to disentangle the effects of pre- and post-copulatory selection to provide insights into individual fitness consequences and reproductive trait evolution under either selective pressure. I made use of the two-spotted field cricket (*Gryllus bimaculatus*), a model organism in the study of sexual selection with a well described mating system including intra- and inter-sexual competition, both pre- and post-copulatory. By implementing a novel experimental design, I was able to experimentally allow either intense pre-copulatory selection, intense post-copulatory selection, and removal (or weakening) of sexual selection. I first investigated the fitness consequences to females (and their offspring) mated with intense pre- or post-copulatory sexual selection (Chapter 1). I then applied the same mating regimes over multiple generations using experimental evolution and measured evolutionary responses for a wide range of pre- and post-copulatory reproductive traits (Chapter 2). Lastly, I examined the costs of male ejaculate production under restraint food availability conditions (Chapter 3).

In **Chapter 1**, I investigated how disentangled pre- and post-copulatory sexual selection contribute to fitness. The debate on whether sexual selection increases or decreases fitness is ongoing, and the effects of each episode of selection remain unexplored. Here, I assessed direct (female reproductive success and survival) and indirect fitness (offspring growth and survival) of cricket females mated with the potential for either intense pre-copulatory selection (monogamously, but with pre-copulatory choice), intense post-copulatory selection (polyandrously, with post-copulatory choice) or under weakened sexual selection (through enforced monogamy, without pre- or post-copulatory choice). I found no differences in fitness of females (or their offspring) that experienced pre- and post-copulatory sexual selection. Yet, those experiencing pre- and post-copulatory sexual selection were more likely to reproduce, their offspring hatched sooner, developed faster, and had higher body mass at adulthood, compared to those mated with weakened selection. This study highlights the potential of sexual selection enhancing indirect female fitness. Nevertheless, these benefits occurred together with direct survival costs to females. Only by potentially outweighing these costs, increased offspring quality could lead to beneficial population-level consequences of sexual selection.

Chapter 2 aimed at investigating whether traits diverge in response to pre- and post-copulatory sexual selection. Literature on pre- and post-copulatory sexual selection is still discrepant about which episode of selection acts on which reproductive traits and whether selection triggers correlated evolution and/or evolutionary trade-offs between multiple reproductive traits. I used the same experimental setup as in the previous chapter (in fact, animals used in Chapter 1 acted as the founding generation for the current study) and applied experimental evolution. I experimentally subjected crickets to disentangled pre- or post-copulatory selection over multiple generations and measured responses of multiple pre- and post-copulatory male traits. Specifically, after three and six generations of experimental evolution a range of pre-copulatory traits were measured such as male calling and courtship song, aggressiveness, attractiveness, morphology (body size, weaponry, and body mass), next to post-copulatory traits, such as sperm viability and testes mass. I show that trait divergence occurred within the evolutionary timeframe studied. Overall, pre-copulatory traits responded positively to pre-copulatory selection, with males under intense pre-copulatory selection evolving - as expected - enhanced pre-copulatory trait values. On the contrary, against theoretical predictions, males under intense post-copulatory selection evolved lower post-copulatory, as well as lower pre-copulatory trait values. Our findings hence suggest post-copulatory selection not promoting phenotypes with higher fertilisation abilities, and a potential underlying positive correlation between certain pre- and post-copulatory traits, instead of allocation trade-off. This chapter emphasizes the potential correlations between pre- and post-copulatory traits and the fact that they can hardly be discussed separately, not even through isolated selection.

In **Chapter 3** I investigated the potential costs of ejaculate production and the trade-off between costs and benefits under constraint resource availability. The fact that males can suffer costs similar to females due to gamete production has been widely acknowledged, but poorly investigated. Male crickets were allocated to varying feeding regimes (to low or high life-lasting food treatments), and then to experimentally increased rates of ejaculate production (high and low ejaculate expenditure rates) by daily spermatophore removal. We measured treatment effects on changes in somatic maintenance, future reproduction, lifetime survival and longevity. I found no evidence that investment in ejaculates impinges upon male somatic maintenance, future reproduction, or longevity. Instead, our study revealed only negative effects of food limitation, suggesting low or undetectable costs of spermatophore production. Nevertheless, the fact that males were impaired due to nutrient deficiency in producing viable ejaculates, suggests condition-dependent costs for ejaculate production. High mating rates may select for males to maximise their capacity of ejaculate production, making ejaculate traits less prone to trade-offs with other fitness-related life history traits. This chapter explores the fundamental theory that gamete production is less costly for males than females and highlights that the expression of reproductive traits is affected by environmental factors such as food availability.

General Introduction

Sexual selection – a retrospective view

The theory of sexual selection was first proposed by Charles Darwin (1871) as an explanation for the evolution of exaggerated dimorphic male traits which did not appear to increase longevity or fecundity, hence would not be favoured by natural selection. According to Darwin, such traits function to increase an individual's reproductive success (specifically, males) when these compete against rivals for access to breeding territories or mates (hence, females). Although Darwin thereby provided a key conceptual framework for thinking about the natural world, his views on sexual reproduction were limited as to generally assuming females to be sexually monogamous (Birkhead, 2010). He declared males to be the active and females to be the passive sex, with the latter only exerting some choice and accepting one male in preference to the others (Darwin, 1871). It is possible that his point of view was biased by a cultural prudery of the time, which did not allow for the potential idea of mate preferences and female promiscuity (Shuker & Kvarnemo, 2021). He seemed to be particularly protective towards his wife Emma and his daughter Henrietta (Etti), since Etti helped him correct the proofs of *Descent* and was not to be offended by suggestive sexual thinking (Birkhead, 2010). However, there are indicators that Darwin must have been aware about females mating with multiple partners (i.e. polyandry) as he possessed literature on extra-pair copulations in pigeons (Girton, 1765) and even mentioned an example of inter-species multiple paternity in white domestic and Chinese geese in his own work (Darwin, 1871).

Intentional or not, Darwin's omission of female promiscuity led to almost a century of behavioural ecologists solely investigating pre-copulatory sexual selection, through mechanisms of male-male competition and monogamous female choice occurring before mating (Parker & Birkhead, 2013). It was not before the work of Geoff Parker (1970) that sexual selection was extended to include the post-copulatory concept of sperm competition, when sperm of multiple males compete within the female reproductive tract to fertilise her eggs, in females which mate with multiple partners within a reproductive cycle (i.e., polyandry). Profiting from improved molecular methods on parentage assignment, studies investigating the various mechanisms of sperm competition were still mainly male oriented, as it was assumed that selection operated more intensely on males than females (Birkhead, 2010). Thornhill (1983) brought the female perspective back on the table, when he first hypothesised that, similar to pre-copulatory choice, females make post-copulatory choices as well, characterising them as cryptic, since these processes take place hidden within the female reproductive tract. However, his idea was not given much attention until 10 years later, when the work of Eberhard (1996) and Birkhead (1998) initiated a multitude of studies exploring the existence of cryptic female choice.

Since then, another 20 years have passed until Shuker and Kvarnemo (2021) outlined that, after extending our views of what sexual selection entails, it was also time to extend our original Darwinian definition of sexual selection to include those views. Hence, instead of focussing only on the differences in reproductive success caused by competition over mates (Andersson, 1994), they proposed a more neutral definition that sexual selection is 'any selection that arises from fitness differences associated with non-random success in the competition for access to gametes for fertilisation' (Shuker & Kvarnemo, 2021).

Pre-copulatory sexual selection: male-male competition & female choice

Both pre- and post-copulatory sexual selection act through traits to shape phenotypes, which can manifest as morphological, physiological, or behavioural. One probable reason why pre-copulatory intrasexual competition was so indisputably accepted was because it is more or less self-evident, since males often openly display these (pre-copulatory) traits or use them to compete against each other to monopolise high quality resources, such as territories or food and consequently gain mating partners (i.e., intra-sexual selection) (Andersson, 1994). They do so by engaging into physical contests, which is observable in a wide range of taxa (Emlen, 2008, 2014; Rico-Guevara & Hurme, 2019) such as insects (Bonduriansky & Brooks, 1999), fish (Grant & Colgan, 1984), amphibians (Robertson, 1986), mammals (Herrera & Macdonald, 1993) and birds (Johnson, 1988). Males have evolved specific appendages that function as weapons to outcompete rivals (Emlen, 2008), such as the horns of the bighorn sheep *Ovis canadensis* (Lundrigan, 1996), the antlers of the red deer *Cervus elaphus* (Currey et al., 2009), the mouthparts of the stag beetle *Cyclommatus metallifer* (Goyens et al., 2015), or the tail of the cichlid fish *Nannacara anomala*, which is, in addition to swimming, also used to beat rivals (Enquist & Jakobsson, 1986). Males across taxa can also compete indirectly, by displaying elaborate secondary sexual traits, such as the red colouration of face, rump and genitalia of male mandrills (Setchell & Wickings, 2005) or the colourful abdomen of the peacock spider *Maratus* (Girard & Endler, 2014). Other males have evolved specific courtship behaviours, such as the courtship song of the field cricket *Gryllus lineaticeps* (Wagner & Reiser, 2000), or the courtship display-phenotypes in paradise birds of the genus *Parotia* (Scholes, 2008). These sexual traits often signal either male dominance or high quality through the ability to retain such a costly trait, referred to as the "handicap", in Zahavi's work (Zahavi, 1975). For males these costs can be metabolic/ energetic but can also come in the form of increased predation risk as colourful courtship displays may lead to overall higher visibility (Kotiaho, 2001; Sakaluk, 1990), a generally high energy expenditure due to multiple aggressive encounters with rivals (Borgia, 1985), or reduced immunocompetence (Gershman et al., 2010; Scharf et al., 2013).

Females remain to be considered the "choosy sex", controlling their reproductive outcome by mating with the best male(s) through mate choice. Mate preferences can result in direct benefits through material gains such as parental care, food, or shelter, which can increase female fecundity as well as female and offspring survival (Møller & Jennions, 2001). It can also entail indirect benefits in the sense of "good genes" which can increase offspring viability and/ or attractiveness, thus making them more successful in reproducing themselves (Head et al., 2005; Wong & Candolin, 2005). An example for direct benefits of female mate choice can be seen in the great tit *Parus major* where males with intense plumage colouration defend their broods with higher attentiveness against predators (Norris, 1990) or in the Hawaiian cricket *Laupala cerasina* where males provide spermless microspermatophores (small sperm packages) to females for pre-copulatory consumption (Shaw & Khine, 2004). Examples for indirect benefits manifest in the grey tree frog *Hyla versicolor*, as offspring of males producing long-duration calls to attract females show better performance during larval and juvenile stages (Welch et al., 1998), and in the peacock *Pavo cristatus* where offspring of males with elaborate trains show increased growth and survival (Petrie, 1994).

Post-copulatory sexual selection: sperm competition & cryptic female choice

On the contrary, a reason why post-copulatory sexual selection was overlooked is that it is not at all self-evident. Post-copulatory processes mostly occur inside the female body after the act of copulation, which makes them particularly hard to study, with some mechanisms only being revealed thanks to progressive molecular techniques (i.e., electron microscopy and DNA analysis). However, the acknowledgment of female promiscuity led to the question which of several males inseminating a female would fertilise her eggs, stimulating the investigation of mechanisms behind post- copulatory intra-, and later also intersexual selection (Birkhead, 2010).

Similar to pre-copulatory selection, sperm from multiple males engage in intrasexual competition to fertilise the female egg(s), resulting in post-copulatory selection acting on a wide variety of sperm traits, such as testes size and sperm phenotype or behavioural traits. Relative testes size was one of the first traits studied and is still regarded as an indicator of the level of sperm competition, as an increased number of produced sperm or production rate, which will lead to bigger relative testes size will provide fertilisation advantages if competition levels are high (Amann, 1970; Lüpold et al., 2020; Simmons & Fitzpatrick, 2012). Examples of increased testes size due to high sperm competition can be seen in fruit flies (Vahed & Parker, 2012) and yellow dung flies (Hosken & Ward, 2001), while an increase in sperm numbers was observed in house mice (Firman et al., 2018). Sperm size, with all its subcategories (i.e. sperm head, midpiece, flagellum and total length) is thought to be associated with increasing sperm competition as well (Gage & Morrow, 2003; García-González & Simmons, 2007; Lüpold et al., 2020, but for conflicting evidence on both testes and sperm size see Chechi et al., 2017 and Crudgington et al., 2009) with traits such as sperm length potentially influencing sperm swimming speed (Wigby & Chapman, 2004b) and velocity (Firman & Simmons, 2010b, but see also Humphries et al., 2008). However, not only sperm itself but also the seminal fluid surrounding it within the ejaculate is known to have effects on paternity outcome (Parker, 1970). The complex mixture of proteins, peptides and other ejaculate substances produced by male accessory glands have the potential to influence the performance of focal and rival sperm, as well as female compatibility and behaviours (Gillott, 2003; Poiani, 2006; Ramm, 2020).

To avoid investing energy into sperm traits related to direct sperm competition in general, some males evolved alternative strategies such as mate guarding (Parker, 2020). Males either closely follow premature females, to ensure they are the very first male to copulate after maturation (Dodson & Beck, 1993; Milner et al., 2010), or sometimes even before (Sentenská et al., 2021). Alternatively, they guard the female post-copulatory, preventing additional copulations with rival males (Córdoba-Aguilar et al., 2010; Sakaluk, 1991; Sherman, 1983). Another strategy that males of some spider species pursue is breaking off parts of their copulatory organ inside the female genitalia, referred to as mating plugs, blocking it from subsequent rival sperm to enter (Sentenská et al., 2018; Uhl et al., 2010). However, it is not always the first male to mate that gains a fertilisation advantage, as in multiple species the last male copulating with the female achieves the highest proportion of offspring sired (Lewis & Austad, 1990; Simmons, 2019). The mechanisms behind this so-called sperm precedence, or P2 can be outlined as incoming sperm displacing previous sperm (Harshman & Prout, 1994; Parker et al., 2010; Parker & Pizzari, 2010), sperm being stratified in a first in-last out system (Lessells & Birkhead, 1990; Parker & Pizzari, 2010) (both connected to the anatomy of the female spermatheca), or sperm loss, where the female either passively loses (Birkhead et al., 1995; Parker & Pizzari, 2010) or actively ejects entered sperm over time (Droge-Young et al., 2016; Peretti & Eberhard, 2010; Snook & Hosken, 2004). All the above-mentioned adaptations highlight the complexity of post-copulatory processes, with males aiming to succeed in fertilisation and females cryptically affecting this outcome.

Cryptic female choice entails female-mediated morphological, behavioural, or physiological mechanisms that operate to bias fertilisation toward the sperm of specific males

(Thornhill, 1983). It has been inherently obscure and even more difficult to study than sperm competition due to it being mediated by subtle molecular interactions and therefore particularly difficult to verify (Firman et al., 2017). Explicit definitions on how to demonstrate cryptic female choice have been provided by Birkhead (1998, 2000) and Firman et al (2017). The mechanisms driven by cryptic female choice are various and can occur during mating, shortly following insemination, over prolonged sperm storage, or around the time of fertilisation. By controlling the timing and order of competing inseminations females can aid the displacement of previous sperm by sperm of a new male, as in the polyandrous moth *Ephestia kuehniella* (Xu & Wang, 2010). By actively terminating or prolonging copulation duration females can either limit or boost the amount of sperm transferred by a male, as shown from our own work in the nuptial gift-giving spider *Pisaura mirabilis* (Matzke et al., 2022) or in the guppy *Poecilia reticulata*, where females copulate longer with more attractive males (Pilastro et al., 2007). Preferential sperm storage, where females bias stored sperm towards unrelated males is shown in the field cricket (*Gryllus bimaculatus,* Bretman et al., 2009 and *Teleogryllus oceanicus,* Tuni et al., 2013). Sperm ejection, which follows shortly after insemination, and differential sperm storage through displacement, stratification, mixing or sperm dumping, reflect the above-mentioned grey zone, as mechanisms of sperm competition and cryptic female choice are not mutually exclusive and often work through a process of male-female interaction (Firman et al., 2017). In the socially polyandrous dunnock *Prunella modularis* the males peck at the female cloaca before mating to induce female ejection of previous sperm (Davies, 1983), while in the feral fowl *Gallus domesticus*, females eject disfavoured inseminations by socially subdominant males (Pizzari & Birkhead, 2000). Another important cryptic role is played by female reproductive fluids, which for example can modulate sperm swimming velocity in external (Rosengrave et al., 2016; Urbach et al., 2005), as well as internal fertilisers (Gasparini & Pilastro, 2011). In some spider species, male sperm appears in an encapsulated form within the female spermatheca (the sperm storage organ) and can be selectively activated and decapsulated by female reproductive fluids (Herberstein et al., 2011).

Sex roles

The sex specific differences in both pre- and post-copulatory processes postulated above are explained by anisogamy, the phenomenon by which females produce a limited number of large gametes compared to males which produce, almost unlimited numbers of smaller gametes. Due to the mere number of available sperm within their reproductive lifetime (Scharf et al., 2013) males can maximise their reproductive success by mating with as many partners as possible (Bateman, 1948). Females on the other hand, are constrained by their limited number of eggs, (Bateman, 1948), as well as their increased gamete biomass production rate (Hayward & Gillooly, 2011) and hence are thought to possess a lower reproductive potential than males (Clutton-Brock & Parker, 1992; Kokko & Jennions, 2008). Choosiness in females has evolved to safeguard their heavy investment in a limited number of costly gametes and offspring in the form of parental care (Kokko & Jennions, 2008; Trivers, 1972). In addition, this has reinforced theoretical explanations as to why females should mate monogamously instead of polyandrously, since female reproductive output increases relatively less from multiple matings than does that of males (Dewsbury, 2005; Parker & Birkhead, 2013). However, decades of research have widely demonstrated that polyandry entails benefits, with females profiting both directly and indirectly from mating with multiple partners, and has far reaching evolutionary consequences (Pizzari & Wedell, 2013), as further discussed below.

Interestingly, the costs of individual gamete cell production in males have traditionally been considered low resulting in high numbers of sperm being produced (Scharf et al., 2013). Yet, we know that sexual traits which increase an individual's reproductive fitness must come with a cost counterbalancing such benefit (Kotiaho, 2001). Resources and energy allocated to sperm production would not otherwise be allocated to soma maintenance or other functions (for example), which may lead to a decrease in body condition or longevity. This can be observed in the two-spot ladybird beetle *Adalia bipunctata*, where mating reduces male lifespan around 50% and spermatophore transfer depletes male nutritional reserves (Perry & Tse, 2013), as well as in the flour beetle *Tribolium castaneum*, where higher levels of sperm competition lead to males evolving longer, but more costly sperm (Godwin et al., 2017). In the soil nematode *Caenorhabditis elegans*, it is the sole production of sperm which decreases male lifespan (Van Voorhies, 1992). Due to these energetic expenses, males can potentially become sperm depleted, either temporarily (Rönn et al., 2008) or permanently (Michalik & Rittschof, 2011), while other males might limit the amount of sperm provided to females in response to female mating status or their relative fecundity (Wedell et al., 2002). Next to gamete production itself, the seminal fluid surrounding the sperm consists of multiple components such as proteins and peptides (Avila et al., 2011; Poiani, 2006), functioning as a unit to ensure male fertility and maximise male siring success (Ramm, 2020; Simmons & Fitzpatrick, 2012). According to a recent meta-analysis, seminal fluid quantity is strongly and consistently condition dependent, (Macartney et al., 2019), suggesting that costs associated to ejaculate production are taxonomically widespread. The empirical evidence for sperm production being costly is mounting, but the number of studies addressing this question is still limited, highlighting the need for more research, especially in species, where we do not have conclusive evidence yet.

Fitness consequences of sexual selection

Sexual selection is an evolutionary process that arises from fitness differences associated with non-random success in the competition for access to females (pre-copulatory sexual selection) or their gametes (post-copulatory sexual selection), but whether it increases or decreases the fitness of individuals is still under debate (Candolin & Heuschele, 2008; Holman & Kokko, 2013; Kokko et al., 2003; Whitlock & Agrawal, 2009). The theory of 'good genes' postulates that sexual selection can have positive effects on individual fitness when female preference is biased towards individuals of overall higher genetic quality, passing on these high quality genes to the next generation (Hamilton, 1982; Whitlock & Agrawal, 2009; Zahavi, 1975). Similar to that, the 'sexy sperm hypothesis' suggests that females gain indirect benefits through mating polyandrously, as they are being fertilised by the most competitive sperm, passing on these competitive fertilisation abilities to their sons (Curtsinger, 1991; Simmons, 2003; Simmons & Kotiaho, 2007; Sivinski, 1984). Polyandrous mating further enables females to select sperm from a larger gene pool to ensure genetic compatibility (Jennions & Petrie, 2000; Zeh & Zeh, 1997). Additionally, females may also directly benefit by obtaining resources from multiple partners, such as nuptial gifts or parental care, which increase female fecundity as well as their ability to rear offspring (Kelly & Alonzo, 2009; Rooney & Lewis, 2002). According to the 'differential allocation hypothesis' (Burley, 1988), females can further invest more into their offspring when mating with preferred high-quality males, for example by providing more resources to their offspring (Kotiaho et al., 2003) or to their eggs (Kolm, 2001).

However, there has been evidence that mate choice and especially mating with multiple partners is not exclusively beneficial but can imply costs to both sexes. Mate searching, competition for mates, courtship and mating activities in general are often associated with time, energy, and increased predation risk (Kvarnemo & Simmons, 2013), together with potential infection by sexually transmitted diseases and costs related to immune function (Innocenti & Morrow, 2009). Such exaggerated costly male traits can be seen in the wolf spider *Hygrolycosa rubrofasciata* where male sexual signaling (drumming) causes an increased metabolic rate, potentially increasing mortality (Kotiaho et al., 1998), or in the damselfly *Mnais costalis* where the two wing colour-morphs of 'fighters' and 'sneaks' are condition dependent (Hooper et al., 1999). In females, the direct benefit of producing attractive offspring through mating with attractive males might be counterbalanced, as in the house cricket *Acheta domesticus*, where such females suffer from impaired viability (Head et al., 2005). Female fitness is also depressed in the fruit fly *Drosophila melanogaster*, when they are mated with larger males (which are deemed more attractive) or when females are raised in a male-biased high-density environment (Friberg & Arnqvist, 2003). In the same species, increased exposure to male seminal fluids will lower female survival rate (Chapman et al., 1995), while the experimental removal of sexual selection through enforced monogamy leads to males evolving to be less harmful to females (Holland & Rice, 1999). This supports the theory that any deviation from strict monogamy leads to sexual conflict, where male and female optima in reproductive interests do not align and hence both sexes can have negative effects on one another's fitness (Arnqvist & Rowe, 2013; Chapman et al., 2003; Fricke et al., 2009; Rankin et al., 2011). Another case of sexual conflict is sexually antagonistic genetic variation when males and females of the same species have different genetic optima (Brommer et al., 2007). This can be seen in the red deer where males with relatively high fitness father, on average, daughters with relatively low fitness, due to a negative genetic correlation between estimates of fitness in males and females (Foerster et al., 2007).

At the population level sexual selection can increase overall population fitness via correlated response if individuals with higher mating and/or fertilisation capacity are also those with higher fecundity and survival-related traits that elevate their progeny's lifetime reproductive success (when, for example, there is genetic variation for genes that determine 'quality') (Tomkins et al., 2004). Further, it may allow selecting for beneficial mutations or purging of harmful alleles, bringing large fitness benefits to both sexes (Rowe & Houle, 1996; Singh et al., 2017; Whitlock & Agrawal, 2009). That sexual selection increases overall population fitness was recently supported by a meta-analysis (Cally et al., 2019), which found that the beneficial population-level consequences of sexual selection outweigh the harmful ones, but these effects can differ between sexes and environment. Hence, it remains an important aspect in sexual selection studies to understand whether the costs to the individual (particularly females) inflicted by sexual conflict are balanced by the benefits of sexual selection (Cordero & Eberhard, 2003; Garcia-Gonzalez & Simmons, 2010; Rundle et al., 2007; Stewart et al., 2008). Yet, our understanding on how specific pre- and post-copulatory mechanisms affect individual fitness is limited and requires the disentanglement of the two selective forces and evaluate them individually and thus understand their relative fitness impact.

Unifying the pre- and post-copulatory 'divide'

With a vastly increasing number of studies on post-copulatory sexual selection in the past decades, the aim of studying both selection episodes together has been attracting more and more attention in recent years (Evans & Garcia-Gonzalez, 2016). So far, we know that the relationship between pre- and post-copulatory sexual selection is similar to the one between mechanisms of inter- and intrasexual selection, potentially overlapping, with male and female interests sometimes concurring and sometimes counteracting each other. We further know that traits involved in both pre- and post-copulatory episodes are unlikely to evolve independently from one another and hence the key to understanding how individual reproductive phenotypes are maintained is by considering both selective pressures and their interaction (Evans & Garcia-Gonzalez, 2016).

Interdependence of pre- and post-copulatory traits is assumed based on their phenotypic, as well as genetic correlations (Evans & Garcia-Gonzalez, 2016). If pre- and post-copulatory traits are positively correlated, it may indicate that individuals can equally invest into both sets of traits, which also implies an underlying high genetic quality of males, due to the high maintenance costs of sexual traits. This is supported by the phenotypic-linked hypothesis, stating that through their mate choices females favour male phenotypes with greater mating and fertilisation abilities (Sheldon, 1994). Negative trait correlation may instead indicate that individuals are constrained by an energetic trade-off, allocating their resources to either pre- or post-copulatory traits (Parker et al., 2013; Simmons et al., 2017). Whether traits are correlated at the genetic level or not entails an evolutionary consequence. If pre- and post-copulatory traits are positively correlated, selective pressures acting exclusively during one episode of selection (pre or post) should generate a corresponding response, facilitating the evolution of on traits involved in the other episode of selection (Lüpold et al., 2014). The contrary is expected if traits are negatively correlated, as selection during one episode of selection (for example, pre-copulatory) will constrain the evolution of traits in the other (for example post-copulatory).

However, in order to assess any trait's response to selection, we first need to understand the relative role that pre- and post-copulatory episodes play in sexual trait evolution in general and reproductive fitness in particular. One approach used to evaluate their relative strength is the statistical decomposition of the overall variance in reproductive success as proposed by Arnold & Wade (1984a, b), which defines pre- and post-copulatory selection as 'selection that occurs through variation in mating success and independently of such variation, respectively'. This analytical framework was later extended (Pélissié et al., 2014; Pischedda & Rice, 2012; Rose et al., 2013) through adding the post-copulatory component, which is the variation in siring success that arises independently of mating sequence. Overall, the evidence in determining the relative importance of pre- and post-copulatory selection on reproductive fitness seems to be scarce, with the existing studies rendering ambiguous results, showing equal contribution of both episodes (Danielsson, 2001; Filice & Dukas, 2019), or either pre- (Pélissié et al., 2014; Pischedda & Rice, 2012; Rose et al., 2013) or post-copulatory (Arnqvist & Danielsson, 1999; Marie-Orleach et al., 2021) selection being of greater relative value. An alternative approach to understand the action of each selective episode is by using quantitative genetics (Evans, 2010; Simmons & Kotiaho, 2007; Tuni et al., 2018). This method makes use of the pedigree within a population to estimate the genetic architecture and predicting the heritability of a trait or the genetic correlations between traits (Wilson et al., 2010). Similarly, these studies show contrasting results (Evans & Garcia-Gonzalez, 2016; Mautz et al., 2013), reporting positive trait correlations between sexual signalling and ejaculate size in the houbara bustard (*Chlamydotis undulata undulata*, Chargé et al., 2013), between testes weight as well as sperm length and male condition in the dung beetle (*Onthophagus taurus*, Simmons & Kotiaho, 2002), and between ejaculate size and aggression as well as body weight in the field cricket (*Gryllus bimaculatus*, Tuni et al., 2018). Yet, negative trait correlations were reported between male attractiveness and mating investment in the scorpionfly (*Panorpa cognata,* Engqvist, 2011), between sexual ornamentation and sperm viability in the guppy (*Poecilia reticulata*, Evans, 2010) and between the amount of trill in the courtship song and male ability for immune responses, as well as sperm viability in the field cricket (*Teleogryllus oceanicus*, Simmons et al., 2010).

While we are convinced that integrating all mentioned perspectives is crucial in gaining optimal insights into the interrelations between pre- and post-copulatory selection, we think there is a key element missing in generating a predictive framework. To unravel the relative strength of pre- and post-copulatory selection, it is necessary to first disentangle both selective episodes by establishing mating conditions that only allow for exclusive pre- or exclusive post-copulatory selection and then, estimate trait divergence on an evolutionary time scale.

Experimental evolution

One way to gain powerful insights in the evolutionary processes underlying trait evolution is by applying experimental evolution. With this tool it is possible to assess traits in response to varying selective pressures in manipulated, evolving laboratory populations (Kawecki et al., 2012; Simmons & García-González, 2008). Experimental evolution was at first mainly used in research on bacteria and cell cultures (Hsu & Kellogg Jr, 1960; Wu & Lin, 1978), but is now well established in the field of reproductive evolutionary biology. Studies have generally shown that sexual selection is key for reproductive trait evolution, through the two most applied manipulations concerning the variation of strength and/or opportunity for sexual selection. The former is accomplished by manipulation of the social environment that leads to enhanced competition. For example, studies manipulating sex ratios show that *Drosophila melanogaster* males from male-biased populations suffer depletion of their accessory glands (Linklater et al., 2007), but increase testes size (Reuter et al., 2008), while females survive longer (Wigby & Chapman, 2004a). Further, males adjust their behavioural responses to rivals (Edward et al., 2010), and fight less often on food patches when raised in a female-biased environment (low competition), while mated females show more frequent aggressive encounters (high competition) (Bath et al., 2021). Others manipulating population densities, report speciation occurring more rapidly in high density populations in *Sepsis cynipsea* due to increased sexual conflict (Martin & Hosken, 2003). Varying the opportunity for sexual selection, is achieved through mating system manipulation (polyandry vs. monogamy) to assess key reproductive traits (Pitnick, Brown, et al., 2001; Pitnick, Miller, et al., 2001). In a polyandrous mating regime, where individuals mate with which and how many mates they prefer, there is both opportunity for pre- and post-copulatory choice, while in a monogamous mating regime post-copulatory selection is entirely removed. Most of these studies, including the pioneer study from Holland and Rice (1999), used enforced monogamy by randomly assigning mating partners to each other, which further also minimizes pre-copulatory sexual selection. The insights gained from these experiments show that sexual selection has effects on fitness, with males evolving under weakened selection (enforced monogamy) harming females less and hence increasing their longevity as well as lifetime productivity (Holland & Rice, 1999), but also on sperm traits, with males evolving polyandrously increasing testes mass (*Scathophaga stercoraria*, Hosken et al., 2001, *Drosophila melanogaster*, Pitnick, Brown, et al., 2001; Pitnick, Miller, et al., 2001; *Onthophagus taurus*, Simmons & García-González, 2008) or sperm production (*Mus domesticus*, Firman & Simmons, 2010a). While all the studies mentioned above demonstrate the key role of sexual selection in reproductive trait evolution, they lack distinguishing between pre-copulatory and post-copulatory selection.

Aims and study system

The aim of this dissertation is to provide insights into:

1) **How disentangled pre-and post-copulatory sexual selection contribute to fitness**.

   The debate on whether sexual selection increases or decreases fitness is ongoing, and the effects of each episode of selection remain unexplored. Experimentally allowing for either pre- or post-copulatory selection to occur, followed by measures of direct and indirect female fitness traits can ease our understanding on the fitness consequences of sexual selection for the individual and its potential to affect populations. (Chapter 1; Matzke et al., 2023, Journal of Evolutionary Biology).

2) **Whether there is correlated evolution and/or evolutionary trade-offs between multiple reproductive traits in response to pre- and post-copulatory sexual selection.**

   Literature on pre- and post-copulatory sexual selection is still discrepant about which episode of selection acts on which reproductive traits and whether it triggers correlated responses or not. Experimentally subjecting crickets to disentangled pre- or post-copulatory selection over multiple generations and measuring responses of multiple (pre- and post-copulatory) male traits to both pre- and post-copulatory selection, will aid our understanding on the interplay between the two selective pressures (Chapter 2; Matzke et al., Manuscript unpublished).

3) **The costs of sperm production: are these really negligible?**
   The fact that males can suffer costs similar to females due to gamete production has been widely acknowledged, but poorly investigated. Exposing male crickets under varying nutritional states to high and low ejaculate expenditure rates and measuring effects on somatic maintenance, future reproduction, and individual longevity provides insights on fitness costs to males due to sexual selection (Chapter 3; McMahon et al., 2021, CELLS).

To investigate these questions, I focussed on the two-spotted field cricket *Gryllus bimaculatus*, a species occurring in the Mediterranean regions of Italy, France, and Spain with a well-studied reproductive biology (Horch et al., 2017). Male crickets engage in physical combats using their mandibles to wrestle each other (Adamo & Hoy, 1995), competing for breeding territories (i.e., small cracks in the ground or gaps under stones). To attract potential mates, the winners of these aggressive fights produce long-distance calling songs from their territory by stridulating their wings (i.e., rapidly rubbing their wings on top of each other, creating sound through the uneven line structure within their wings) (Montealegre-Z et al., 2011; Simmons, 1986b; Tachon et al., 1999). As soon as a female approaches, this song is converted into a courtship song accompanied by additional courtship behaviours (**Figure 1a**), such as contact of the antennas and positioning of the male body 'backwards' to ease female mounting (Sakai et al., 2017). Females exert mate choice based on the quality of male calling and/ or courtship song (Rantala & Kortet, 2003; Verburgt et al., 2011), male body size (Bateman et al., 2001; Simmons, 1986a) and odour based CHC (cuticular hydrocarbon) profiles (Iwasaki & Katagiri, 2008; Tregenza & Wedell, 1997). When a female accepts a male, she mounts him and the male extrudes his spermatophore, a round sperm package stored within his genital pouch (**Figure 1b**) and attaches it onto the base of the female ovipositor (egg laying apparatus, a long spike-like duct attached to her posterior) (Sakai et al., 2017). The sperm then flows through a connecting tube into the female sperm storage organ (spermatheca), where it is stored together with sperm from other mating partners. This gives rise to intense sperm competition (Simmons, 1987b) before the female eventually lays hundreds of eggs at a time within a sheltered, moist location. Females are further known to bias fertilisation towards preferred males by removing the spermatophores before sperm transfer is completed with their hindlegs (Simmons, 1986a) or by controlling sperm stores within the spermatheca (Simmons, 1987a). By doing so females can improve hatching success rates (Tregenza & Wedell, 1998) and avoid inbreeding (Bretman et al., 2009; Tregenza & Wedell, 2002). Males on the other hand are known to engage in pre- copulatory mate guarding to monopolise a female until they have successfully produced a spermatophore (Parker & Vahed, 2010) as well as post-copulatory mate guarding, to avoid female remating with another male or her discarding his spermatophore (Wynn & Vahed, 2004). Further, males producing relatively small but numerous sperm have increased fertilisation success (Gage & Morrow, 2003). We know that in the closely related Australian field cricket *Teleogryllus oceanicus* olfactory (CHCs) and acoustic (courtship song) cues have equal weight in predicting male mating success (Simmons et al., 2013), while an increased proportion of live sperm within a male's ejaculate entails higher paternity success rates (Garcia-Gonzalez & Simmons, 2005).

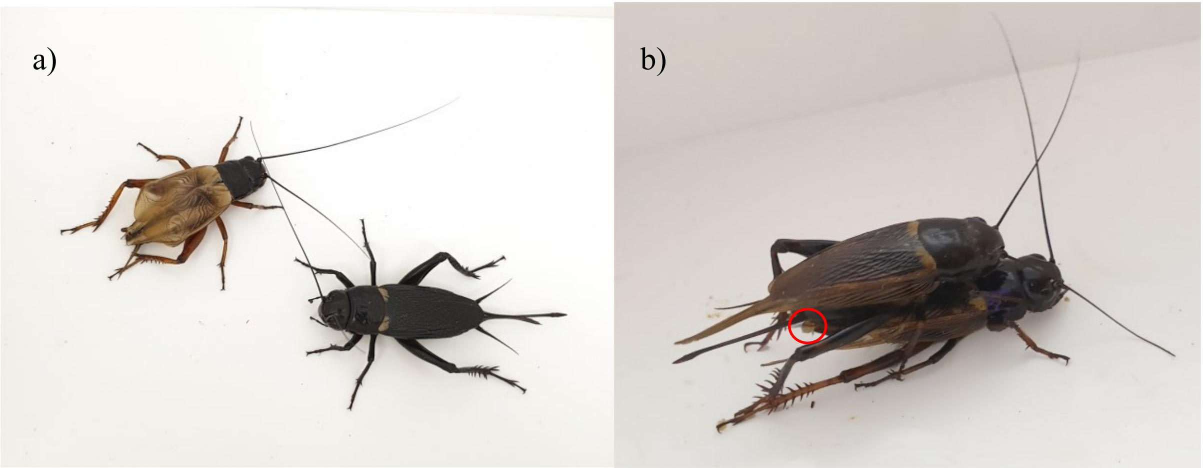

Figure 1: a) male (left) courting a female (right), by stridulating his wings; b) female (top) mounting a male (bottom), while the male is in process of transferring his spermatophore (red circle)

Overall, this species represents an excellent model organism to investigate mechanisms of sexual selection since pre-copulatory male-male competition and female choice occur to shape traits involved into dominance behaviour, calling and courtship song, and post-copulatory sperm competition and cryptic female choice to shape ejaculate size and sperm quality. In addition, they are common insects, well suited for long-term experiments over multiple generations due to their relatively short generation time (approximately 3-4 months) and their easy rearing in laboratory conditions.

Chapter I

Publication I: Pre- and post-copulatory sexual selection increase offspring quality but impose survival costs to female field crickets

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RESEARCH ARTICLE

Pre- and post-copulatory sexual selection increase offspring quality but impose survival costs to female field crickets

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Abstract

Whether sexual selection increases or decreases fitness is under ongoing debate. Sexual selection operates before and after mating. Yet, the effects of each episode of selection on individual reproductive success remain largely unexplored. We ask how disentangled pre- and post-copulatory sexual selection contribute to fitness of field crickets *Gryllus bimaculatus.* Treatments allowed exclusively for (i) pre-copulatory selection, with males fighting and courting one female, and the resulting pair breeding monogamously, (ii) post-copulatory selection, with females mating consecutively to multiple males and (iii) relaxed selection, with enforced pair monogamy. While standardizing the number of matings, we estimated a number of fitness traits across treatments and show that females experiencing sexual selection were more likely to reproduce, their offspring hatched sooner, developed faster and had higher body mass at adulthood, but females suffered survival costs. Interestingly, we found no differences in fitness of females or their offspring from pre- and post-copulatory sexual selection treatments. Our findings highlight the potential for sexual selection in enhancing indirect female fitness while concurrently imposing direct survival costs. By potentially outweighing these costs, increased offspring quality could lead to beneficial population-level consequences of sexual selection.

KEYWORDS
fitness, *Gryllus bimaculatus*, pre- and post-copulatory, sexual selection, survival

1 | INTRODUCTION

Sexual selection is an evolutionary process that arises from fitness differences associated with non-random success in the competition for access to gametes for fertilization (Andersson, 1994; Darwin, 1871; Shuker & Kvarnemo, 2021). It operates through mechanisms occurring before and/or after mating (hence, pre- and post-copulatory selection) (Birkhead & Pizzari, 2002; Evans & Garcia-Gonzalez, 2016). These include pre-copulatory male–male competition (i.e. males fighting for breeding territories or access to females; Andersson & Iwasa, 1996) and female choice (i.e. females selecting one or multiple partners) (Andersson & Simmons, 2006) as well as post-copulatory sperm competition (i.e. sperm of multiple males competing to fertilize the female's eggs) (Parker, 1970; Simmons, 2019) and cryptic female choice (i.e. females biasing fertilization towards a preferred male) (Eberhard, 1996; Pizzari & Birkhead, 2000). Whether sexual selection increases or decreases the fitness of individuals, ultimately affecting the mean fitness of the population, is still under debate (Candolin & Heuschele, 2008; Holman & Kokko, 2013; Kokko et al., 2003; Whitlock & Agrawal, 2009) and fuels research interested in understanding to which extent natural and sexual selection align (Locke Rowe & Rundle, 2021). Theory postulates that reproductive success is biased towards individuals of overall higher genetic quality ('good genes models'; Hamilton, 1982; Houle & Kondrashov, 2002; Kokko et al., 2002; Whitlock & Agrawal, 2009; Zahavi, 1975), through mechanisms of mate choice and/or competition. The process of sexual selection may hence allow females to derive indirect benefits to their progeny, by siring offspring that are themselves more successful in survival and reproduction. The net effects of sexual selection on fitness may also be enhanced by the direct benefits derived from the transfer of resources (e.g. nuptial gifts, parental care) from the mating partner, acting on female's fecundity and ability to rear offspring (Kelly & Alonzo, 2009; Rooney & Lewis, 2002). Maternal effects may also be at play as females that mate with preferred high-quality males may also increase their re- productive investment in offspring ('differential allocation hypothesis'; Burley, 1988), for example by providing more resources to their offspring (Kotiaho et al., 2003) or to their eggs (Kolm, 2001). Finally, if individuals with higher mating and/or fertilization capacity are also those with higher fecundity and survival-related traits that elevate their progeny's lifetime reproductive success (when, for example, there is genetic variation for genes that determine 'quality'), sexual selection would increase population fitness via correlated responses (Tomkins et al., 2004). At the population level, through good gene processes sexual selection may allow selecting for beneficial mutations or purging of harmful alleles, bringing large fitness benefits to both sexes (Rowe & Houle, 1996; Singh et al., 2017; Whitlock & Agrawal, 2009).

On the other hand, if there is conflict over reproduction, one sex (generally males) may increase their reproductive efficiency through harmful harassment (Gay et al., 2009), and/or seminal proteins (Wigby & Chapman, 2005) at the other's (generally females) expense (Chapman et al., 2003; Gavrilets et al., 2001; Rankin et al., 2011). Not only sexual conflict can lead to reduced female survival (Wigby & Chapman, 2005), but by resisting male harm females may suffer energetic costs, investing in defensiveness rather than offspring (Perry & Rowe, 2018). Successful males may also transfer sexually antagonistic genes that elevate their sons' reproductive success while lowering their daughters' (Brommer et al., 2007; Foerster et al., 2007). In this scenario, higher reproductive capacity of males reduces female productivity, with sexual conflict potentially decreasing the benefits of sexual selection (Pischedda & Chippindale, 2006). A key aspect in sexual selection studies is therefore understanding whether the costs to females inflicted by sexual conflict are countered by the benefits of sexual selection (Cordero & Eberhard, 2003; Garcia- Gonzalez & Simmons, 2010; Head et al., 2005; Rundle et al., 2007; Stewart et al., 2008).

A powerful empirical approach for studying the fitness effects of sexual selection has been manipulating the presence and absence of selection, with females having access to many mating partners (i.e. polyandrous matings) or only one (i.e. breeding through enforced monogamy; Partridge, 1980). Many of these studies are often originally aimed at understanding the evolution and maintenance of female mate choice, nevertheless allowing the operation of both mechanisms of sexual selection (Wong & Candolin, 2005). Using experimental evolution to measure differences in female reproductive output between breeding lines differing in mating systems (Cally et al., 2019; Edward et al., 2010; Power & Holman, 2014) has yielded mixed results, with sexual selection shown to elevate certain fitness traits (e.g. offspring viability (Partridge, 1980; Petrie, 1994; Power & Holman, 2014; Simmons & García-González, 2008); and adult survivorship (Promislow et al., 1998)), but not others (e.g. offspring emergence (Martin & Hosken, 2003)). Analysing these findings with a meta-analytic approach revealed a general positive effect of sexual selection on fitness, with yet only a trend for direct measures of female reproductive success, such as number of offspring and pro- portion of viable offspring (Cally et al., 2019). Single-generational experiments have also been pivotal in contributing to our general understanding of the fitness consequences of sexual selection. For example, numerous studies investigating the fitness benefits of mating multiply with different males or repeatedly with the same male, have highlighted the fitness-enhancing role of post-copulatory sexual selection (Arnqvist & Nilsson, 2000; Simmons, 2005). Nevertheless, costs to females from exposure to multiple matings are extensively described (Chapman et al., 1995; Crudgington & Siva-Jothy, 2000; den Hollander & Gwynne, 2009; Martin et al., 2004). Pre-copulatory sexual selection has also shown to affect female fit- ness. For example, females mated to attractive males benefit from elevated offspring fitness (Head et al., 2005). Other studies instead have shown that, despite male attractiveness being heritable, there is no association with fitness traits (Prokop et al., 2012), and mating with preferred males may even be detrimental for females (Friberg & Arnqvist, 2003).

When investigating the fitness consequences of sexual selec-tion, distinguishing simultaneously between the specific effects of pre- and post-copulatory sexual selection on reproductive output is rarely applied, despite many studies have focused on male fitness- related traits (Evans et al., 2015; Gasparini et al., 2019; McDonald et al., 2017; Travers et al., 2016, but see Gómez-Llano et al., 2021). Mechanisms operating before and after mating may contribute differently to the overall variance in reproductive success (Evans & Garcia-Gonzalez, 2016; Simmons et al., 2017), with growing evi- dence reporting either a higher contribution of pre-copulatory selec- tion (Pélissié et al., 2014; Pischedda & Rice, 2012; Rose et al., 2013), of post-copulatory selection (Arnqvist & Danielsson, 1999; Marie- Orleach et al., 2021) or an equal contribution (Danielsson, 2001; Devigili et al., 2015; Filice & Dukas, 2019). The above-mentioned studies (Marie-Orleach et al., 2021; Pélissié et al., 2014; Pischedda & Rice, 2012; Rose et al., 2013) address variance partitioning by statisti- cally evaluating the relative contribution of pre- and post-copulatory mechanisms of selection to fitness, rather than documenting abso- lute fitness values. Hence, interestingly, how each episode of selection relates to fitness output remains largely uncovered.

In this study, we test the assumption that sexual selection can enhance female reproductive success (Cally et al., 2019), and specifically test for differential effects of pre- and post-copulatory sexual selection by experimentally varying the potential for sexual selection before and/or after mating. We used the two-spotted field cricket *Gryllus bimaculatus* to estimate the fitness consequences of mating either under exclusive pre- or post-copulatory mechanisms of sexual selection, compared to mating with weakened or no sexual selection. The field of reproductive biology in this species is well-studied (Alexander, 1961; Gage & Barnard, 1996; Judge & Bonanno, 2008; Parker, 2009; Simmons et al., 2006; Sturm, 2011; Tuni, Beveridge, & Simmons, 2013; Wagner & Reiser, 2000). Pre-copulatory selection occurs in the form of aggressive male–male combats when competing for access to sheltered breeding territories (i.e. cracks in the ground and gaps under stones), from where they perform long-distance calling songs to attract females (Simmons, 1986; Tachon et al., 1999). Females wander in search for sedentary calling males and exert mate choice based on the quality of male calling or courtship song (Rantala & Kortet, 2003; Verburgt et al., 2011), as well as male body condition (Bateman et al., 2001; Simmons, 1986). Field crickets are polyandrous (Bretman & Tregenza, 2005; Simmons, 1987b), hence post-copulatory sexual selection occurs in the form of sperm competition where ejaculates from multiple males are stored in the female sperm storage organ, and females bias fertilization towards preferred males by controlling such stores of sperm (Bretman et al., 2009; Tregenza & Wedell, 2002). Pre- and post- copulatory traits, respectively male body mass, aggressiveness and ejaculate size, are positively correlated on a genetic level, indicating the existence of genetic variation in male quality (Tuni et al., 2018). Previous studies show that pre-copulatory selection contributes to female fitness as egg laying is increased in females that are allowed to choose their mating partners compared with those allocated to mates (Simmons, 1987a) and in females mating with dominant males (Bretman et al., 2006). We also know that multiple mating leads to increased offspring-hatching success (Tregenza & Wedell, 1998). However, since the relative contribution of pre- and post-copulatory processes to selection has not been resolved, we refrain from giving a priori predictions on whether selection occurring before or after mating should lead to higher fitness.

We established three mating treatments, with potential for exclusive (i) pre-copulatory sexual selection, (ii) post-copulatory sexual selection and (iii) weakened or no sexual selection. We are aware that pre-mating competition is unlikely to prevent post-mating selection (Fisher et al., 2016) as there is no mate monopolization in this system. Yet, our aim was to experimentally fully disentangle the effects of pre- and post-copulatory selection on female reproductive success by varying the potential for sexual selection to occur before or after mating. This inevitably entailed mating system manipulation. While standardizing the number of matings, pre-copulatory sexual selection was ensured by allowing multiple males to interact with rivals (i.e. fight) and court females (i.e. sing), females to exert mate choice for one of them and breed monogamously; post-copulatory sexual selection was enabled by preventing any pre-copulatory interactions (male–male fights and/or female choice) and breeding females sequentially with multiple males, hence, polyandrously; and sexual selection was minimized through enforced monogamy, by breeding females to one male only repeatedly. We measured direct fitness by estimating female lifetime survival, the likelihood of reproducing and the number of offspring produced, as well as indirect fitness by estimating offspring growth rates and survival. Females that experience repeated courtship and copulation are known to suffer from reduced longevity (Bateman et al., 2006), yet we predict that the benefits of sexual selection may offset these costs. We expect an overall reproductive benefit for individuals mated with the potential for sexual selection as choice and/or competition may lead to males of superior quality (Tuni et al., 2018; Wedell & Tregenza, 1999) to produce higher quality offspring, and explore the differences derived by selection being pre- or post-copulatory with no strong a priori expectation. However, given that our experimental procedure also manipulates the mating system (polyandry vs. monogamy) we expect indirect fitness to be higher following polyandrous matings, hence with post-copulatory selection.

2 | MATERIALS AND METHODS

2.1 | Animal breeding

Approximately 200 nymphs of *Gryllus bimaculatus* were collected from a large wild population in Tuscany (Giardino, 42°26′18.5″N 11°20′16.3″E, Italy) in July 2018 and transported to the laboratory at LMU Munich (Germany). Nymphs (20–30 individuals each) were placed in several large plastic tanks (23 × 15 × 17 cm) furbished with pieces of egg carton for shelter and provided with *ad libitum* food consisting of dry cat food (Ja! Knusper-Mix Rind & Gemüse), dry fish flakes (sera® Pond flakes Flockenfutter) and fresh apple slices, and water through plastic water vials plugged with cotton stoppers. Once animals reached adulthood, small plastic cups (5 × 7 × 7 cm) containing moist soil were provided for mated females to lay eggs (2 per tank). Cups were removed after 1 week and replaced with novel cups for oviposition. Removed cups were placed in new tanks provided with food and water and were left for eggs to hatch. Offspring were mixed and new tanks were formed, for rearing and breeding. This procedure generated a large, outbred population. Animals were housed in a climate room with 60% relative humidity under a 12:12 h light:dark photoperiod that was kept at 26°C for two generations, and then 28°C.

After three generations, when nymphs reached their penultimate or last instar, we transferred approximately 600 males individually to containers (10 × 10 × 9 cm) equipped with shelter, water and dry cat food. Males were housed individually to control for mating and social (e.g. fighting) experience until sexual maturation. Last instar females (approximately 400) were instead kept in small tanks (12 × 14 × 23 cm) in groups of 5. All animals were checked twice a week for moults to determine whether they reached adulthood, and hence age post-eclosion. On these occasions, females were relocated to all-adult tanks of same densities and feeding conditions. Approximately 2 weeks after reaching sexual maturity animals were allocated to the treatments described below. For logistic reasons, age at mating was not strictly standardized and therefore controlled statistically. The day before the mating, males and females were weighed using a digital scale (KERN & SOHN GmbH, Balingen, 208 Germany, accurate to 0.001 g), and males had their spermatophore removed to standardize sperm age, as males produce replacement spermatophores (McMahon et al., 2021). Females were individually isolated as described for males.

2.2 | Experimental treatments

We established the following treatments: (i) Pre-copulatory sexual selection and monogamous matings (PRE-M), in which each female was placed together with three random males inside an arena sized 32 × 15.5 × 13 cm. Males were left to fight against each other and court (i.e. sing) the female. Once females exerted mate choice, meaning they successfully mounted the male and the latter transferred a spermatophore, the other males were removed from the arena, and the pair was mated again twice. In this species, dominant crickets tend to achieve higher mating success (Bretman et al., 2006; Rantala & Kortet, 2004; but see Tuni et al., 2016; Vedenina & Shestakov, 2018). (ii) Post-copulatory sexual selection and polyandrous matings (POST-P), in which each female was randomly paired to one male inside a 16 × 15.5 × 13 cm sized arena, until successful mounting and spermatophore transfer, for a total of three matings with three different males. (iii) Weakened sexual selection and enforced monogamy (NO-SEL), in which each female was randomly assigned to a male inside a 16 × 15.5 × 13 cm arena until successful mounting and spermatophore transfer. The pair was mated again twice. We standardized number of matings (i.e. three) across treatments not only to exclude sperm limitation but also to balance potential direct benefits females could gain from multiple matings (e.g. nutritious ejaculates) and males were not used interchangeably between treatments. Due to logistics (i.e. handling of a large number of individuals), not all trials were conducted simultaneously, with those from the NO-SEL treatment being conducted once the previous were completed. Identical conditions for animals of all treatments were ensured by maintaining identical temperature and humidity settings within the climate room at all times (for rearing, mating and fitness measurements).

In all the trials, males and females were left together for 10 min after mating to allow mate guarding and prevent females from removing the spermatophore sooner (Simmons, 1986). They were then briefly returned to their individual housing containers, and mated for two additional times as described above, at 2 h intervals to allow males enough time to produce a novel spermatophore (Parker & Vahed, 2010). Individual sperm traits (e.g. number and viability) appear to be repeatable across spermatophores in this species (Gage & Morrow, 2003; McMahon et al., 2021), likely minimizing sperm depletion effects in PRE-M and NO-SEL males. Only animals that successfully completed three matings were included in the study, resulting in a total of 133 females (and 133 males) for the PRE-M treatment, 88 females (and 264 males) the POST-P, and 103 females (and 103 males) the NO-SEL (whereas 3 POST-P, 3 PRE-M and 3 NO-SEL females were excluded).

After mating, females were kept individually in small tanks (12 × 14 × 23 cm) equipped with water, food, egg carton and a small plastic cup (5 × 7 × 7 cm) filled with moist soil, to lay eggs in. After 1 week the cup was placed in a new box (6 × 12 × 12 cm) with food and water for the offspring to hatch and was replaced with a new one to allow females to prolong oviposition in a novel substrate without simultaneous hatching of eggs. Similarly, the second cup was removed after 1 week and females returned in their housing containers.

2.3 | Fitness estimates

The likelihood of reproducing of each female was assessed based on whether any offspring from the two egg batches (oviposition cups 1 and 2) had hatched or not. Latency to hatching was scored as the time from providing an oviposition substrate to the female until hatching of the first offspring. After 2 weeks of egg laying, females were inspected twice a week to score lifetime survival. Approximately 3 weeks from hatching, the number of offspring produced by each female was counted by gently transferring the offspring to new containers using a paintbrush.

After counting, offspring of females within the same treatment and egg batch were mixed. We randomly selected nymphs off egg batch 1 from the PRE-M and POST-P treatments (n = 64 nymphs each) and from NO-SEL treatment (n = 48) and placed them individually in containers (10 × 10 × 9 cm) equipped with shelter and *ad libitum* water and food. Their body mass was measured twice a week until maturation (i.e. final eclosion to adulthood) and three additional times after maturation. Age at maturation was recorded, and all individuals were then inspected twice a week in their housing container to score offspring lifetime survival.

2.4 | Statistical analysis

To test whether our experimental treatments (PRE-M, POST-P and NO-SEL) affected female fitness we used generalized linear models with binomial distribution (glm-b) for likelihood of female reproduction (i.e. likelihood of producing any viable offspring) and negative binomial distribution (glm-nb, accounting for overdispersion) to investigate the total number of offspring hatched (i.e. sum of total number of offspring from oviposition cup 1 and 2) and mean latency to hatching (i.e. number of days from providing the oviposition cup to hatching, averaged for both oviposition cups). Treatment, female mass (mean-centred within treatment) and female age at mating were fitted as factors in the model. In case of a significant treatment effect, we used Tukey post hoc tests to understand differences between the treatments. Given the unintended variation in female age at mating (mean number of days from adult enclosure, PRE-M 22.73 ± 0.5, range = 14–37, *n* = 96; POST-P 22.3 ±0.5, range = 14–30, *n* = 78; NO-SEL 9.8 ±0.4, range = 8–17, *n* = 84; Kruskal-Wallis, $x^2 = 166.84$, df = 2, $p < 0.0001$) we excluded females younger than 1 week (*n* = 16 females from the NO-SEL) from our analyses of female fitness and survival (i.e. a 6 day pre-oviposition period is also recommended to ensure reproductive maturity (Simmons, 1987b) and reference within). We also mean-centred age at mating by treatment to account for all variation among and within treatments before introducing it as a covariate in the models (Enders & Tofighi, 2007). We additionally ran a sequential ANOVA (Type II) on estimates of latency to hatching and number of offspring to specifically test the effect of treatment subsequent to the effect of female age as well as their interaction. Since reproductive success is represented by binomial data, sequential ANOVA was not possible here.

To analyse whether lifetime survival probabilities of females were affected by our experimental treatments, we carried out a Kaplan–Meier survival analysis to create survival curves and tested significance using a multivariate cox regression analysis on our lifetime data. Time (in days) was defined as the response variable with treatment (PRE-M, POST-P and NO-SEL), female mass and female age at mating as the independent variables. This is a non-parametric test to estimate the probability of survival at any given time interval in the data.

To test whether our experimental treatments (PRE-M, POST-P and NO-SEL) affected offspring growth we used a generalized linear mixed model (GLMM) with gaussian distribution, including body mass measured over time as response variable. Treatment and age (timepoint of each measurement) as well as their interaction were fitted as fixed effects (Model 1), as the latter will give information about differences between treatments across the entire growth period (=growth rates). To test whether offspring growth was further affected by sex, we conducted a second model including sex as well as the interactions between sex, treatment and age as fixed effects. Cricket ID was fitted as random effect in both models.

To test whether offspring body mass at maturation differed between treatments and sexes, we used analysis of variance (ANOVA). Kruskal–Wallis test was used to test for treatment differences in offspring age at maturation.

All analyses were conducted in R (version 4.1.1, R core Team, 2021) using the package 'MASS' for negative binomial generalized linear models (logistic regression), the package 'DHARMa' (Hartig, 2020) for checking model assumptions, the package 'multcomp' for conducting Tukey post hoc testing and the package 'survminer' (Kassambara et al., 2021) for survival analysis. Data in the text are reported in mean ± standard errors.

3 | RESULTS

3.1 | Likelihood of female reproduction

The likelihood of successfully reproducing in females (i.e. females with viable offspring) was significantly affected by the experimental treatment, as it was 99.1% (*n* = 130) in the PRE-M treatment, 100% (*n* = 85) in the POST-P treatment and 92.8% (*n* = 84) in the NO-SEL treatment (Table 1, Figure S1). However, post hoc analyses revealed no significant differences between treatments (Tukey Post-Hoc; POST-P – PRE-M: *p* = 1.0, NO-SEL – PRE-M: *p* = 0.11, NO-SEL – POST-P: *p* = 1.0). We also found a marginal positive effect of female body mass, with heavier females being more likely to successfully reproduce, but no effect of age at mating (Table 1).

3.2 | Latency to hatching

Offspring of PRE-M and POST-P females hatched significantly sooner than offspring of the NO-SEL group (Table 1, Figure 1; Tukey Post-Hoc; NO-SEL - PRE-M: *p*-value = 0.001; NO-SEL - POST-P: *p*-value = 0.002), but there was no difference between POST-P and PRE-M offspring (Tukey *post hoc*: *p*-value = 0.99). Latency to hatching was not affected by female body mass or age at mating (Table 1). The sequential ANOVA provided similar results (Table S1).

3.3 | Number of offspring

The total number of offspring hatched from each female was not affected by the treatments (mean number of total offspring, PRE-M 187.3 ± 8.7, *n* = 118; POST-P 182.7 ± 13.5, *n* = 79; NO-SEL 200.2 ± 16.2, *n* = 77; Table 1) nor by female body mass and age at

TABLE 1 Results from the GLMs testing the effects of treatment, female body mass (mean-centred within treatment), female age at mating (mean-centred within treatment), on (i) female reproductive success (hatching probability of entire egg batch, binomial), (ii) latency to hatching (negative binomial) and (iii) number of offspring (negative binomial).

	Reproductive success			Latency to mating			N offspring		
	GLM-Binomial			GLM-NB			GLM-NB		
	X^2	df	*p*-Value	X^2	df	*p*-Value	X^2	df	*p*-Value
Treatment	10.87	2	**0.004**	15.48	2	**<0.001**	0.59	2	0.74
Female body mass	3.89	1	**0.048**	0.11	1	0.74	2.68	1	0.1
Female age at mating	2.29	1	0.13	0.39	1	0.53	0.5	1	0.48

Note: Significant effects are shown in bold.

mating (Table 1), which was supported by results from the sequential ANOVA (Table S1).

3.4 | Female survival

Survival probabilities were significantly higher for NO-SEL females compared with PRE-M (HR = 2.76, df = 2, p = 0.0001) and POST-P females (HR = 2.91, df = 2, $p < 0.0001$, Figure 2) but did not differ between PRE-M and POST-P females. They were neither affected by female age at mating (HR = 0.98, df = 2, p = 0.14) nor by fe- male mass (HR = 1.25, df = 2, p = 0.6). The average age at death was 61.3 ± 1.7 days (n = 80) for NO-SEL females, 53.7 ± 1.1 days (n = 95) for PRE-M females and 53.4 ± 1.2 days (n = 77) for POST-P females.

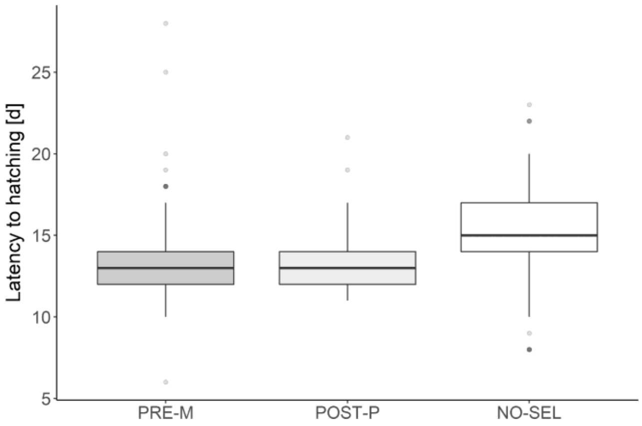

FIGURE 1 Differences across treatment (PRE-M = pre-copulatory sexual selection and monogamy; POST-P = post-copulatory sexual selection and polyandry; NO-SEL = no sexual selection and enforced monogamy) for latency to hatching of offspring, with NO-SEL offspring hatching significantly later compared with PRE-M and POST-P offspring.

3.5 | Offspring growth

Individuals of the NO-SEL treatment (n = 41) grew significantly slower than individuals of the PRE-M (n = 59) and POST-P (n = 63) treatment (Table 2 Model 1, Figure 3). Overall, females grew faster than males, with this effect being strongest in the PRE-M treatment compared with the NO-SEL treatment (Table 2 Model 2).

Offspring body mass at maturation was significantly lower for offspring of NO-SEL treatments (ANOVA; F = 9.01, df = 2, p = 0.0002; mean body mass at maturation [g] PRE-M 0.94 ± 0.03, n = 59; POST-P 0.91 ± 0.03, n = 63; NO-SEL 0.78 ± 0.03, n = 41), as well as for males compared with females (ANOVA; F = 28.8, df = 1, $p < 0.0001$; mean body mass [g] males 0.81 ± 0.02, n = 83; females 0.97 ± 0.02, n = 79) (Figure S2). Offspring age at maturity neither differed between treatments (Kruskal–Wallis; x^2 = 0.76, df = 2, p = 0.69; mean offspring age [d] PRE-M 31.4 ± 1.2, n = 59; POST-P 31.6 ± 0.8, n = 62; NO-SEL 31.2 ± 1.5 n = 41), nor between sexes (Kruskal–Wallis; x^2 = 2.02, df = 1, p = 0.16; mean offspring age [d] females 32.6 ± 0.9, n = 79; males 31 ± 0.9, n = 83).

3.6| Offspring survival

Survival probabilities of offspring did not differ between NO-SEL and PRE-M offspring (HR = 1.48, df = 2, p = 0.12) or POST-P offspring (HR = 1.38, df = 2, p = 0.16) nor were they affected by sex (HR = 0.9, df = 1, p = 0.57) or size at maturity (HR = 1.42, df = 2, p = 0.46) (Figure S3). The average age at death was 88 ± 2.7 days (n = 46) for PRE-M offspring, 86 ± 3.2 (n = 49) for POST-P offspring and 93.6 ± 3.5 days (n = 42) for NO-SEL offspring.

4 | DISCUSSION

We measured reproductive outcome of females exposed to treatments allowing for exclusive pre-copulatory and post-copulatory

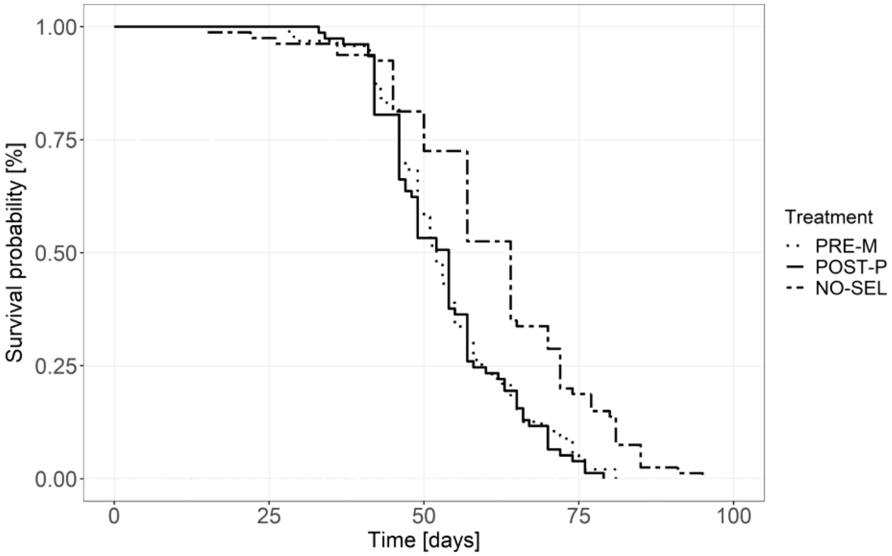

FIGURE 2 Survival probability for females of the parental generation across treatments (PRE-M = pre-copulatory sexual selection and monogamy; POST-P = post-copulatory sexual selection and polyandry; NO-SEL = no sexual selection and enforced monogamy). POST-P (straight line) and PRE-M (dotted line) treatments died sooner compared with NO-SEL the treatment (dashed line).

TABLE 2 Estimated effect sizes and 95% credible intervals around the mean of predictors of body mass; predictors model 1: Treatment (PRE-M, POST-P and NO-SEL), age (timepoint when body mass was measured) and their interaction; model 2: Treatment, age and sex (male or female) and their interactions; treatment effects are indicated as contrasting PRE-M and POST-P against NO-SEL (intercept); random effect: Cricket ID.

	Model 1: Treatment*age	Model 2: Treatment*age*sex
Fixed effects	β estimate (95% CI)	
Intercept	0.021 (−0.026, 0.07)	−0.039 (−0.112, 0.032)
Treatment PRE-M	0.025 (−0.04, 0.09)	**0.099 (0.001, 0.195)**
Treatment POST-P	−0.02 (−0.08, 0.041)	−0.019 (−0.075, 0.115)
Age	**0.018 (0.017, 0.019)**	**0.021 (0.02, 0.023)**
TreatmentPRE-M:Age	**0.009 (0.008, 0.01)**	**0.007 (0.005, 0.009)**
TreatmentPOST-P:Age	**0.011 (0.01, 0.013)**	**0.011 (0.009, 0.013)**
Sex Male		**0.11 (0.01, 0.212)**
TreatmentPRE-M:SexMale		−0.134 (−0.262, 0.0003)
TreatmentPOST-P:SexMale		−0.059 (−0.192, 0.076)
Age:SexMale		**−0.005 (−0.007, −0.003)**
TreatmentPRE-M:Age:SexMale		**0.003 (0.0002, 0.006)**
TreatmentPOST-P:Age:SexMale		0.0001 (−0.003, 0.003)
Random effects	σ^2 (95% CI)	
Cricket ID	**0.028 (0.026, 0.03)**	**0.028 (0.026, 0.031)**
Residual variance	**0.021 (0.02, 0.022)**	**0.02 (0.019, 0.022)**

Note: Significance shown in bold.

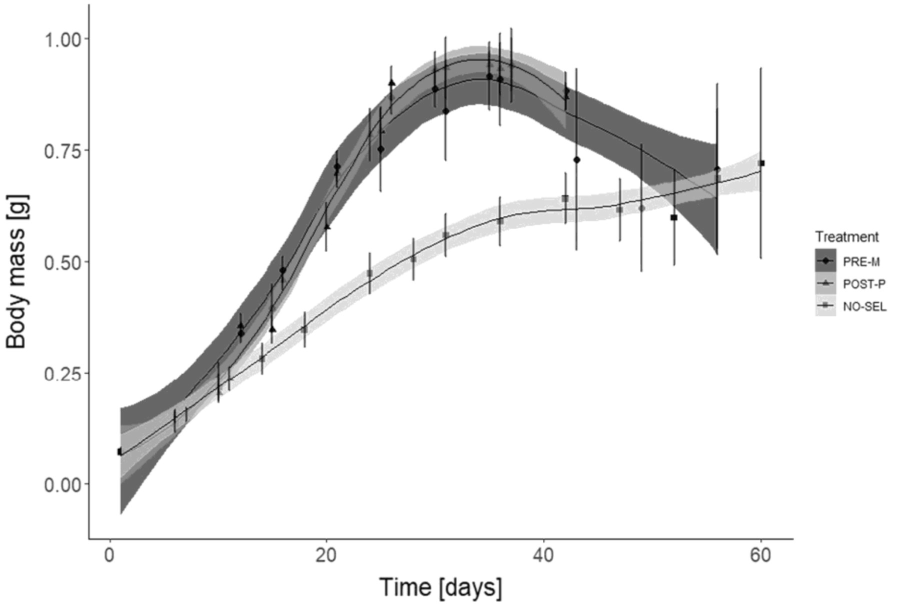

FIGURE 3 Differences across treatment (PRE-M = pre-copulatory sexual selection and monogamy; POST-P = post-copulatory sexual selection and polyandry; NO-SEL = no sexual selection and enforced monogamy) for growth curves of average weight measurements per days.

sexual selection or weakened sexual selection. We expected fitness benefits to arise when sexual selection was present, with no a priori expectation for pre-copulatory selection weighing more than post-copulatory selection on fitness outcome. Yet, when considering the different mating systems (monogamy vs polyandry) that were experimentally applied to obtain the two treatments, we hypothesised polyandry, and hence post-copulatory selection, to provide fitness advantages to the offspring (Arnqvist & Nilsson, 2000; Tregenza & Wedell, 1998). We found that, despite suffering higher survival costs, females benefit from sexual selection as those from the no-selection treatment were less likely to reproduce, had offspring that hatched later in time, development was slower and body mass at maturation lower. There was, however, no difference in how either pre- or post-copulatory selection affected the fitness estimates studied. We can exclude an effect of the mating system, as monogamy was present in treatments with (i.e. pre-copulatory treatment) and without selection (i.e. no selection treatment) and these nevertheless yielded different reproductive outcomes. These findings suggest that sexual conflict may potentially be at play, negatively affecting female life-time reproductive success but not offspring fitness given that sexual selection, regardless of whether it occurs before or after mating, ap- pears to provide indirect reproductive benefits in the form of faster and higher quality offspring development.

The likelihood of females reproducing was overall high in our study, and given that we controlled for sperm limitation, reproductive failure may derive from naturally non-viability of gametes (Simmons, 1987b) or incompatibility (García-González, 2004; Zeh & Zeh, 1996, 1997). Yet, it was lowest in females that did not exert mate choice. Female crickets that have a choice may have the means to avoid mating with unfertile and/or incompatible partners. By mating with preferred males these may also invest more in their offspring (Burley, 1988), as previously shown by Simmons (1987a; but see also Arnqvist & Danielsson, 1999; Kotiaho et al., 2003; Sheldon, 2000). It is possible that females use certain male traits as quality indicators, such as body size (Simmons, 1986) and/or song traits (Rantala & Kortet, 2003; Verburgt et al., 2011) to assess relative male quality, including fertility and allocate resources to re- production accordingly. Female crickets of *Gryllus lineaticeps* mating to males producing preferred song types are, for example shown to live longer, produce more eggs and these have higher hatching success (Wagner & Harper, 2003). Although there is contrasting evidence, female preference for dominant males has also been re- ported (Bretman et al., 2006) and dominance has been identified as eliciting higher egg production in *G. bimaculatus* females in some studies (Bretman et al., 2006), but not others (Tuni et al., 2016). In our study, mate assessment could take place either with many partners present simultaneously in pre-copulatory selection treatments, or sequentially in post-copulatory selection treatments. Given that only females that successfully mated were included in our study, assessment and choice may have potentially translated into post- copulatory allocation to increase offspring quality in terms of sooner hatching, faster and better development. The positive genetic correlations between multiple reproductive traits in males of this system (Tuni et al., 2018) also suggests that females may be selecting for the same overall male phenotype. For example, dominance could be assessed in only one of our treatments, the one with pre-copulatory selection, but given that aggressive individuals are those with larger body mass (Tuni et al., 2018), these may have been preferred even in the absence of contest competition when assessed sequentially. In the case of *G. bimaculatus*, larger males (i.e. with higher body mass), which are also the most aggressive, possess ejaculates of greater size (i.e. sperm numbers; Tuni et al., 2018).

Increased maternal investment when sexual selection had the potential to occur may have led to the more successful offspring re- ported in our study. A study on *G. bimaculatus* indeed shows that offspring of females allowed to choose their mates developed faster and had higher survival compared with those that were allocated mates (Simmons, 1987a). Developing faster at higher growth rates brings large reproductive advantages to the offspring as these individuals will be able to reproduce sooner and will possess superior body condition at the time of mating. Opposing to this, off- spring with delayed growth might be disadvantaged when females are choosing to mate with fully developed males in better body condition (Bateman et al., 2001; Simmons, 1986). Given that females mating without sexual selection were also the youngest, we cannot fully exclude that the differences documented between the treatments with and without selection are driven by differences in female age at mating. Specifically, younger (no-selection) females may have been delayed in their oviposition if egg development was not complete, leading to later hatching of offspring. Maternal age effects are also known to potentially influence offspring development and performance (Mousseau & Fox, 1998). Whereas numerous studies report negative transgenerational effects of senescing mothers (e.g. offspring of older females hatch later), develop more slowly or have shorter lifespan (Benton et al., 2008; Bock et al., 2019; Lind et al., 2015; Priest et al., 2002), others report positive effects due to an increase in investment in reproduction by older mothers (Froy et al., 2013; Kroeger et al., 2020; Part et al., 1992; Poizat et al., 1999; Travers et al., 2021). In the case of field crickets, a recent study on *Gryllus bimaculatus* shows that offspring of older females experience longer latencies to hatching and have lower hatching success; these were bigger at adulthood but had shorter lifespans (Noguera, 2021). While these findings may suggest that the effects reported in our study are unlikely driven by the younger female age, maternal age effects should be explored further.

Not all of our estimates of fitness differed between treatments, in particular offspring number. A study from Gómez-Llano et al. (2020) on the fruit fly *Drosophila melanogaster*, which to our knowledge is the only other study using an experimental design that attempts to simultaneously discriminate between the effects of pre- and post-copulatory selection on fitness, similarly found that the number of emerging adults per female did not differ among different sexual selection regimes, although in the context of adaptation to novel thermal environments, evolving with pre-copulatory sexual selection proved beneficial.

We found that sexual selection also entails costs, as removal of sexual selection led to longer female survival. All females across treatments were exposed to the stress of male harassment, as males are known to court vigorously (Bateman, 2000; Bateman et al., 2006). Yet, there are some differences between the treatment groups. In our pre-copulatory sexual selection treatments these costs may have been particularly exacerbated by male behaviour, as males were aggressively fighting against each other and approaching females simultaneously. Aggressive behaviours were often ob- served to be directed to females (Bateman et al., 2006; Vedenina & Shestakov, 2018; personal observations). On the other hand, in our post-copulatory sexual selection treatment, being exposed to several different males may have potentially increased disease transfer (Simmons, 2005; Thrall et al., 2000) or transfer of harmful chemicals from multiple ejaculates, as known in *Drosophila melanogaster*, where males evolve seminal fluids that increase their mating success but are harmful to females and even increase female mortality (Chapman et al., 1995). This could result in reduced longevity (Friberg & Arnqvist, 2003), which is a cost that promiscuous females suffer from more likely with strong negative implications for female lifetime reproductive success. The lower survival observed in females mated with the opportunity for sexual selection instead does not appear to be a consequence of trade-offs in resource allocation between reproduction and soma-maintenance (Roff et al., 2002; Stearns, 1992), as if for example females had increased offspring production. Variation in female age at mating, with those from the no-selection treatment being unintentionally younger, may also potentially explain our results. Younger females may bet- ter cope with costly mating, such as multiple inseminations and/or multiple encounters with courting males, when these occur earlier in life, rather than pointing to trade-offs between reproduction in early life and survival (Wilson & Walker, 2019). This is also shown in the field cricket *Gryllus assimilis* (Limberger et al., 2021), where older mated females had an increased mortality rate compared to females mated at younger age and virgin females. Limberger et al. (2021) suggest that late-mated crickets increase their resource allocation towards current reproduction, since future reproduction might not be as likely as it is for younger females, leading to an acceleration of senescence and death.

Interestingly, the direct survival costs of mating with males selected either pre- or post-mating, may be potentially compensated by the indirect offspring viability benefits gained from sexual selection. Despite contrasting theoretical arguments (Cameron et al., 2003) and empirical evidence (Pischedda & Chippindale, 2006; Stewart et al., 2008; Taylor et al., 2008), indirect benefits arising from harmful matings have been suggested to outweight the costs imposed by males (Cordero & Eberhard, 2003). In the field cricket *Acheta domesticus*, females mating with attractive partners experience survival costs that are counterbalanced by producing offspring with greater fitness (Head et al., 2005). Similarly, in the Australian field cricket *Teleogryllus oceanicus*, females mated to males that invested more in the pro- duction of accessory glands incurred longevity costs due to harm- ful ejaculate-driven effects, but yet their offspring survival was improved (Garcia-Gonzalez & Simmons, 2010). Despite not being able to define the target of female choice and/or male manipulative traits, our findings are similarly suggestive of indirect benefits counteracting direct costs of sexual selection.

Overall, our findings suggest that the action of sexual selection overrides any benefit inherently linked to the mating system, monogamous or polyandrous, as we importantly uncover differences in fitness outcomes of monogamous matings occurring with and without sexual selection. Several studies investigating whether polyandry is adaptive have reported fitness benefits of mating with multiple partners (Tregenza & Wedell, 1998; Tuni et al., 2013a; Tuni & Bilde, 2010). Yet, these studies commonly compare fitness of polyandrous females to monogamous matings in which they al- locate males to females, limiting female choice and resembling our enforced monogamy treatment with no sexual selection. Our findings are therefore in line with most literature, as polyandrous matings from the post-copulatory treatment yielded higher fitness outcomes compared to monogamous mating lacking selection (no selection treatment). It would be interesting to investigate whether the magnitude of such indirect benefits would be equally high, if se- lection was allowed to operate.

4 | CONCLUSIONS

Whether sexual selection enhances fitness or not fuels an active field of research, with studies reporting positive effects such as increased population growth rates (Fox et al., 2019) and accelerated rates of adaptation to novel environments (Gómez-Llano et al., 2020; Parrett et al., 2019; Parrett & Knell, 2018; Servedio & Boughman, 2017), while others suggest higher extinction rates due to costly sexual traits or reduction of effective population size and genetic drift (Kokko & Brooks, 2003; Kokko & Jennions, 2008; Whitlock, 2000). Our results are in line with current literature, which states that fit- ness values are often higher in populations evolving under sexual selection, relative to populations where sexual selection is experimentally removed or weakened (Cally et al., 2019). Pre- and/or post- copulatory sexual selection may lead to a comparable magnitude of indirect fitness benefits to females, as revealed by measuring a range of fitness estimators encompassing offspring viability, growth and survival but impose direct survival costs to females. By outweighing the costs of reduced longevity, the increased developmental ability reported for offspring has the potential to contribute to the beneficial population-level consequences of sexual selection (Cally et al., 2019; Gómez-Llano et al., 2020; Parrett et al., 2019), highlighting the relevance of indirect benefits of sexual selection in maintaining population viability.

AUTHOR CONTRIBUTIONS

Magdalena Matzke: Data curation (lead); formal analysis (lead); investigation (lead); methodology (equal); validation (equal); visualization (lead); writing – original draft (lead); writing – review and editing (equal). **Aurora Rossi:** Investigation (supporting); writing – original draft (supporting). **Cristina Tuni:** Conceptualization (lead); formal analysis (supporting); funding acquisition (lead); investigation (supporting); methodology (equal); resources (lead); supervision (lead); validation (equal); writing – original draft (supporting); writing – review and editing (equal).

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CONFLICTS OF INTEREST

All authors gave final approval for publication and declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available on Dryad at https://doi.org/10.5061/dryad.wm37pvmrj and on Zenodo at https://doi.org/10.5281/zenodo.7340773.

PEER REVIEW

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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Supporting Information (S1) for:

Pre- and post-copulatory sexual selection increase offspring quality but impose survival costs to female field crickets

Magdalena Matzke, Aurora Rossi, Cristina Tuni

Supporting analysis:

Table S1: Results of sequential ANOVA (Type II) as alternative analysis to test the effect of treatment subsequent to the effect of female age at mating (mean-centred), as well as their interaction and female mass (mean-centred) for i) latency to hatching and ii) total number of offspring. Significant effects are shown in bolt

	Effect	DF	Sum of squares	F-value	p-value
Latency to hatching	Female age at mating	1	4.9	1.78	0.18
	Treatment	2	219.4	39.6	<0.001
	Female mass	1	1.6	0.56	0.45
	Female age* Treatment	2	8.5	1.54	0.22
Number of offspring	Female age at mating	1	0	0.02	0.88
	Treatment	2	17	0.46	0.63
	Female mass	1	24	1.31	0.25
	Female age* Treatment	2	85	2.33	0.1

Supporting results

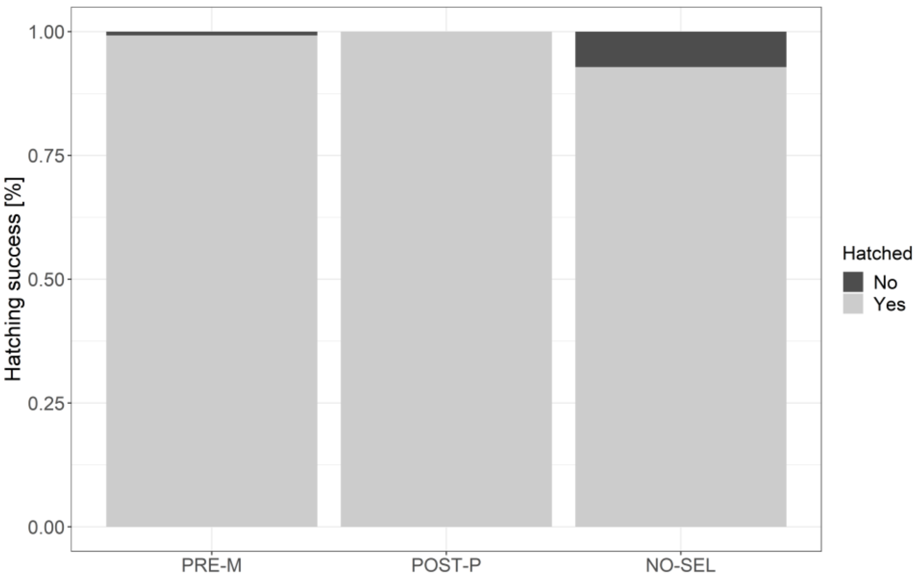

Figure S1: Reproductive success was lower for NO-SEL females compared to PRE-M and POST-P females (PRE-M= pre-copulatory sexual selection and monogamy; POST-P= post-copulatory sexual selection and polyandry; NO-SEL= no sexual selection and enforced monogamy).

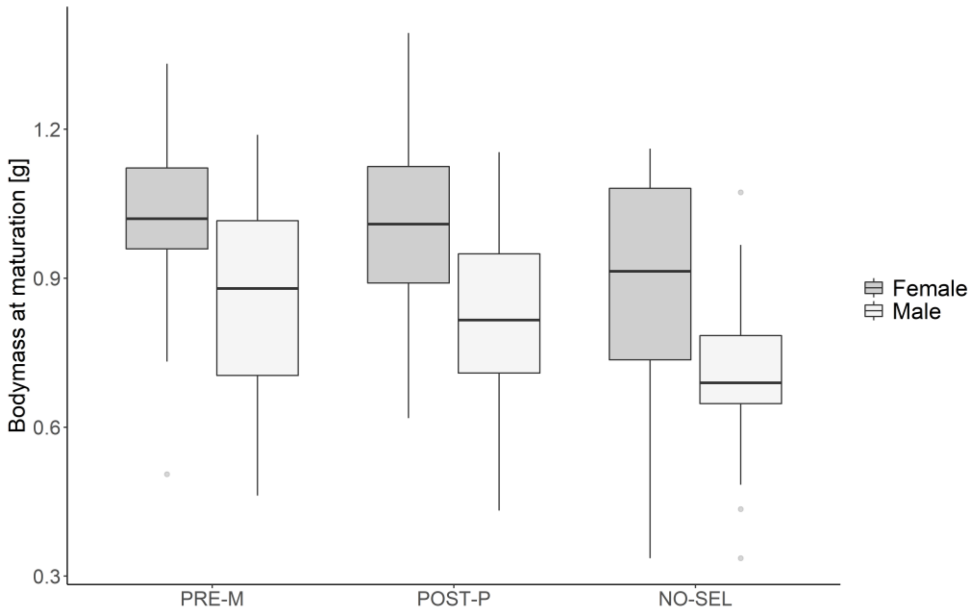

Figure S2: Body mass at maturation for both sexes was higher for females compared to males across treatments (PRE-M= pre-copulatory sexual selection and monogamy; POST-P= post-copulatory sexual selection and polyandry; NO-SEL= no sexual selection and enforced monogamy).

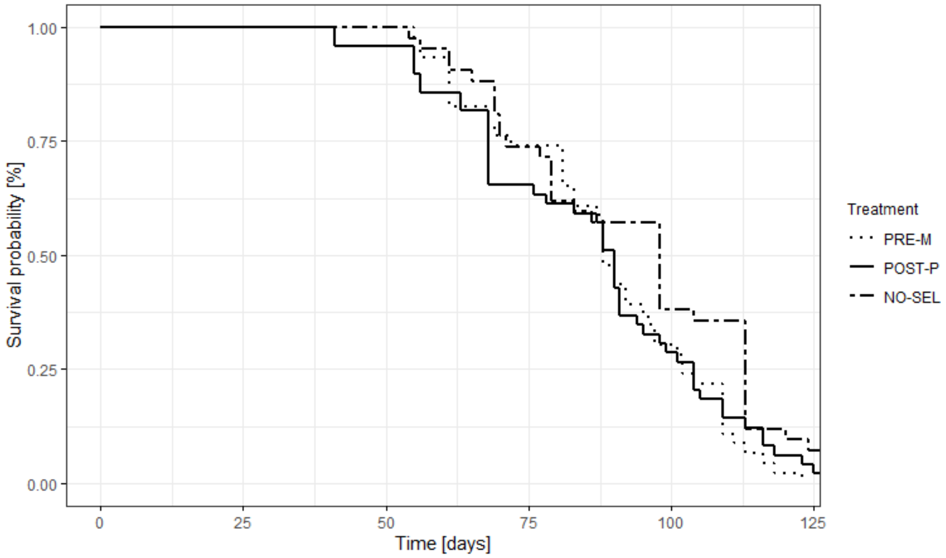

Figure S3: Offspring survival probabilities across treatments (PRE-M= pre-copulatory sexual selection and monogamy; POST-P= post-copulatory sexual selection and polyandry; NO-SEL= no sexual selection and enforced monogamy). There was no difference between treatments.

Chapter II

Manuscript I: Evolutionary responses of key reproductive traits under manipulated pre- and post-copulatory selective pressures: from ejaculates to behaviour

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Unpublished manuscript

Abstract

Pre- and post-copulatory sexual selection shape the evolutionary diversification of a wide range of reproductive traits. Males should evolve better mate acquisition abilities (pre-copulatory traits) under intense pre-copulatory selection, and better fertilization abilities (post-copulatory traits) under intense post-copulatory selection. Yet, disentangling the effects of pre- and post-copulatory selection remains largely unexplored. Given that reproductive traits are often correlated, selection acting on pre-copulatory traits may facilitate the evolution of post-copulatory traits if these are positively correlated or may impair their evolution if the latter are negatively correlated. To improve our understanding of correlated evolution and/or constraints on adaptation (genetic trade- offs) we measured the evolutionary responses of key pre- and post-copulatory reproductive traits to experimentally manipulated pre- and post-copulatory selective pressures. We established experimental evolution lines of field crickets *Gryllus bimaculatus*, with animals evolving under i) intense pre-copulatory selection, through monogamous matings following male fights, courtship and female choice, ii) intense post-copulatory selection, through sequential polyandrous matings allowing for sperm competition and cryptic female choice, and iii) relaxed selection through enforced monogamy. After three and six generations, we measured several key pre-copulatory traits, such as male song, aggressiveness, attractiveness, weaponry, body mass and size, and post- copulatory traits such as sperm viability and testes mass. We show that trait divergence occurs within the studies' evolutionary timeframe. Specifically, after six generations intense pre- copulatory selection led to increased pre-copulatory trait values, while post-copulatory selection to lowered pre- and post-copulatory trait values. Our findings point to both, positive and negative trait responses, further suggesting potential underlying positive correlations between pre- and post- copulatory traits, such as song and testes, instead of trade-offs. Results not only provide insight into how individual pre- and post-copulatory traits respond to differential selective pressures, but also shed light onto the relative role pre- and post-copulatory sexual selection play on the evolution of complex male phenotypes.

Keywords: pre- and post-copulatory, sexual selection, *Gryllus bimaculatus*, experimental evolution, disentangled selection

Introduction

Sexual selection is responsible for the evolution of some of the most diversified traits observed in the animal kingdom, spanning from behaviour to morphology and physiology. Our understanding of trait evolution has been largely shaped by the Darwinian view of selection giving rise to male traits that increase their mating success (namely, pre-copulatory traits) such as ornaments (e.g., bright colorations, long feathers) and armaments (e.g., antlers and horns) (Darwin, 1871). These traits are selected through mechanisms of male-male competition, i.e., males competing for access to mates (Andersson & Iwasa, 1996), and female choice, i.e., females choosing the most attractive male (Andersson & Simmons, 2006). However, as females in many species mate with multiple males, sexual selection continues after mating (Birkhead, 1998; Parker & Pizzari, 2010), through mechanisms of sperm competition, i.e., sperm of multiple males compete for fertilization of the eggs (Parker, 1970; Simmons, 2019), and cryptic female choice, i.e., females bias fertilizations towards preferred males (Eberhard, 1996; Pizzari & Birkhead, 2000). Post-copulatory sexual selection shapes a wide array of traits involved in increasing male's fertilization success (namely, post-copulatory traits), such as sperm phenotype or ejaculate size.

Pre- and post-copulatory traits are unlikely to evolve independently as traits are nearly always correlated due to pleiotropy and/or linkage (Evans & Garcia-Gonzalez, 2016; Lande & Arnold, 1983). If correlations between pre- and post-copulatory traits are positive, selection on one trait (for example, ornaments) will facilitate the evolution of its correlated trait (for example, ejaculate size) (Lande & Arnold, 1983; Lynch & Walsh, 1998; Stearns, 1992). Positive correlations may be due to genetic variation in male quality or environmental variation in body condition. In this scenario, males might be able to equally invest in both, pre- and post-copulatory traits (Chargé et al., 2013; Tuni et al., 2018). In turn, post-copulatory traits may be favoured by female mate choice ("phenotypic linked hypotheses") (Sheldon, 1994). On the other hand, if traits are negatively correlated, selection on one trait (for example, ornaments) may constrain the evolution of the other trait (for example, ejaculate size). Negative trait correlations may derive from genetic or environmental trade-offs in resource allocation, with allocation of resources towards traits involved in one episode of selection (e.g., pre-copulatory traits), limiting the investment in traits of the other (e.g., post-copulatory traits) (Engqvist, 2011; Evans, 2010; Simmons et al., 2010). Studies investigating the genetic architecture of reproductive traits do not find a prevailing direction (positive or negative) of trait correlations.

For example, studies show negative correlations between male attractiveness and sperm quality in guppies (Evans, 2010) or male attractiveness and copulation investment in scorpionflies (Engqvist, 2011). Other studies show positive correlations between male aggressiveness and sperm number, as in the field cricket (Tuni et al., 2018) or between male sexual signalling and ejaculate size, as in the houbara bustard (Chargé et al., 2013). There are several potential factors that can underlie this heterogeneity, as reviewed by Evans & García-González (2016). These include resource acquisition and allocation, when trade-offs are only exposed under limited conditions (Parker, 1998; Van Noordwijk & De Jong, 1986), ecological factors such as seasonal variation in resource quality or availability (Gillespie et al., 2014; Gwynne & Simmons, 1990; Miller & Svensson, 2014) or social experience (i.e., population density and sex ratio (Miller & Svensson, 2014; Punzalan et al., 2010)), which can all affect patterns of sexual selection. Additionally, genotype-by-environment interactions, when genetic covariances of traits change across environmental gradients (Bussiere et al., 2008; Evans et al., 2015), fine-scale population structures (i.e., local "clumping" of phenotypes within a population (Kasumovic et al., 2008; McDonald et al., 2013)) or methodological factors (i.e., confounding variance in fertilization success with variance in offspring viability (García-González, 2008)) can further complicate studying the relationship between pre- and post-copulatory selection. Yet, our overall understanding of how mating and fertilization traits respond to pre- and post-copulatory sexual selection, and whether there is correlated evolution or constraints on adaptation (genetic trade- offs), remains limited.

One of the most powerful tools providing insights into the evolutionary processes underlying trait evolution is experimental evolution, where trait values are measured in response to variation in selection pressures in evolving laboratory populations (Kawecki et al., 2012; Simmons & García-González, 2008). Experimental evolution studies have importantly shown that sexual selection is key for reproductive trait evolution by varying the strength and opportunity for selection through manipulation of sex ratios and/ or population densities (Linklater et al., 2007; Martin & Hosken, 2003; Wigby & Chapman, 2004), or the opportunity for post-copulatory sexual selection through mating system manipulation (monogamy vs. polyandry) (Holland & Rice, 1999; Pitnick, Brown, et al., 2001; Pitnick, Miller, et al., 2001). One of the first studies applying the latter approach implemented the removal (or weakening) of sexual selection through enforced monogamy in *Drosophila melanogaster,* a naturally polyandrous species with occurring sexual conflict, showing that weakened selection led to lower male courtship rates, males harming females less, hence increasing female longevity as well as lifetime productivity (Holland & Rice, 1999). This experimental setup has since been used repeatedly to investigate trait evolution driven by sexual selection, showing for example that males from polyandrous selection lines evolved larger testes (*Scathophaga stercoraria*, Hosken et al., 2001a; Hosken & Ward, 2001; *Drosophila melanogaster*, Pitnick, Brown, et al., 2001; Pitnick, Miller, et al., 2001; *Onthophagus taurus*, Simmons & García-González, 2008), increased sperm production (*Mus domesticus*, Firman & Simmons, 2010), or initiated courtship songs sooner with shorter interval durations between chirp pulses (*Drosophila pseudoobscura*, Snook et al., 2005). On the contrary, other traits, such as body size, as well as horn size did not diverge from those of monogamously mated males (*Onthophagus taurus,* Simmons & García-González, 2008). Despite the undisputable value of such design, it is nearly impossible to attribute the observed trait responses to either pre- or post-copulatory selection, due to the applied experimental setup: whereas in a treatment with enforced monogamy, post-copulatory selection will be disabled, and pre-copulatory selection will also be weakened through the absence of mate choice opportunities, in a polyandrous treatment where number of matings and/or mating partners are not controlled for, pre- and post-copulatory selection will both occur. Although these studies strongly suggest that sexual selection is a non-neglectable force in shaping male reproductive traits, they fail to infer the relative importance of pre- and post-copulatory selection.

With the aim of measuring evolutionary responses of key reproductive traits to exclusive pre- copulatory and post-copulatory selective pressures, we present a novel experimental evolution study where pre- and post-copulatory selection are experimentally disentangled. We focus on the two-spotted field cricket *Gryllus bimaculatus,* a system with a very well-studied reproductive biology (Horch et al., 2017). Pre-copulatory sexual selection occurs in the form of aggressive male- male fights over breeding territories (i.e., cracks in the ground and gaps under stones), in which males interact aggressively using their mandibles as weapons to wrestle against competitors (Adamo & Hoy, 1995). Fight winners chase away losers and sing to attract wandering females (Simmons, 1986b; Tachon et al., 1999). These songs are long-distant attractive songs, which are followed by close-range courtship songs performed once the female approaches the male (Simmons, 1986a). Females exert mate choice based on the quality of male calling and/or courtship song (Rantala & Kortet, 2003; Verburgt et al., 2011), as well as on male body size (Bateman et al., 2001; Simmons, 1986a) and odour (Iwasaki & Katagiri, 2008; Tregenza & Wedell, 1997). Field crickets are polyandrous (Bretman & Tregenza, 2005; Simmons, 1987), with ejaculates from multiple males being stored in the female sperm storage organ giving rise to intense sperm competition. Sperm viability, the proportion of viable sperm in the ejaculate, is considered one of the main determinants of fertilization success (Garcia-Gonzalez & Simmons, 2005). Females are also known to bias fertilization towards preferred males by controlling stores of sperm (Bretman et al., 2009; Tregenza & Wedell, 2002). Most of the above-mentioned reproductive traits are known to be heritable (body mass, ejaculate size, and aggressiveness in *Gryllus bimaculatus* (Tuni et al., 2018), calling rate and male attractiveness in *Teleogryllus oceanicus* and *commodus* (Hall et al., 2008; Lailvaux et al., 2010; Simmons et al., 2010)) and to be under sexual selection (Bretman et al., 2009; Gage & Morrow, 2003; Shestakov & Vedenina, 2015; Simmons, 1986a, b, 1987; Tachon et al., 1999; Verburgt et al., 2011). A recent study on *G. bimaculatus* has further shown that pre- and post-copulatory traits, respectively male body mass, aggressiveness and ejaculate size (sperm numbers), are all genetically positively correlated, indicating the existence of genetic variation in male quality (Tuni et al., 2018) and the potential for correlated responses to selection. However, other studies have reported negative correlations between sperm quality (sperm viability) and male aggressiveness (Tuni et al., 2016). Studies from other cricket species also report mixed evidence for positive and negative phenotypic correlations between other pre- and post-copulatory traits, for example negative correlations between body mass and sperm numbers (*Acheta domesticus*, Klaus et al 2011), between the amount of trill in courtship songs and sperm viability (*Teleogryllus oceanicus*, Simmons et al., 2010), or between male dominance and pheromones (*Teleogryllus oceanicus,* Thomas and Simmons 2009), hence two pre-copulatory traits. What all these studies suggest, is that there are ample possibilities in this system for pre- and post-copulatory selection to act on different traits in a correlated matter, or conversely independently.

In this study, we established three experimental evolution lines by mating field crickets with exclusive i) pre- copulatory selection, ii) post- copulatory selection, and iii) weakened or relaxed selection, and measured a wide range of pre- and post-copulatory traits after three and six generations of selective regimes, aiming to experimentally disentangle the effects of each episode of selection on specific reproductive traits. Exclusive pre-copulatory sexual selection was ensured by allowing multiple males to interact with rivals (i.e., fight) and court females (i.e., sing) and females to exert mate choice for one of them. The pair was then bred monogamously three times. Post-copulatory sexual selection was attained, while minimizing pre-copulatory sexual selection, by preventing any pre-copulatory interactions and breeding females sequentially with three different males, hence, polyandrously. Finally, pre-copulatory selection was minimized while at the same time removing post-copulatory selection through enforced monogamy, by breeding females to one male only repeatedly for 3 times. We standardized number of matings (i.e., three) across treatments not only to exclude sperm limitation but also to balance potential direct benefits females could gain from multiple matings (e.g., nutritious ejaculates). After three and six generations of experimental evolution, we measured a wide range of pre-copulatory and post- copulatory traits (summarized in Table 1). Specifically, pre-copulatory traits, including male calling and courtship song, aggressiveness against other males and attractiveness to the female, body mass, weaponry and body size, are hypothesized to respond to intense pre-copulatory sexual selection, whereas post-copulatory traits, including sperm viability and testes mass, to intense post- copulatory sexual selection. How pre-copulatory traits respond to intense post-copulatory selection, and *vice versa* (how post-copulatory traits respond to intense pre-copulatory selection), will inform us on correlated evolution and/or evolutionary trade-offs. While we do not expect trait divergence under relaxed sexual selection, the highest trait responses to selective pressures should occur in the sixth generation compared to the third due to a longer evolutionary history of differential selective pressures.

Methods

Collecting and rearing

Animal breeding procedures are also described in Matzke et al., 2023. In brief, approximately 200 wild-caught *G. bimaculatus* nymphs of a wild Italian population were transported to climate chambers (26 C°, 60% relative humidity, 12:12 hour light:dark photoperiod) at the LMU. Nymphs (n=20-30) were placed in several large plastic tanks (23 x 15 x 17 cm) furbished with carton and provided with ad libitum food consisting of dry cat food (Ja! Knusper-Mix Rind & Gemüse), dry fish flakes (sera® Pond flakes Flockenfutter) and fresh apple slices, and water vials. Once adults, cups (5 x 7 x 7 cm) containing moist soil were added, for mated females to lay eggs. After a week, cups were moved to new tanks for nymphs to hatch and replaced with new ones. Nymphs were mixed and allocated to new tanks, and the breeding procedure was repeated as above.

Experimental evolution lines

After three generations, several hundreds of randomly collected nymphs, which would constitute the parental generation for the experimental evolution lines, were moved at 28 C° (with the same humidity and photoperiod as above). Once nymphs reached their penultimate or last instar, we transferred approximately 600 subadult males individually to containers (10 x 10 x 9 cm) equipped with shelter, water, and dry cat food, to control for mating and social experience until sexual maturation. Subadult females (approx. 400) were instead kept in small tanks (12 x 14 x 23 cm) in groups of five individuals. Animals were checked twice a week to determine adulthood, with females molting on the same day being rearranged in tanks of same density (n=5 individuals).

Approximately two weeks after reaching sexual maturity animals were allocated to mating treatments to initiate the experimental evolution lines. Their body mass was measured using a digital scale (KERN & SOHN GmbH, Balingen, 208 Germany), and subsequently males had their spermatophore removed to control for sperm age. We established the following breeding treatments to generate: 1) Pre-copulatory sexual selection lines (hereafter, Pre-M), by placing one female with three males in a 32 x 15.5 x 13 sized arena. Males could fight against each other and court the female. Once successful mounting and transfer of spermatophore occurred with the preferred male, the others were removed and the couple was re-mated monogamously two more times, with a two- hour interval in between. Mated females (n= 133) were randomly allocated to four replicate Pre-M lines of 33 or 34 females each. 2) Post-copulatory sexual selection lines (hereafter, Post-P), by pairing a female and a male in a 16 x 15.5 x 13 cm sized arena until successful mounting and spermatophore transfer. The male was then replaced with two consecutive new partners, with a two-hour interval in between, for the female to mate polyandrously. Mated females (n= 88) were randomly allocated to four replicate Post-P lines, with 22 females each. 3) Weakened sexual selection lines (hereafter, No-Sel), by pairing a female and a male, and mating them sequentially for 3 times monogamously, as described above. Mated females (n= 103) were randomly allocated to three No-Sel lines, with

34 or 35 females each.

Only females successful in all three matings were included and moved to individual tanks (12 x 14 x 23 cm) equipped with water, food, egg carton and a small plastic cup (5 x 7 x 7 cm) filled with moist soil for oviposition. After one week the cup was placed in a new box (6 x 12 x 12 cm) for the eggs to hatch and replaced with a new one to allow further oviposition. Offspring were counted as part of a separate study (Matzke et al., 2023), and then nymphs were mixed within their replicate lines and raised collectively as described above for the stock population until nymphs reached the subadult stage. These were then isolated individually to control for age and social experience, and the three breeding treatments were implemented as described above, for a total of six generations.

Trait measurements

At generation three and six we measured a range of traits in males two-three weeks after adult eclosion (number of days from adulthood; F3 generation, Pre-M 19 ± 0.5, Post-P 21.25 ± 0.23, No- Sel 19.8 ± 0.79; F6 generation, Pre-M 18.5 ± 0.38, Post-P 18.8 ± 0.34, No-Sel 16.8 ± 0.32 days of age). Traits are summarized in Table 1 and were measured in the order described below from the same individuals (i.e., focal males). Courtship song was not measured at generation three.

Calling and courtship song

In order to record calling song, males were placed within their housing container inside individual sound chambers (built of egg carton walls, approx. 20 x 25 x 30 cm). A linear PCM audio recorder (TASCAM DR-05) was placed on top of the container, with the microphone facing the netted opening within the lid of the box, and their song activity was recorded overnight. Recordings started between 6-8 pm and lasted a maximum of 12 hours. The procedure was conducted on a total of 222 F3 males (n=80 Post-P, n=80 Pre-M, n=62 No-Sel) and 252 F6 males (n=92 Post-P, n=92 Pre-M, n=68 No-Sel). Courtship song was recorded on the following day during mating assays between males and females (described below), only for F6 males (n=54 Post-P, n=58 Pre-M, n=53 No-Sel). A TASCAM DR-05 audio recorder was placed on the side of the arena in which a pair was present to record male song during courtship.

Audio files were subsequently analysed with the Open-Source Software Audacity (2.4.2.). Calling song consists of a series of repeated chirps, further constructed out of single iterated pulses produced at high speed (Simmons, 1988; Verburgt et al., 2011). The following song characteristics were measured from a five second interval of recording that contained continuous singing: chirp number, pulses per chirp, average chirp duration, interval duration between chirps, maximum frequency (peak), and maximum amplitude (peak). In the F3 generation four five second intervals were chosen at different time points of the recording for each male (two intervals during the first two hours after the start of singing, two after 6-8 hours). Due to repeatability estimates between 0.24 and 0.54 it was assumed appropriate to continue analysis of calling song with averaged estimates.

Recordings from courtship songs often only lasted between one to two minutes, from which a two second interval containing continuous courtship song was chosen for analysis. Courtship song consists of a series of repeated chirps (phrases) containing one high frequency pulse followed by multiple low frequency pulses (Rantala & Kortet, 2003). The following song characteristics were measured: number of high frequency pulses per two second interval, high frequency pulse duration, phrase length, peak amplitude of high and low frequency pulses and peak frequency of high and low frequency pulses.

Mating assays: male courtship and attractiveness

Following overnight song recordings, focal males were paired to females belonging to the stock population. Females had been bred following general procedures described above and had been isolated in same-sex tanks at their last instar (n=5 per tank) to control for age at testing (number of days from adulthood; females from F3: 17.1 ± 0.05, n=191; females from F6: 19.04 ± 0.2, n=91). A stock female and a male (either a Pre-M, Post-P or No-Sel), for which we ensured by visual inspection the presence of a spermatophore, were placed in a test arena (16 x 15.5 x 13 cm). Mating trials were video recorded using a Logitech web camera located at a fixed distance on top of the arena (30 cm) and connected to a laptop. Courtship in crickets usually consists of a sequence of behaviours such as juddering, stridulation of the wings (courtship song, but also with alternating songs), following the female and presenting the male's back to her so that he can be mounted by the female (Bateman and Fleming 2006). As soon as the female mounted the male, we prevented males from transferring a spermatophore: the couple was gently separated with a soft brush and videorecording was stopped. In case the male did not court, the trial was terminated after a maximum of 20 minutes.

Table 1: Summary and description of pre- and post-copulatory traits of male field crickets *Gryllus bimaculatus* measured at generation F3 and F6 of experimental evolution under intense pre-copulatory, intense post-copulatory and weakened sexual selection. *only measured for F6.

Trait Class	Function	Measure	References
Pre-copulatory:			
Calling song	Produced by sexually mature males to attract females over long distances ("long distance calls")	Chirp number, pulses per chirp, chirp duration, chirp interval duration, peak frequency, peak amplitude	Verburgt et al., 2011; Zhemchuzhnikov et al., 2017 in *G. bimaculatus*
Courtship song*	Produced by males to court females in their direct (physical) or indirect (body odor) presence ("short distance song")	Number of high frequency pulses (HFP), average HFP duration, average phrase length, peak frequency (LFP, HFP), peak amplitude (LFP, HFP)	Rantala & Kortet, 2003; Shestakov & Vedenina, 2015 in *G. bimaculatus*
Attractiveness	Male ability to elicit a positive female response in mate acceptance	Latency to female mounting from performance of first male courtship song	Holzer et al., 2003 in *G. campestris*
Aggressiveness	Physical combats used to defend territories, with higher aggression levels increasing winning chances	Absolute and relative chasing time of the focal male towards its opponent	Adamo & Hoy, 1995; Tachon et al., 1999 and Santostefano et al., 2017 in *G. bimaculatus*
Body mass	Indicator of body condition, increases male mating and fighting success	Body weight	Hack, 1997a; Judge & Bonanno, 2008, in *G. pennsylvanicus*
Body size	Increases fighting and male mating success	Pronotum width (dorsal & ventral), femur length, Pronotum length, Body length, head width	Bateman et al., 2001 in *G. bimaculatus*
Weaponry size	Mouthparts used in male fights to grasp and bite the opponent	Mandible length, maxilla length	Adamo & Hoy, 1995; Tachon et al., 1999 in *G. bimaculatus*
Post-copulatory:			
Testes mass	Proxy for sperm production, increasing sperm competitiveness and siring success	Wet weight of dissected testes	Parker, 2016; Simmons, 1987; Tuni et al., 2018 in *G. bimaculatus*
Sperm viability	The number of viable sperm, increasing sperm competitiveness and siring success	Percentage of live sperm cells in the spermatophore	García-González & Simmons, 2005, Simmons & Fitzpatrick, 2012 in *G. bimaculatus*

Video recordings were inspected and manually scored for i) latency to sing, as the time from the beginning of the trial to start of male courtship song, interpreted as male propensity to court, and ii) latency to female mount, as the time interval from first courtship song to the first mounting from the female, interpreted as a proxy for male attractiveness. Additional behaviours were estimated, such as latency to first physical contact between sexes from start of the trial and latency to female mount from first physical contact.

Aggressiveness

On the day following mating assays focal males from the experimental evolution lines were paired against males belonging to the stock population (hereafter, opponents). Opponents were bred following general procedures described above and had been isolated individually concurrently to focal males in order to obtain males of similar age for testing (number of days from adulthood of opponents in F3 generation: 20.6 ± 0.05, n=119; and F6 generation: 20.5 ± 0.18, n=109). One focal and one opponent male were both colour-marked with acrylic paint and shortly after placed in a test arena (16 x 15.5. x 13 cm) and left to fight for 10 minutes. Male-male interactions were video recorded using a video-surveillance system of a camera fixed on top of 2 arenas, allowing multiple trials to be conducted at the same time. If necessary, opponents that were not injured, were reused in other fights for a maximum of three times in total, on consecutive days, as effects of the social experience (winner-loser effects) are known to fade within 24 hours (Khazraïe & Campan, 1999). When reusing opponents, they were randomly distributed across lines and treatments.

Fighting in crickets consists of a quick escalated sequence of behaviours (e.g., antennae fencing, mandible spreading, singing, wrestling, and biting) and ends with the winner chasing a fleeing loser (Adamo & Hoy, 1995; Tuni et al., 2019). Videos were scored with the tracking software EthoVision XT (Noldus) from which we extrapolated the cumulative duration of proximity (the time both individuals spent within the proximity of 2 cm), of relative and absolute movement towards the other male (“chasing”) and of relative and absolute movement away from the other male (“fleeing”) of focal males (Santostefano et al., 2017b).

Sperm viability

On the following day male spermatophores were sampled for sperm viability assays (number of days from adulthood; males from F3 generation: 26 ± 0.1, n=248, and F6 generation: 21.19

± 0.17, n=332). We followed established procedures for this species (Gasparini et al., 2018; McMahon et al., 2021; Tuni et al., 2016). In brief, using soft forceps we removed the spermatophore from all males on the day before the assay in order to standardise spermatophore age. Spermatophores to be tested were collected and placed into a 0.5ml Eppendorf tube with beadle saline (200µL) for 10 minutes to allow sperm to exit, removing the evacuation tube to ease release of sperm. A total of 5µL of the solution was pipetted on a microscopy glass slide and stained with the LIVE/DEADTM sperm viability kit (Invitrogen, Molecular Probes Inc, Eugene, OR, USA). We used 5µL SYBR (1:50) and 2µL of propidium iodide (PI), incubating the sample for five minutes in darkness after each addition. A cover slip was added, and the solution was then viewed under a fluorescent microscope (Olympus BX61; Olympus, Tokyo, Japan) with live sperm displaying as green (due to SYBR) and dead sperm as red (due to PI). Live and dead cells were counted in a total amount of 300 cells.

Males were frozen in individual tubes at -20° C for further morphological dissections.

Morphology: Body size, weaponry & testes size

Morphological trait measures were conducted on a total of 243 F3 males (n=92 Post-P, n=86 Pre- M, n=65 No-Sel) and 221 F6 males (n=81 Post-P, n=80 Pre-M, n=60 No-Sel). Male crickets were removed from the freezer, thawed at room temperature for at least 20 minutes and placed on mm square paper. Using a camera (Logitech) connected to a laptop and fixed at 30cm height we photographed the entire body of the cricket in dorsal view and took a second photo of the entire body in ventral view, adding the right leg, which was dissected at the most proximal joint with micro scissors, and placed alongside the cricket body. Dissections were then further performed under a stereomicroscope (Zeiss). First, the head was pinned down and the maxillae and mandibles were removed at the joint using micro scissors. These were placed on a mm square paper and photographed using a camera (Moticon) connected to the stereomicroscope (Zeiss) at 0.65 magnification. Second, the abdominal cavity was cut open with micro scissors and pinned, and right and left testes were removed using forceps. Each testis was rolled along a paper towel to remove excess moisture from its surface, and extraneous tissue was removed using forceps and then weighed to the nearest 0.001 g using a Mettler Toledo Semi-Micro Balance.

Images of morphological samples were analysed using ImageJ Image analysis software following the protocol of Judge & Bonanno, 2008. We measured multiple traits indicating body size, such as head width, full body length, pronotum length and width from both dorsal and ventral views, and femur length. Mouthparts that are weaponry used during fights included left and right mandible and maxilla lengths. All traits were measured in millimetres using the scale measurements for reference. Since body size and weaponry measures taken at generation six were highly correlated (see supporting information, Table S1 and Figure S1), we focus on femur length as a proxy for body size and mandible size as a measure of weaponry.

Statistical analysis

To test whether evolving under differential selective pressures (Pre-M, Post-P, No-Sel treatments) for six generations affects evolutionary responses concerning individual pre- and post-copulatory traits we used linear mixed effect models (i.e., with gaussian distribution) (LMM) for calling and courtship song, behavioural traits latency to sing (attractiveness) and absolute chasing time of focal male (aggressiveness), as well as for all morphological traits (pre- and post-copulatory). We used generalised linear mixed effect models (GLMM) with Poisson distribution for the traits chirp number (calling song) and number of high frequency pulses (courtship song) due to count values and binomial distribution (GLMM-b) to investigate relative chasing time of focal male (aggressiveness) and sperm viability. In both binomial models the cbind function was applied to include focal and opponent chasing time, as well as life and dead sperm cells. Treatment and male age (mean-centred) were fitted as factors in the model, except for morphological measures. In case males were tested against another individual (courtship song, attractiveness, and aggressiveness), female or opponent age (mean-centred) was added as factor as well. Replicate line ID was fitted as random effect in all models, as well as an individual observation level-ID to account for overdispersion in the two binomial models (Harrison, 2015). In case of a significant treatment effect, we used Tukey post hoc tests to understand differences between the treatments.

To illustrate comparable effect sizes across traits we conducted two meta-analyses. In the first analysis, standardised estimates of traits were pooled by trait class (i.e., all calling song traits, all morphological traits) and tested in several linear mixed models, with trait estimates as response variable, treatment and trait as factors and line ID as well as male ID as random factors. In the second analysis, standardised estimates of traits were pooled by selection episode (i.e., all pre- copulatory traits and all post-copulatory traits). To contrast pre- vs. post-copulatory traits, two single models, with the first one including all pre- and the second one all post-copulatory trait estimates, were run with identical model factors as in the trait class models. Effect sizes for each meta-analysis (beta estimate intercept ± beta estimate treatment factor) and their standard errors are reported as supporting information (Table S4). These effect sizes were then utilised to create two forest plots, where they are depicted along a vertical zero line, with the horizontal position (positive or negative) indicating the direction, and the distance between effect sizes of different treatments indicating the strength of the effect.

As done for generation six, to understand differences in pre- and post-copulatory traits evolving under pre-copulatory, post-copulatory or weakened selection for three generations the meta-analyses were repeated for all traits except courtship song, with traits first pooled by trait class and then pooled by selection episode. Effect sizes for each meta-analysis (beta estimate intercept ± beta estimate treatment factor) and their standard errors are reported as supporting information (Table S6), with both results being illustrated in two separate forest plots (Figure S2 and S3).

Statistical analyses were conducted in RStudio (R 4.1.1, RStudio 2021.09.0) using the package "lme4" (Bates et al., 2015) for running linear mixed models, the package "DHARMa" (Hartig, 2020) for checking model assumptions, the package "multcomp" for conducting Tukey post hoc testing (Hothorn et al., 2008) and the package "ggforestplot" (Scheinin et al., 2021) for creating forest plots. Data are either reported as mean ± standard errors in the text as well as in Tables S2 and S5 as supporting information, or as beta estimate and 95% credible intervals (Tables 2, 3 and 4).

Results

We chose to report only results of traits measured from animals at the F6 generation as these were exposed to selective treatments for longest time. Results on the meta-analyses of traits from the F3 generation are shown as supporting information (**Table S6, Figure S2** and **S3**).

Pre-copulatory traits

Calling and courtship song

Chirp duration, pulses per chirp and peak amplitude of calling song differed between treatments (**Table 2, Figure 1a-c**) with Post-P males producing lower chirp durations, as well as less pulses per chirp than Pre-M and No-Sel males (Tukey Post-Hoc test: chirp duration No-Sel – Post-P: estimate 16.1 ± 4.3, $p<0.001$; pulses per chirp No-Sel – Post-P: estimate 0.43 ± 0.12, $p=0.001$) and a lower peak amplitude than Pre-M males (**Table S2**). Chirp number, interval duration between chirps and peak frequency were not affected by treatment (**Table 2**).

Pre-M males produced their courtship song low frequency pulses in a slightly higher frequency than Post-P (**Table 2, Figure 1d**). Further, Pre-M males produced a longer high frequency pulse duration than No-Sel males, as well as a higher peak amplitude within their high frequency pulses. Reversely, No-Sel males produced a marginally longer average phrase length than Pre-M males as well as a higher peak frequency within their high frequency pulses than Pre-M and Post-P males (Tukey Post-Hoc test No-Sel – Post-P: estimate 1.36 ± 0.52, $p=0.02$; **Tables 2** and **S2**).

Male age at the day of song recording differed between treatments (linear regression: $R^2=0.5$, $F(2,162)=84.52$, $p<0.0001$; Pre-M 21.62 ± 0.22 ($n=58$), Post-P 21.74 ± 0.23 ($n=54$), No-Sel 18.26 ± 0.18 ($n=53$) days of age), which did not affect any of the traits measured. Males increased the peak amplitude of their low frequency pulses when mated to older females (**Table 2)**.

Table 2: Estimated beta values and 95% credible intervals around the mean of predictors of pre-copulatory calling and courtship song measures; predictors: Treatment (Pre-M, Post-P, No-Sel), male age and female age (only courtship song); treatment effects indicated as contrasting Post-P and No-Sel compared against Pre-M (intercept); treatment effects between Post-P and No-Sel are reported in the text via Post-Hoc testing; random effect: Replicate line (Line). Male and female age were standardized (mean-centred). Significance shown in bold. LFP: Low frequency pulse; HFP: High frequency pulse; [1]Poisson distribution.

Calling song

	Chirp number[1]	Pulses per chirp	Chirp duration [ms]	Chirp interval duration [s]	Peak frequency [kHz]	Peak amplitude [-dB]
Fixed effects (β estimate (95% CI))						
Intercept	17.85 (17.2, 18.5)	4.03 (3.9, 4.15)	128.79 (124.28, 133.33)	0.17 (0.15, 0.18)	5.22 (5.15, 5.29)	-19.21 (-20.68, -17.63)
Post-P	-0.17 (-1.05, 0.68)	**-0.29** **(-0.47, -0.11)**	**-12.94** **(-19.55, -6.43)**	0.01 (-0.01, 0.03)	-0.09 (-0.18, 0.01)	**-3.24** **(-5.47, -1.03)**
No-Sel	-0.13 (-1.07, 0.84)	0.14 (-0.06, 0.34)	3.14 (-3.69, 9.92)	-0.01 (-0.03, 0.02)	-0.04 (-0.15, 0.06)	-0.73 (-3.16, 1.66)
Male age	-0.03 (-0.11, 0.05)	0.01 (-0.01, 0.03)	0.53 (-0.07, 1.15)	0.0004 (-0.001, 0.002)	0.002 (-0.01, 0.08)	0.07 (-0.1, 0.23)
Random effects (σ2 (95% CI))						
Line ID	0.34 (0.16, 0.58)	0.01 (0.005, 0.02)	18.55 (8.97, 33.09)	0.0003 (0.0001, 0.0004)	0.006 (0.004, 0.01)	2.89 (1.6, 4.77)
Residual variance	5.65 (4.89, 6.56)	0.31 (0.27, 0.37)	334.47 (291.34, 391.43)	0.002 (0.002, 0.003)	0.03 (0.026, 0.035)	23.07 (19.92, 26.8)

Courtship song

	Number of HFPs[1]	HFP duration [ms]	Phrase length [s]	LFP: peak frequency [kHz]	LFP: peak amplitude [-dB]	HFP: peak frequency [kHz]	HFP: peak amplitude [-dB]
Fixed effects (β estimate (95% CI))							
Intercept	2.05 (1.96, 2.15)	46.31 (44.1, 48.51)	0.28 (0.26, 0.3)	4.77 (4.66, 4.9)	-78.77 (-80.85, -76.68)	14.32 (13.77, 14.9)	-63.05 (-64.5, -61.61)
Post-P	-0.09 (-0.21, 0.03)	-2.7 (-5.61, 0.31)	0.01 (-0.01, 0.04)	-0.17 (-0.32, -0.02)	1.69 (-1.05, 4.47)	-0.29 (-1.09, 0.46)	-0.76 (-2.73, 1.11)
No-Sel	-0.16 (-0.33, 0.006)	-5.24 (-9.05, -1.47)	0.04 (0.003, 0.07)	0.04 (-0.16, 0.23)	0.73 (-2.88, 4.13)	1.06 (0.11, 1.99)	-3.17 (-5.66, -0.63)
Male age	-0.02 (-0.05, 0.008)	-0.41 (-1.12, 0.28)	0.005 (-0.002, 0.01)	0.02 (-0.01, 0.06)	-0.52 (-1.1, 0.05)	0.12 (-0.05, 0.29)	0.04 (-0.44, 0.5)
Female age	-0.004 (-0.02, 0.01)	0.32 (-0.04, 0.68)	0.003 (-0.001, 0.01)	0.006 (-0.01, 0.02)	0.58 (0.27, 0.87)	-0.03 (-0.12, 0.05)	-0.02 (-0.26, 0.22)
Random effects (σ^2 (95% CI))							
Line ID	0.014 (0.01, 0.03)	3.88 (1.64, 7.51)	1.95e-05 (7.33e-06, 4.24e-05)	0.01 (0.003, 0.015)	2.06 (0.88, 4.12)	0.11 (0.04, 0.22)	0.52 (0.2, 1.11)
Residual variance	3.06 (2.54, 3.69)	64.9 (54.13, 78.35)	0.006 (0.054, 0.008)	0.16 (0.14, 0.2)	40.18 (33.51, 49.33)	3.75 (3.15, 4.58)	29.23 (24.33, 35.67)

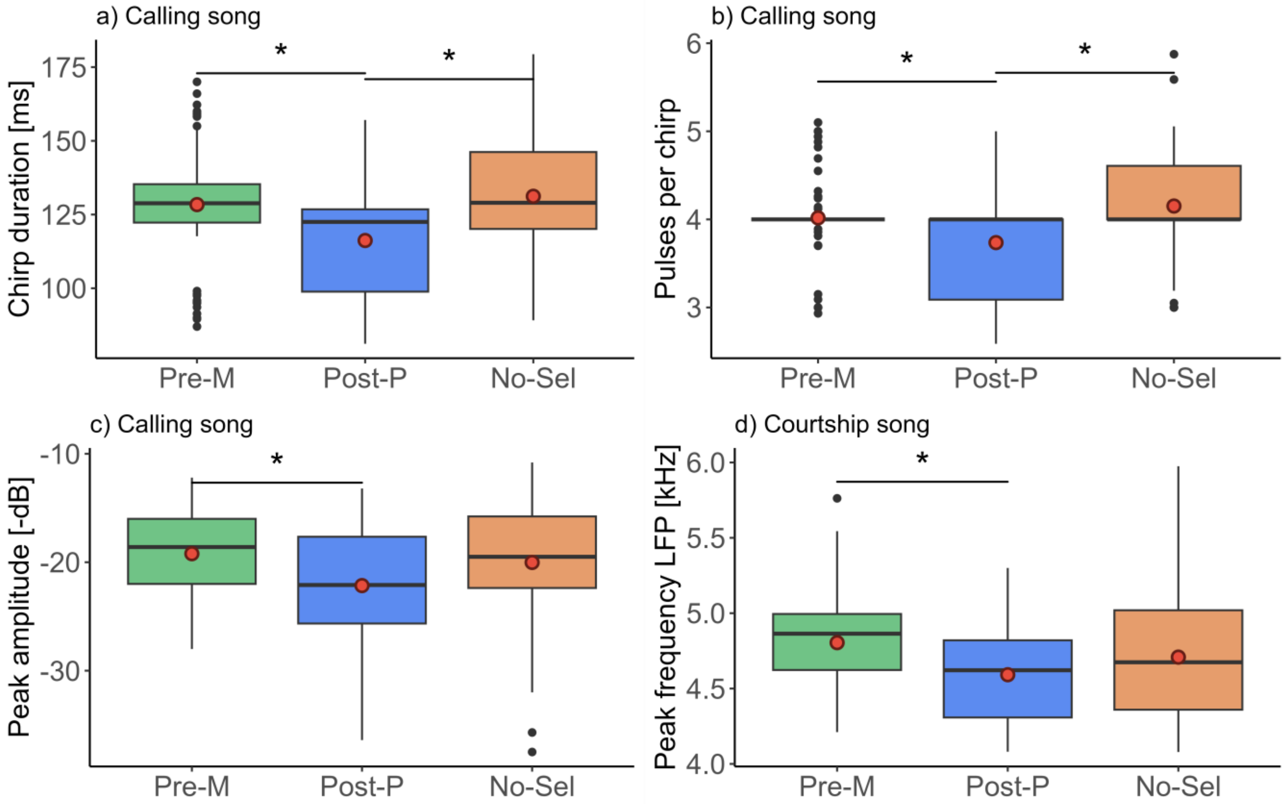

Figure 1: Illustration of significant (*) differences between treatments in calling (a-c) and courtship song (d) traits. Post-P males produced a shorter chirp duration and less pulses per chirp compared to Pre-M and No-Sel males in their calling songs (a & b), as well as a lower peak amplitude than Pre-M males (c). Post-P males also produced courtship songs with a lower peak frequency within their low frequency pulses (LFP) (d). Red dots indicate mean estimates.

Attractiveness and aggressiveness

Male attractiveness, measured as latency to female mount from first male courtship song, did not vary between treatments, neither was it affected by male or female age (**Table 3**). Further, treatment did not affect additional behaviours estimated at mating, such as latency to first courtship song, latency to first physical contact or latency to female mount from first physical contact (**Tables S2** and **S3**).

Male aggressiveness (i.e., absolute and relative time spent chasing the opponent) was not affected by treatment, neither by focal nor opponent age (**Tables 3** and **S2**).

Body mass, weaponry & body size

Males from the Pre-M and Post-P treatment marginally differed in body mass, with Pre-M males evolving higher body mass compared to both Post-P and No-Sel males (**Table 3, Figure 2a**).

Pre-M and Post-P males did not differ in their mandible size or femur length (**Table 3**). While there was only a trend that Post-P males evolved smaller mandibles than Pre-M males (**Table 3**), their mandible sizes were reduced compared to No-Sel males (Tukey Post-Hoc Post-P – No-Sel: Estimate=0.29 ± 0.11, p=0.02; **Figure 2b**). Additionally, No-Sel males evolved longer femurs than Pre-M (**Table 3**) and Post-P males did (Tukey Post-Hoc Post-P – No-Sel: Estimate=0.63 ± 0.21, p=0.006; **Figure 2c**).

Table 3: Estimated beta values and 95% credible intervals around the mean of predictors of pre-copulatory behaviours, such as attractiveness (latency to mate) and aggressiveness (absolute and relative chasing time), and morphology measures such as body mass, weaponry (mandible size) and body size (femur length); predictors: Treatment (Pre-M, Post-P, No-Sel), male age and female (attractiveness) or opponent age (aggressiveness), treatment effects indicated as contrasting Post-P and No-Sel compared against Pre-M (intercept); treatment effects between Post-P and No-Sel are reported in the text via Post-Hoc testing; random effect: Replicate Line (Line ID), Observation level ID (glmer-b). Age was standardized (mean-centred). Significance shown in bold; [1]GLMM-b.

Behaviours

	Latency to mating [min]	Absolute chasing time [min]	Relative chasing Time[1] [%]
Fixed effects (β estimate **(95% CI))**			
Intercept	3.49 (3.25, 3.74)	1.86 (1.63, 2.11)	0.31 (0.06, 0.55)
Post-P	-0.11 (-0.44, 0.2)	-0.12 (-0.45, 0.2)	-0.07 (-0.4, 0.26)
No-Sel	-0.15 (-0.57, 0.25)	-0.29 (-0.69, 0.11)	-0.16 (-0.52, 0.21)
Male age	0.03 (-0.03, 0.08)	-0.01 (-0.05, 0.03)	-0.001 (-0.03, 0.03)
Female/ opponent age	-0.04 (-0.08, 0.005)	-0.03 (-0.07, 0.02)	**0.04** **(0.001, 0.07)**
Random effects **(σ^2 (95% CI))**			
Line ID	0.007 (0.003, 0.01)	0.03 (0.01, 0.05)	0.04 (0.03, 0.07)
Observation level ID			0.55 (0.52, 0.59)
Residual variance	1.03 (0.86, 1.25)	0.018 (0.016, 0.02)	(π2/3)

Body mass, weaponry & body size

	Male body mass [g]	Mandible size [mm]	Femur length [mm]
Fixed effects (β estimate **(95% CI))**			
Intercept	0.75 (0.72, 0.78)	3.42 (3.29, 3.56)	12.54 (12.31, 12.76)
Post-P	**-0.05** **(-0.09, -0.004)**	-0.16 (-0.33, 0.005)	-0.21 (-0.51, 0.11)
No-Sel	**-0.07** **(-0.12, -0.02)**	0.13 (-0.08, 0.34)	**0.43** **(0.07, 0.77)**

Random effects **(σ^2 (95% CI))**			
Line ID	0.002 (0.001, 0.003)	0.02 (0.01, 0.04)	0.05 (0.03, 0.1)
Residual variance	1.1 (0.96, 1.28)	0.1 (0.08, 0.11)	0.89 (0.76, 1.05)

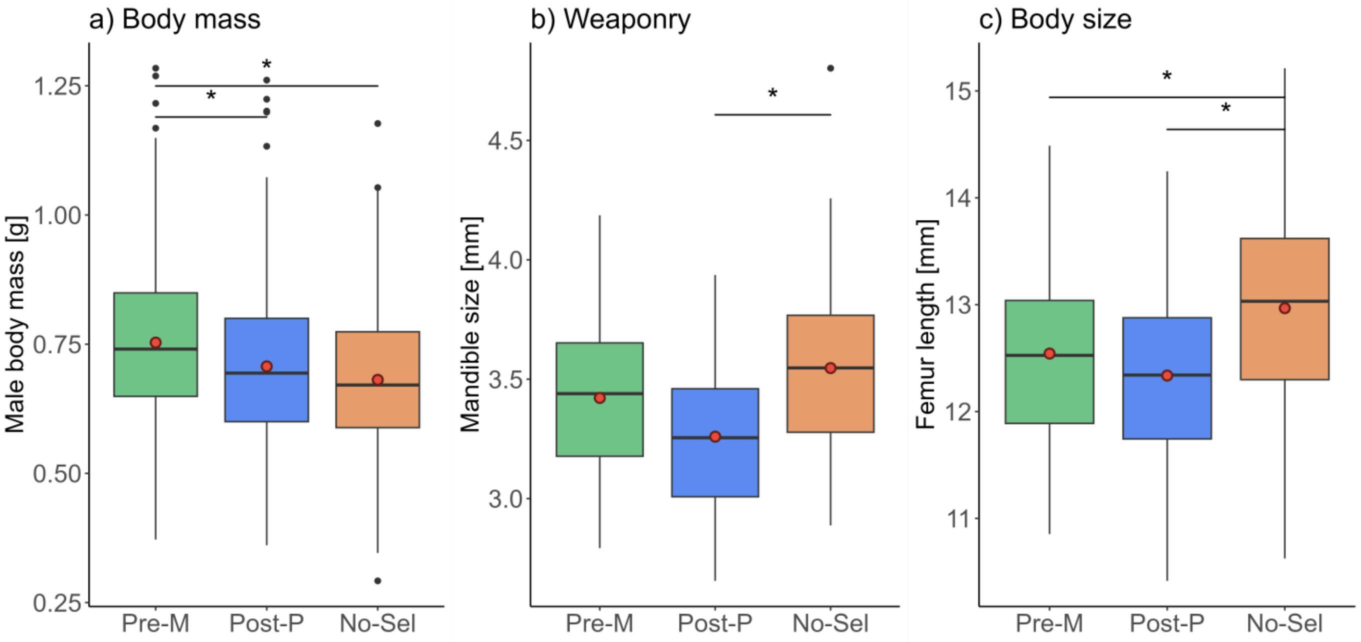

Figure 2: Illustration of significant (*) differences between treatments in pre-copulatory body mass, weaponry (mandible size), and body size (femur length). Pre-M males evolved higher body mass than Post-P and No-Sel males (a); No-Sel males evolved bigger mandibles compared to Post-P males (b) and longer femurs compared to Pre-M and Post-P males (c). Red dots indicate mean estimates.

Post-copulatory traits

Testes mass & sperm viability

Post-P males evolved lower testes mass compared to both Pre-M and No-Sel males (Tukey Post-Hoc Post-P – No-Sel: Estimate= 3.02 ± 1.13, p=0.02; **Table 4, Figure 3a**).

Pre-M and Post-P males did not differ in their percentage of live sperm cells (**Table 4**). However, No-Sel males evolved a higher sperm viability than both Pre-M and Post-P males (Tukey Post-Hoc Post-P – No-Sel: Estimate= 0.38 ± 0.16, p=0.04; **Table 4, Figure 3b**). Sperm viability was not affected by male age.

Table 4: Estimated beta values and 95% credible intervals around the mean of predictors of post-copulatory morphology (testes mass) and sperm viability (percentage of live sperm); predictors: Treatment (Pre-M, Post-P, No-Sel), male age, treatment effects indicated as contrasting Post-P and No-Sel compared against Pre-M (intercept); treatment effects between Post-P and No-Sel are reported in the text via Post-Hoc testing; random effect: Replicate line (Line ID), Observation level ID (glmer-b). Age was standardized (mean-centred). Significance shown in bold; [1]GLMM-b.

	Testes mass [mg]	Sperm viability[1] [%]
Fixed effects (β estimate **(95% CI))**		
Intercept	14.41 (13.16, 15.58)	0.44 (0.29, 0.57)
Post-P	**-2.6** **(-4.28, -0.93)**	0.13 (-0.08, 0.36)
No-Sel	0.43 (-1.54, 2.23)	**0.51** **(0.27, 0.77)**
Male age		-0.03 (-0.13, 0.06)
Random effects **(σ^2 (95% CI))**		
Line ID	1.55 (0.8, 2.54)	0 (0.00, 0.00)
Observation level ID		1.03 (0.98, 1.08)
Residual variance	11.73 (10.03, 13.75)	(π2/3)

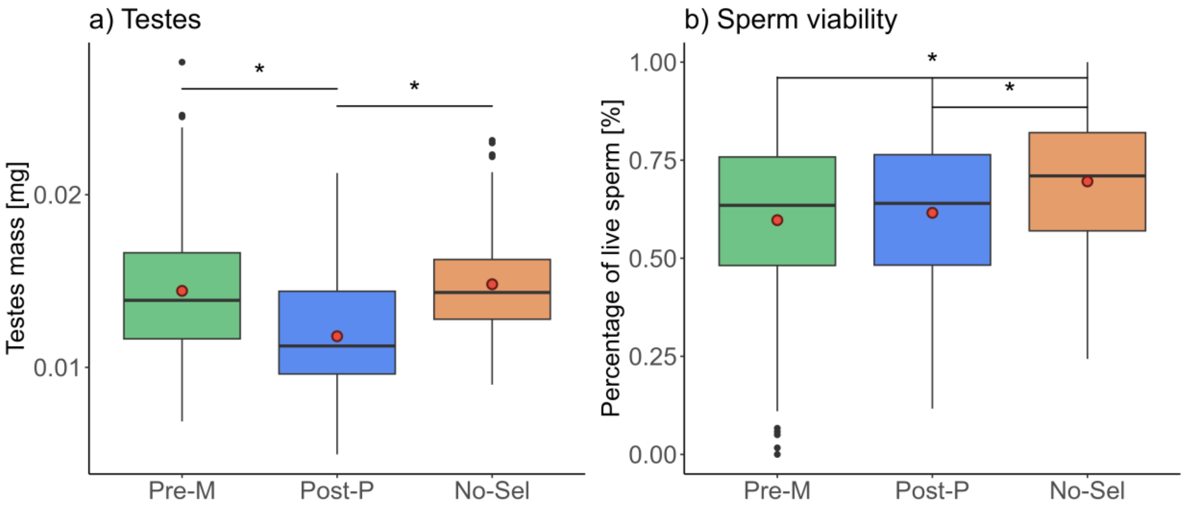

Figure 3: Illustration of significant (*) differences between treatments in post-copulatory testes mass and sperm viability. Post-P males evolved lower testes mass than Pre-M and No-Sel males (a); No-Sel males evolved a higher percentage of live sperm cells compared to Pre-M and Post-P males (b). Red dots indicate mean estimates.

Meta-Analyses

Analyses of pooled trait classes show that males under post-copulatory selection evolved lower calling song estimates than males under pre-copulatory selection (Tukey Post-Hoc Post-P – Pre-M: estimate=-0.33 ± 0.14, p=0.04, **Figure 4**), but not courtship song. Pooled estimates for weaponry traits (mandibles and maxillae), body size measures (head and prosoma width, femur, prosoma and body length), attractiveness (courtship and latency to mate) and aggressiveness

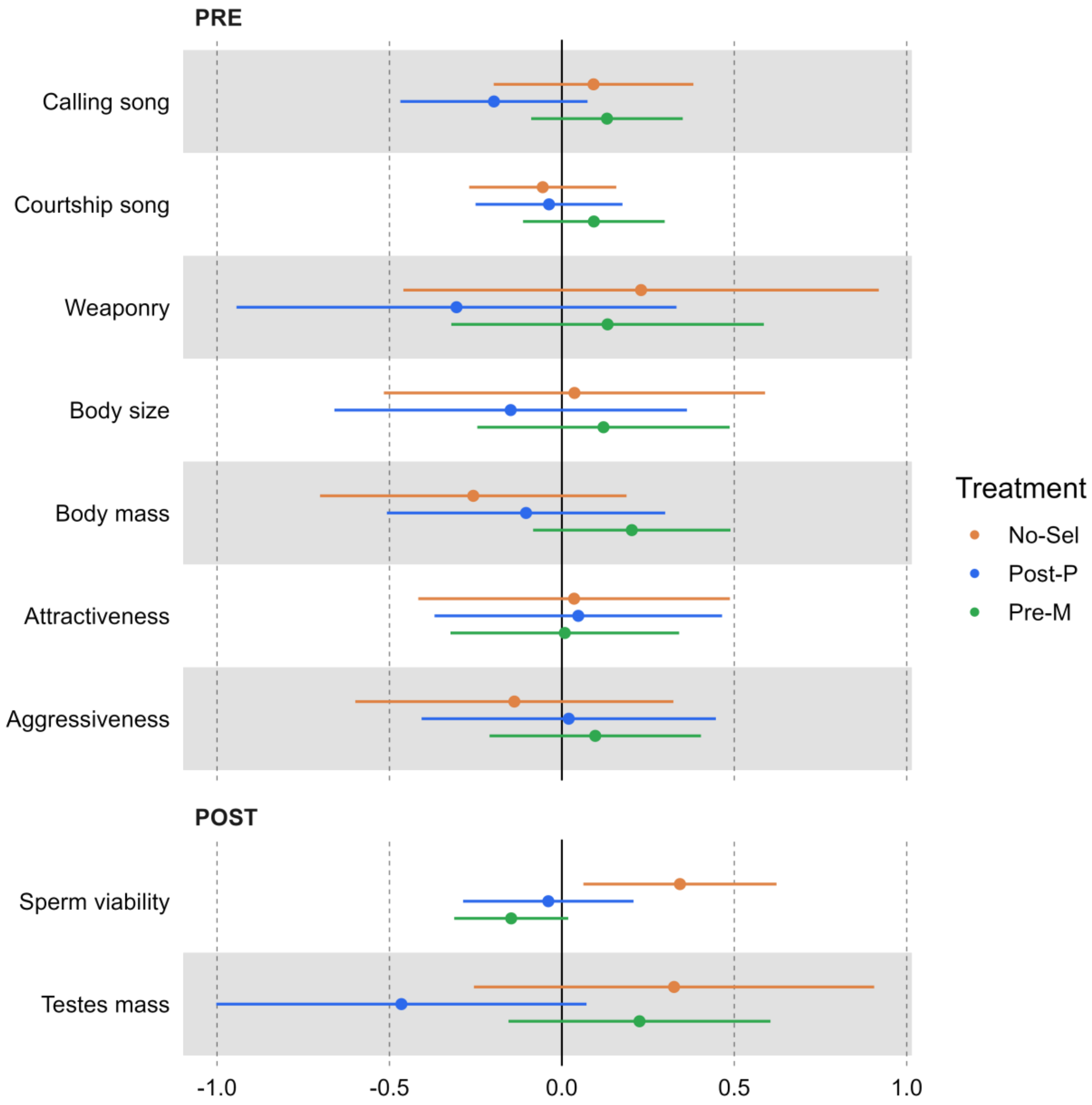

Figure 4: Meta-analysis of pre- and post-copulatory traits sorted by trait class; standardized effect sizes (± standard errors) are depicted along a vertical zero line with the horizontal position (positive or negative) indicating the direction, and the distance between effect sizes of different treatments indicating the strength of the effect. Calling song estimates were higher for Pre-M than Post-P males, while body mass was higher in Pre-M than both Post-P and No-Sel males. No-Sel males evolved higher sperm viability compared to both Pre-M and Post-P males, while Post-P males had the lowest testes mass.

(absolute and relative chasing time) did not show any differences between treatments (**Figure 4**). Estimates for body mass, sperm viability and testes mass were not pooled, but effect sizes are shown in **Figure 4**.

Meta-analysis of pooled pre-copulatory (calling song, courtship song, behaviours, body size, body mass, weaponry) and post-copulatory traits (testes mass and sperm viability) showed that males under pre-copulatory selection evolved overall higher estimates of pre-copulatory traits than males under post-copulatory selection (Tukey Post-Hoc Post-P – Pre-M: estimate -0.2 ± 0.08, p=0.03; **Figure 5**). While there was only a trend for the same relationship between Pre-M and Post-P in overall estimates of post-copulatory traits, males under relaxed selection had higher post-copulatory trait measures compared to both pre- and post-copulatory selection treatments (Tukey Post-Hoc No-Sel – Pre-M: estimate 0.35 ± 0.13, p=0.02; No-Sel – Post-P: estimate 0.56 ± 0.13, p<0.001; **Figure 5**).

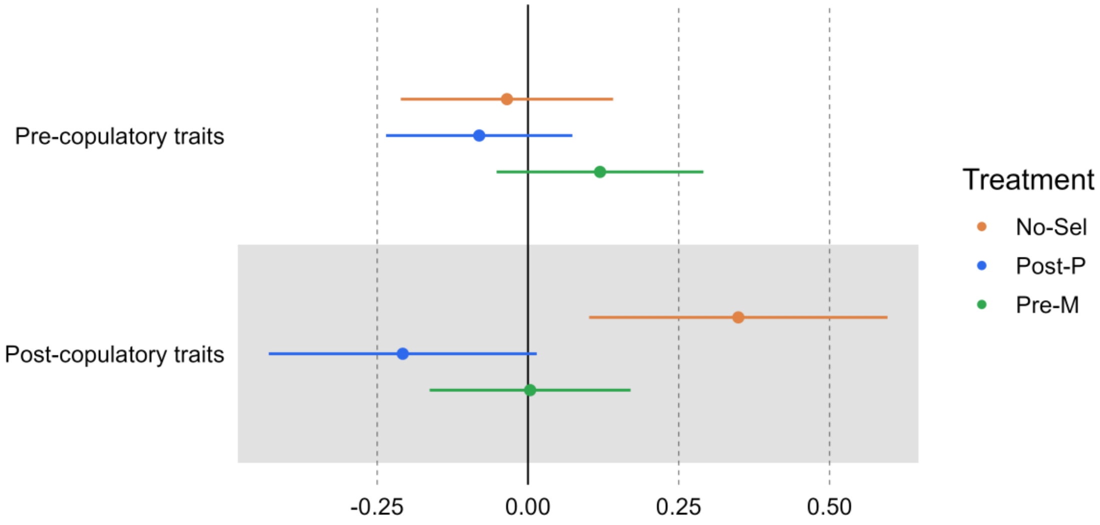

Figure 5: Meta-analysis of pre- vs. post-copulatory traits; standardized effect sizes (± standard errors) are depicted along a vertical zero line with the horizontal position (positive or negative) indicating the direction, and the distance between effect sizes of different treatments indicating the strength of the effect; in the pre-copulatory traits, estimates were higher for Pre-M than for Post-P; in the post-copulatory traits, estimates in No-Sel were higher than in Pre-M and in Post-P.

Meta-analysis of traits measured after three generations revealed that body mass estimates were higher for males evolving under weakened selection compared to males evolving under pre- or post-copulatory selection (**Table S6, Figure S2**). However, there were no differences in overall pre- or post-copulatory traits between the Pre-M, Post-P and No-Sel treatment (**Table**

S6, Figure S3). Visual trends observed in the post-copulatory traits support the effects reported in post-copulatory traits after six generations. Yet, visual trends observed in the pre-copulatory traits do not concur with effects reported in pre-copulatory traits after six generations, as pre-copulatory selection yielded lowest trait values after post-copulatory and weakened selection.

Discussion

After six generations of experimental evolution with crickets evolving exclusively under pre-copulatory selection (Pre-M), post-copulatory selection (Post-P) or under relaxed sexual selection (No-Sel), we expected to find higher estimates of pre-copulatory traits in the pre-copulatory evolution lines, as well as higher estimates of post-copulatory traits in the post-copulatory evolution lines. The responses (higher or lower values) of post-copulatory traits in Pre-M lines and vice versa, of the pre-copulatory traits in the Post-P lines would importantly be suggestive of either correlated evolution or evolutionary trade-offs.

We observed evolutionary trait responses after few generations of experimental evolution, reinforcing the notion that sexual selection can drive the rapid evolution of sexual traits. While some traits evolved according to our expectations, such as higher body mass in males under intense pre-copulatory selection, others did not, such as lighter testes in males under post-copulatory selection. When measuring post-copulatory traits evolving in males under pre-copulatory selection, we did not find any change in mean trait values. However, certain pre-copulatory traits diverged in males under post-copulatory selection, as these males evolved calling and courtship song traits of lower quality. This may overall suggest correlated evolution between testes mass and song. In addition, we found that males under relaxed selection evolved longer femurs and higher sperm viability, also indicating a potential correlation between pre- and post-copulatory traits. Our meta- analytic approach overall revealed a positive effect of pre-copulatory sexual selection on pre- copulatory traits, and, surprisingly, a mildly negative effect of post-copulatory sexual selection on post-copulatory traits (**Figure 5**). Our results suggest that both episodes of sexual selection trigger, respectively, the rapid evolution of reproductive traits, and below we discuss the implications for our findings.

In accordance with our predictions, we found that males under pre-copulatory selection evolved higher trait values for pre-copulatory traits: male crickets undergoing male-male agonistic interactions and courtship over multiple generations evolved higher body mass. Although this effect was only minor, it may indicate that females exerted pre-copulatory mate choice for heavier males. *Gryllus bimaculatus* females are known to preferably mate with larger males (Simmons, 1986a, 1986b), which may also represent those with higher body mass. It may further suggest that higher body mass enables males to endure energy expenditures connected to pre-copulatory mating behaviours, such as singing or fighting. Song production is known to be costly, with various evidence indicating that it might be condition dependent. For example, in *G. bimaculatus*, we know that courtship song increases male haemolymph lactate level, which is a commonly used index of anaerobic respiration in signalling systems, hence a measure of muscle fatigue and exhaustion during energetically demanding activities (Mowles, 2014). Further, courtship song is shown to be approximately two and a half times more energetically exhausting than the calling song in the house cricket *Acheta domesticus* (Hack, 1998; Rantala & Kortet, 2003). Yet, evidence on condition dependence of male courtship song is ambiguous, as shown by a study on the field cricket *Gryllus texensis* (Gray & Eckhardt, 2001). Nevertheless, next to having more resources for song production, an increased body mass is also potentially beneficial for withstanding energetic costs sustained during aggressive fights (Adamo & Hoy, 1995; Simmons, 1986b) as males wrestling with an opponent were shown to consume oxygen at a rate more than 40 times that of stridulation (Hack, 1997b). Aggressiveness and body mass are traits that are genetically correlated in this species (Tuni et al., 2018). Heavier males may have therefore been better at outcompeting rivals and courting females, siring offspring themselves of heavier body mass.

Interestingly, while males evolving under intense pre-copulatory sexual selection had highest song trait values, this effect appears to be driven by a negative effect of post-copulatory sexual selection. Namely, Post-P males had lower quality calling songs (less pulses per chirp, lower chirp duration and amplitude). The quality of courtship song did not generally differ, if not for one specific song trait (lower frequency of low frequency pulses). The ability to acoustically attract females and induce mating through courtship is essential in this species, were females wander long distances to encounter males (Simmons, 1988). Louder songs are under preference by *Gryllus bimaculatus* females (Zhemchuzhnikov et al., 2017) and it is known from other cricket species that females prefer males producing higher chirp rates and longer chirp durations (i.e., the field cricket *Gryllus lineaticeps*, Wagner, 1996). Further, song has previously been shown to generally evolve rapidly under different mating system structures in other insects (Snook et al., 2005). In our study males evolving under intense post-copulatory selection were not exposed to any form of social interaction before mating over multiple generations. Females chose their mate within three competitors, however, not based on song quality, but based on post-copulatory criteria. Our findings suggest, that through this mating scenario, selective pressures decreased on producing high quality calling or courtship songs, since competition between males shifted to a post- copulatory level. Hence these males evolved shorter calling song chirp durations, potentially caused by a lower pulse rate per chirp, as well as lower chirp amplitudes and lower courtship song frequencies.

Interestingly, we found lack of trait divergence for behavioural pre-copulatory traits, such as aggressiveness and latency to mating (a proxy for male attractiveness). This could be due to behavioural traits being less responsive to selective pressures as they are multivariate (Santostefano et al., 2016). Previous studies on *G. bimaculatus* show that heritability for aggression is significant, but notably low (Santostefano et al., 2017a; Tuni et al., 2018). Hence, both behavioural traits are potentially overshadowed by multiple other traits, such as body and mandible size affecting male fighting ability or wing morphology and courtship song abilities affecting male attractiveness. Furthermore, the social context might have also biased both behavioural measures. Aggressive behaviour in *G. bimaculatus* was shown to be subject to indirect genetic effects, as genotypes predisposed to be aggressive (due to direct genetic affects) strongly decrease aggressiveness in opponents (Santostefano et al., 2017b). It is rather likely, that similar effects might be at play when males court females, as the mating success derived from courtship investment greatly depends on the female response (Moore et al., 1997; Schneider et al., 2017). Additionally, it is possible that longer periods of time under more generations of experimental evolution are needed for behavioural traits to respond.

Contrarily to our predictions, males under intense post-copulatory selection did not evolve higher post-copulatory trait values. In fact, post-copulatory sexual selection appeared to have a negative effect on post-copulatory traits (**Figure 5**). Specifically, Post-P males evolved lowest testes mass, and lower sperm viability compared to males evolving under relaxed selection. Polyandrous mating increases sperm competition risk and is commonly reported to enhance post- copulatory traits, such as sperm morphology (Firman & Simmons, 2010b), number (Firman et al., 2013), motility (Firman et al., 2013; Firman & Simmons, 2010a) and/or sperm viability (García- González & Simmons, 2005; Hunter & Birkhead, 2002). Testes size or mass is often used as an approximation for sperm production or numbers with high sperm competition leading to an increased testes size in yellow dung flies (Hosken et al., 2001; Hosken & Ward, 2001), horned beetles (Simmons & García-González, 2008) and fruit flies (Pitnick, Miller, et al., 2001). Similar to these studies, we would have expected enhanced post-copulatory traits - sperm viability and testes mass - in polyandrous post-copulatory treatments. Interpretation of these findings is puzzling as it would imply that sperm competition and/or cryptic female choice select for males with lowest fertilization abilities, in the form of reduced ejaculate size and quality (i.e., sperm number and viability). The results shown in our study therefore raise the question whether testes mass is an appropriate predictor of sperm number in *G. bimaculatus*. Studies on house mice (Firman et al., 2018; Firman & Simmons, 2010a), show that males evolving under sperm competition for eight generations produced more sperm as well as sperm of higher motility and velocity in absence of changes in testes size or morphology (testes size did also not diverge neither after 16 or 18 generations (Firman & Simmons, 2011)). In some studies on *Drosophila melanogaster* neither sperm numbers nor testes size increased in males evolving under high sperm competition risk (Chechi et al., 2017; Crudgington et al., 2009), possibly indicating a lack of genetic variance in testes mass, leading to testes not responding to selection. This is supported by a study on the field cricket *Teleogryllus oceanicus* (García-González & Simmons, 2005), where no genetic variance in testes mass was found. However, in our study, testes mass did vary and respond to differential selective pressures, although unexpectedly. One alternative explanation could be provided by Reuter et al. (2008), who showed that testes size of fruit fly males responded to mating rate and sperm depletion, rather than sperm competition. This would explain higher testes mass in the monogamous pre-copulatory and relaxed selection treatment, since males were mated three times to the female, unlike males from the post-copulatory treatment that were each mated only once. Another explanation could be that high sperm competition induces either the development of more sperm-producing tissue within the testes, increasing sperm production efficiency (Firman, 2014), or the growth of accessory glands (Crudgington et al., 2009; Linklater et al., 2007, also in females: Hosken et al., 2001), which would not be detected from our measures of testes mass.

Removing the opportunity for sexual selection via enforced monogamy leaves individuals with a limited potential of exerting pre- or post-copulatory choice. We hence anticipated no trait divergence for both pre- and post-copulatory traits, as any remaining selection potential in these evolution lines would be extremely weakened. Contrary to our expectations, we found that males under relaxed selection generally evolved higher post-copulatory trait values (**Figure 5**). Specifically, these evolved higher sperm viability compared to both pre- and post-copulatory treatments. Body size did not generally differ, if not for one specific morphological trait (femur length). Since these results are rather puzzling, we speculate that they might be due to adjustment to non-natural conditions when monogamy is enforced in a naturally polyandrous species (Simmons, 1986a). It seems that the occurring adaptations ought to maintain a stable population fitness level, as males with low sperm viability would not essentially contribute to the following generations.

We do not find any evidence for trade-offs between traits in our study. Instead, the findings of reduced calling song and testes mass within our Post-P treatment could potentially indicate that these traits are correlated, similarly to findings of enhanced femur length and sperm viability in the No-Sel treatment. In both cases, a potential correlation between traits could have led to the selection on one trait facilitating a similar response in the correlated trait. Although deeper understanding of the genetic architecture of these traits would be needed to draw such conclusions, we hypothesise that traits may be responding in a correlated manner, to specific selective pressures. This scenario would go against current literature proposing evolutionary trade-offs between pre- and post- copulatory traits (Simmons et al., 2017), rather supporting the 'phenotypic linked hypotheses', which states that individuals can invest in multiple traits due to genetic variation in male quality (Chargé et al., 2013; Sheldon, 1994; Tuni et al., 2018).

Overall, our meta-analysis of pooled pre- and post-copulatory traits indicates that evolving under intense pre-copulatory selection elicits positive trait responses in multiple pre-copulatory traits, while evolving under post-copulatory selection elicits negative trait responses in both pre- and post-copulatory traits. Other studies found positive trait divergence among multiple post- copulatory traits after just eight (Firman & Simmons, 2010), twelve (Firman & Simmons, 2011) or fourteen (Firman & Simmons, 2012) generations of house mice evolving polyandrously compared to those evolving monogamously. Unlike our study, such polyandrous mating systems may have allowed for pre-copulatory and post-copulatory selection to co-occur, selecting overall superior phenotypes with higher mating and fertilization abilities. This could suggest that the effects of post- copulatory selection are too closely intertwined with the ones of pre-copulatory selection, for them to fully unfold their evolutionary potential if pre-copulatory selection is suppressed. Supportive evidence comes from another study disentangling pre- and post-copulatory selection similar to our study, which found thermal adaptations in fruit flies evolving under pre-copulatory selection, but not under post-copulatory selection (Gómez-Llano et al., 2021).

Selection is thought to either act gradually over time with constant selective pressures or in fluctuating waves due to varying environments (Bell, 2010). However, there is no a priori prediction of how much time selection requires to start acting on traits or more specifically, after how many generations trait divergence is measurable. This might depend on the organism, its reproductive cycle, the type of trait investigated (and its underlying genetic variance) and most notably, the strength of selection. Certain traits could simply not respond to post-copulatory selection pressures at the same speed as other traits do to pre-copulatory selection. This would be supported by studies investigating the evolutionary rates of divergence in reproductive traits, which show that pre-copulatory traits (i.e., weaponry) evolve faster than post-copulatory traits (i.e., testes mass and sperm morphometrics) in Onthophagine dung beetles (Simmons & Fitzpatrick, 2016), as well as in bovids and cervids (Reuland et al., 2021). Interestingly, while post-copulatory traits evolved more slowly than body size in the first study, they evolved at similar speed with body mass in the second. A possible explanation lies in polyandry weakening the strength of selection, as recently suggested by Cramer et al (2023), where simulated higher promiscuity levels led to either stabilizing, null or disruptive selection on sperm size. In our study, when investigating whether overall values of pre- and post-copulatory traits differed between treatments after three generations of experimental evolution, we show that they did not, if not for one individual pre-copulatory measure (body mass). Yet, after three generations there was a slight tendency that post-copulatory traits evolved in the same manner as after six generations, with post-copulatory selection yielding the lowest values. However, since the trends observed in generation three were only marginal, we refrain from giving any conclusive statement on either gradual or fluctuating selection in our study. We are aware that certain reproductive traits might respond only after a higher number of generations of experimental evolution. Yet, other studies have shown that some traits respond after a fairly short amount of time. While sexual selection removed mutations that affected male strength after just two generations in the dung beetle *Onthophagus taurus*, (Almbro & Simmons, 2014), selection was shown to be more efficient in removing deleterious alleles in male than in female seed beetles after three generations (*Callosobruchus maculatus*, Grieshop et al., 2016). In populations of *Drosophila melanogaster*, the resistance to desiccation was higher when sexual selection was allowed to operate after applying five generations of artificial selection (Gibson Vega et al., 2020). In a study with comparable experimental setup (animals evolving with pre-, post- copulatory or no sexual choice), fruit flies showed thermal adaptation, as well as decreased sexual dimorphism due to increased temperatures after six generations (*Drosophila melanogaster*, Gómez-Llano et al., 2021). In house mice, evolving under sperm competition led to males evolving diverged sperm traits after eight generations (*Mus domesticus*, Firman & Simmons, 2010). These are all examples of selection eliciting responses already after a short period of time. We hence encourage researchers to not be daunted by enormous experimental evolution setups of 20-50 generations (or more) in flies (Crudgington et al., 2005; Holland & Rice, 1999; Linklater et al., 2007), beetles (Michalczyk et al., 2011; Simmons & García-González, 2008) or mice (Firman, 2014; Firman et al., 2015), which are nevertheless extremely important to aid our understanding of long-term sexual selection mechanisms. Yet, we need more studies investigating the relationship between pre- and post-copulatory selection, which can already be achieved after few generations of intensified selection.

Conclusion

We provide evidence for evolutionary trait responses after six generations of experimental evolution aimed at disentangling the effects of pre- and post-copulatory sexual selection. Overall, pre-copulatory traits responded positively to pre-copulatory selection, while post-copulatory traits did so negatively to post-copulatory sexual selection. Further, the similar negative response of certain pre-copulatory traits to post-copulatory selection is suggestive of positive correlation between pre- and post-copulatory traits (song and testes), instead of allocation trade-off. Surprisingly, evolving under weakened sexual selection seems to facilitate a strong positive response in post-copulatory traits, potentially acting as adaptation to ensure population survival. Our results not only provide insight into how individual pre- and post-copulatory traits respond to differing selective pressures, but also shed light onto the relative role pre- and post-copulatory sexual selection play on the evolution of complex male phenotypes.

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Supporting Information for:
Evolutionary responses of key reproductive traits under manipulated pre- and post-copulatory selective pressures: from ejaculates to behaviour

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Supporting results

i) Correlations between morphological traits of generation F6

Results from PCA on morphological traits, including weaponry (mandible and maxillae size) and body size (head width, pronotum length and width, femur and body length) are shown below (**Table S1, Figure S1**).

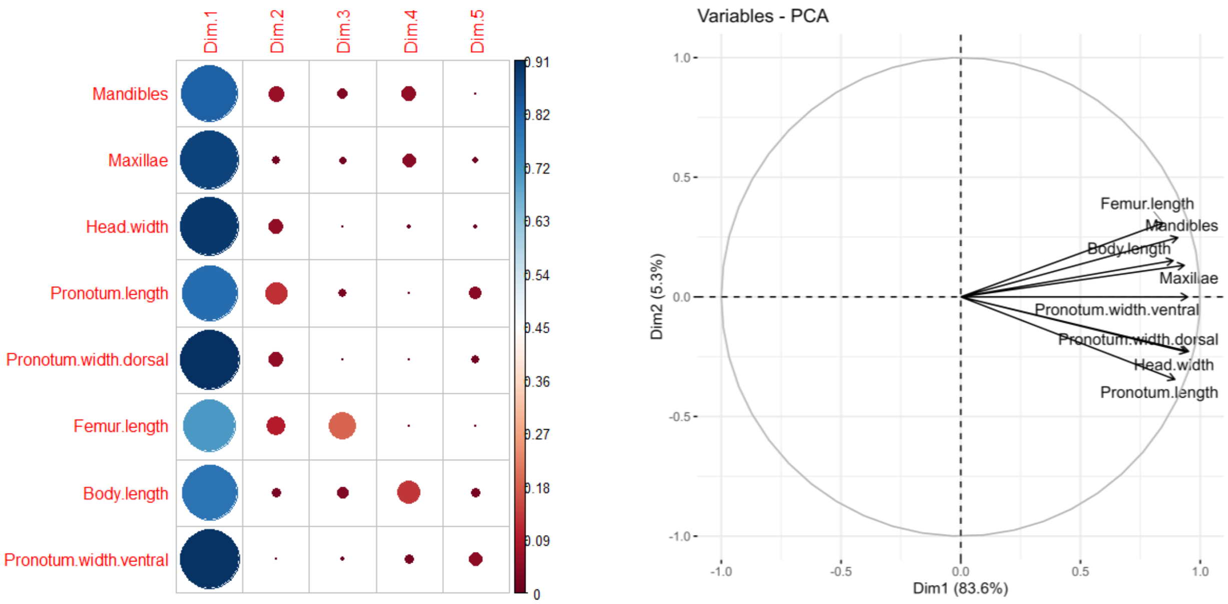

Figure S1: Display of PCA results; a) the quality of representation of each variable by the first five dimensions through percentages; dark blue colours represent high percentages (=good representation), dark red colours represent low percentages (=poor representation); b) 2-D directions of variables between Dimensions one and two; arrows in similar directions represent high correlation between single variables.

Table S1: Eigenvalue and variance estimates (+ cumulative) of PCA including measurements of mandibles, maxillae, head width, pronotum length and width (dorsal and ventral), femur and body length. Dimension 1 is represented by 83.56% of all variances.

	Eigenvalue	**Variance (%)**	**Cumulative variance (%)**
Dimension 1	6.68	83.56	83.56
Dimension 2	0.42	5.26	88.82
Dimension 3	0.29	3.63	92.46
Dimension 4	0.26	3.24	95.69
Dimension 5	0.14	1.76	97.46
Dimension 6	0.09	1.1	98.55
Dimension 7	0.08	0.96	99.52
Dimension 8	0.04	0.48	100

ii) Trait values for generation F6

Table S2: Mean estimates of all pre- and post-copulatory measurements at generation six pooled by trait class, reported as means, standard errors (SE) and sample size (n) for each Pre-M, Post-P and No-Sel treatment.

Pre-copulatory

Trait Class	Measure	**PRE-M**		**POST-P**		**NO-SEL**	
		Mean ± SE	n	Mean ± SE	n	Mean ± SE	n
Calling song	Chirp number	17.66 ± 0.3	93	17.94 ± 0.3	87	17.78 ± 0.3	76
	Pulses per chirp	4.02 ± 0.06	93	3.74 ± 0.06	87	4.15 ± 0.07	76
	Chirp duration [ms]	128.4 ± 1.9	93	116.3 ± 1.9	87	131.2 ± 2.4	76
	Chirp interval duration [ms]	166.2 ± 4.4	93	173 ± 6.2	87	158.5 ± 5.4	76
	Peak frequency [kHz]	5.22 ± 0.02	93	5.13 ±0.02	87	5.18 ± 0.02	76
	Peak amplitude [-dB]	-19.21 ± 0.41	93	-22.16 ± 0.6	87	-20.02 ± 0.63	76
Courtship song	Number of HFPs	7.56 ± 0.24	55	6.91 ± 0.23	54	7.12 ± 0.25	51
	Average HFP duration [ms]	46.24 ± 1.28	58	43.54 ± 1.05	53	41.1 ± 52	52
	Average phrase length [ms]	286.4 ± 9.8	58	301.1 ± 10.6	52	299.5 ± 12.9	50
	LFP: peak frequency [kHz]	4.8 ± 0.05	54	4.63 ± 0.06	47	4.74 ± 0.07	49
	LFP: peak amplitude [-dB]	-78.52 ± 1.02	54	-77.02 ± 0.94	48	-78.34 ± 0.85	49

	HFP: peak frequency [kHz]	14.4 ± 0.26	58	14.13 ± 0.28	53	15.21 ± 0.24	53
	HFP: peak amplitude [-dB]	-63.07 ± 0.79	58	-63.77 ± 0.7	53	-66.24 ± 0.68	53
Morphology weaponry	Mandible size [mm]	3.42 ± 0.04	80	3.26 ± 0.04	81	3.55 ± 0.05	60
	Maxilla size [mm]	4.82 ± 0.08	80	4.59 ± 0.08	81	4.96 ± 0.09	60
Morphology body size	Femur length [mm]	12.54 ± 0.1	80	12.34 ± 0.11	81	12.97 ± 0.14	60
	Head width [mm]	7.7 ± 0.09	80	7.42 ± 0.09	81	7.47 ± 0.1	60
	Pronotum width (dorsal) [mm]	8.91 ± 0.079	80	8.68 ± 0.088	81	8.65 ± 0.089	60
	Pronotum width (ventral) [mm]	8.46 ± 0.09	80	8.22 ± 0.09	81	8.38 ± 0.1	60
	Pronotum length [mm]	5.49 ± 0.08	80	5.38 ± 0.08	81	5.33 ± 0.09	60
	Body length [mm]	29.09 ± 0.16	80	28.38 ± 0.16	81	28.84 ± 0.19	60
Body mass	Male body mass [g]	0.75 ± 0.01	532	0.71 ± 0.01	599	0.68 ± 0.01	231
Attractiveness	Latency to mating from first song [s]	52.83 ± 8.53	59	47.7 ± 7.27	71	51.43 ± 12	46
	Latency to first courtship song [min]	2.2 ± 0.3	59	3.02 ± 0.42	77	2.16 ± 0.27	48
	Latency to first physical contact [s]	9.9 ± 1.42	64	15.11 ± 2.4	89	15 ± 2.9	59
	Latency to mating from first physical contact [min]	2.98 ± 0.35	60	3.1 ± 0.37	73	2.68 ± 0.39	50
Aggressiveness	Absolute chasing time [min]	1.82 ± 0.12	92	1.72 ± 0.11	92	1.64 ± 0.13	68
	Relative chasing time [%]	57.66 ± 1.92	92	56.17 ± 1.82	92	51.6 ± 2.42	68

Post-copulatory

Morphology testes	Testes mass [mg]	14.43 ± 0.46	80	11.8 ± 0.35	81	14.81 ± 0.43	60
Sperm viability	Percentage of live sperm [%]	59.72 ± 1.83	138	61.59 ± 1.75	120	69.6 ± 1.95	73

iii) Analyses of additional pre-copulatory behaviours (F6 generation)

Latency to sing (the time from the beginning of the trial to start of male courtship song), latency to first physical contact between the sexes and latency to female mount from first physical contact were not affected by treatment, as shown in **Table S3**.

Table S3: Beta estimates and 95% credible intervals around the mean of predictors of pre-copulatory behaviours (latency to first courtship song, latency to first physical contact, latency to mating from first physical contact); predictors: Treatment (Pre-M, Post-P, No-Sel), male and female age, treatment effects indicated as contrasting Post-P and No-Sel compared against Pre-M (intercept)); random effect: Replicate Line (Line ID). Age was standardized (mean-centred). Significance shown in bold.

	Latency to sing [s]	Latency to physical contact [s]	Latency to mating from physical contact [min]
Fixed effects (β estimate (95% CI))			
Intercept	4.52 (4.25, 4.79)	1.51 (1.12, 1.88)	4.91 (4.62, 5.2)
Post-P	0.15 (-0.19, 0.51)	0.29 (-0.18, 0.78)	-0.16 (-0.55, 0.21)
No-Sel	0.05 (-0.38, 0.46)	0.27 (-0.31, 0.87)	-0.17 (-0.61, 0.26)
Male age	-0.03 (-0.08, 0.02)	0.01 (-0.06, 0.08)	-0.03 (-0.08, 0.02)
Female age	0.01 (-0.03, 0.05)	0.01 (-0.05, 0.07)	0.01 (-0.03, 0.05)
Random effects (σ^2 (95% CI))			
Line ID	0.03 (0.01, 0.07)	0.04 (0.02, 0.07)	0.06 (0.03, 0.1)
Residual variance	0.83 (0.7, 0.99)	2.35 (1.99, 2.79)	0.75 (0.63, 0.9)

iv) Meta-analysis F6

The following table reports standardized effect sizes ± standard errors, for traits of generation six pooled by trait class (i.e., all calling song traits, all morphological traits), and pooled overall.

Table S4: Standardized effect sizes and standard errors (F6) around the mean of predictors of precopulatory calling and courtship song, weaponry, body size and body mass, as well as attractiveness and aggressiveness, and post-copulatory testes mass and sperm viability. Pooled estimates represent standardized effect sizes of all pre- and post-copulatory estimates pooled together, respectively.

	Pre-M	**Post-P**	**No-Sel**
Pre-copulatory traits		Effect sizes ± SE	
Calling song	0.131 ± 0.112	-0.197 ± 0.139	0.092 ± 0.148
Courtship song	0.093 ± 0.105	-0.037 ± 0.109	-0.056 ± 0.109
Weaponry	0.133 ± 0.231	-0.306 ± 0.326	0.23 ± 0.352
Body size	0.121 ± 0.187	-0.15 ± 0.261	0.037 ± 0.282
Body mass	0.203 ± 0.146	-0.104 ± 0.206	-0.257 ± 0.227
Attractiveness	0.008 ± 0.169	0.048 ± 0.213	0.035 ± 0.231
Aggressiveness	0.097 ± 0.156	0.02 ± 0.218	-0.138 ± 0.235
Pooled	**0.12 ± 0.087**	**-0.081 ± 0.079**	**-0.035 ± 0.09**
Post-copulatory traits			
Testes mass	0.225 ± 0.193	-0.465 ± 0.274	0.325 ± 0.296
Sperm viability	-0.147 ± 0.084	-0.039 ± 0.126	0.342 ± 0.143
Pooled	**0.004 ± 0.085**	**-0.208 ± 0.113**	**0.349 ± 0.126**

v) Trait values for generation F3

Table S5: Mean estimates of all pre- and post-copulatory measurements at generation three pooled by trait class, reported as means, standard errors (SE) and sample size (n) for each Pre-M, Post-P and No-Sel treatment.

Pre-copulatory

Trait Class	Measure	PRE-M		POST-P		NO-SEL	
		Mean ± SE	n	Mean ± SE	n	Mean ± SE	n
Calling song	Chirp number	17 ± 0.25	72	17.07 ± 0.24	64	17.91 ± 0.36	50
	Pulses per chirp	3.94 ± 0.05	72	3.84 ± 0.05	64	3.82 ± 0.06	50
	Chirp duration [ms]	129.2 ± 1.6	72	126.6 ± 1.6	64	127 ± 2.15	50
	Chirp interval duration [ms]	172.7 ± 5.9	72	174.9 ± 4.9	64	164.1 ± 8.1	50
	Peak frequency [kHz]	5.24 ±0.02	72	5.17 ± 0.02	64	5.18 ± 0.02	50
	Peak amplitude [-dB]	-19.8 ± 0.5	72	-20 ± 0.5	64	-19.29 ± 0.7	50
Morphology weaponry	Mandible size [mm]	3.29 ± 0.03	92	3.17 ± 0.04	85	3.36 ± 0.04	63
	Maxilla size [mm]	4.58 ± 0.05	92	4.43 ± 0.06	86	4.69 ± 0.06	64
Morphology body size	Femur length [mm]	12.15 ±0.11	92	11.77 ± 0.12	85	12.4 ± 0.13	64
	Head width [mm]	7.27 ± 0.07	92	7.11 ± 0.09	86	7.56 ± 0.09	64
	Pronotum width (dorsal) [mm]	8.38 ± 0.08	92	8.26 ± 0.09	86	8.63 ± 0.01	64
	Pronotum width (ventral) [mm]	7.98 ± 0.07	92	7.78 ± 0.08	86	8.19 ± 0.09	64
	Pronotum length [mm]	5.07 ± 0.05	92	4.98 ± 0.06	86	5.31 ± 0.06	64
	Body length [mm]	27.82 ± 0.2	92	27.57 ± 0.24	86	29.01 ± 0.26	64
Body mass	Male body mass [g]	0.67 ± 0.01	540	0.67 ± 0.01	483	0.74 ± 0.02	248
Attractiveness	Latency to mating from first song [s]	37.73 ± 7.29	75	53.28 ± 14.31	75	93.21 ± 22.3	55
	Latency to first courtship song [min]	1.64 ± 0.27	73	2.21 ± 0.31	72	1.67 ± 0.36	54
	Latency to first physical contact [s]	26.62 ± 9.1	80	24.35 ± 5.51	77	11.13 ± 2.2	60
	Latency to mating from first physical contact [min]	1.51 ± 0.17	74	2.64 ± 0.4	71	2.8 ± 0.51	55
Aggressiveness	Absolute chasing time [min]	1.59 ± 0.08	80	1.61 ± 0.09	80	1.61 ± 0.08	62
	Relative chasing time [%]	51.29 ± 1.78	80	53.94 ± 1.92	80	53.47 ± 2.1	62

Post-copulatory

Morphology testes	Testes mass [mg]	8.18 ± 0.4	80	9.1 ± 0.33	81	10.46 ± 0.46	62
Sperm viability	Percentage of live sperm [%]	66.57 ± 2.23	84	62.51 ± 2	85	64.95 ± 3.5	45

vi) Meta-analysis F3

The following table reports standardized effect sizes ± standard errors, for traits of generation three pooled by trait class (i.e., all calling song traits, all morphological traits), and pooled overall.

Table S6: Standardized effect sizes and standard errors (F3) around the mean of predictors of precopulatory calling song, weaponry, body size and body mass, as well as attractiveness and aggressiveness, and post-copulatory testes mass and sperm viability. Pooled estimates represent standardized effect sizes of all pre- and post-copulatory estimates pooled together, respectively.

	Pre-M	**Post-P**	**No-Sel**
Pre-copulatory traits		Effect sizes ± SE	
Calling song	0.071 ± 0.131	-0.081 ± 0.162	-0.005 ± 0.174
Weaponry	-0.242 ± 0.224	0.066 ± 0.315	0.263 ± 0.341
Body size	-0.229 ± 0.184	-0.013 ± 0.256	0.343 ± 0.278
Body mass	-0.103 ± 0.084	-0.096 ± 0.119	0.411 ± 0.133
Attractiveness	-0.174 ± 0.109	0.123 ± 0.134	0.049 ± 0.142
Aggressiveness	0.049 ± 0.142	-0.006 ± 0.181	0.046 ± 0.191
Pooled	**-0.117 ± 0.14**	**0.013 ± 0.172**	**0.106 ± 0.183**
Post-copulatory traits			
Testes mass	-0.011 ± 0.249	-0.279 ± 0.352	0.414 ± 0.38
Sperm viability	0.096 ± 0.109	-0.103 ± 0.154	0.011 ± 0.185
Pooled	**0.051 ± 0.154**	**-0.183 ± 0.207**	**0.269 ± 0.227**

Analysis of pooled trait classes showed that males under weakened selection evolved higher body mass than males under pre- and post-copulatory selection (Tukey Post-Hoc test Post-P – Pre-M: estimate=0.007 ± 0.12, p=1; No-Sel – Pre-M: estimate=0.51 ± 0.13, p=0.0003; No-Sel – Post-P: estimate=0.51 ± 0.13, p=0.0004; **Table S6, Figure S2**). Pooled estimates for all other trait classes did not differ between treatments.

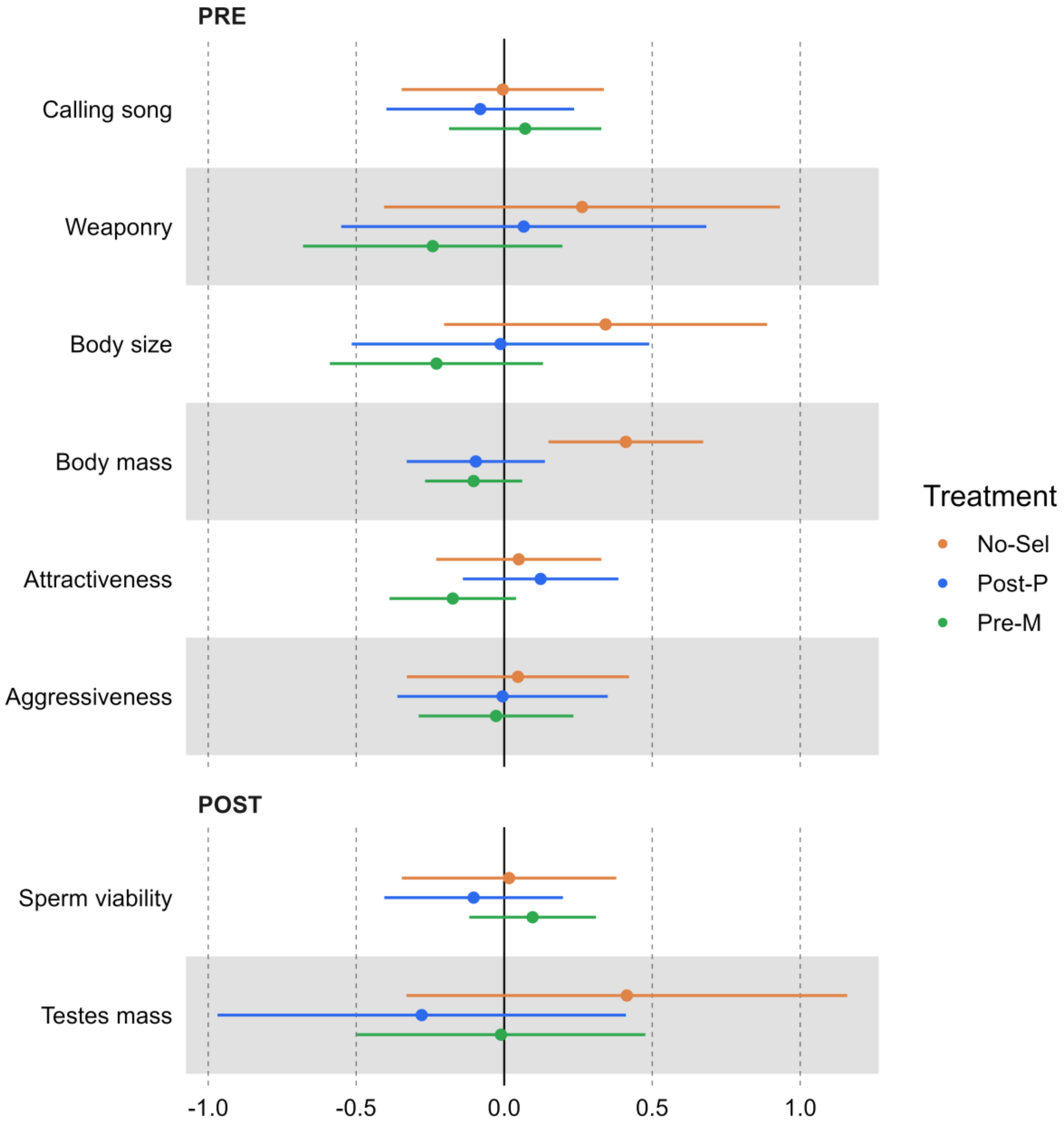

Figure S2: Meta-analysis of pre- and post-copulatory traits measured at F3 sorted by trait class; standardized effect sizes (± standard errors) are depicted along a vertical zero line with the horizontal position (positive or negative) indicating the direction, and the distance between effect sizes of different treatments indicating the strength of the effect. Body mass estimates were higher in males evolving under weakened selection than for males evolving under pre- and post-copulatory selection.

Meta-analysis of pooled pre-copulatory (calling song, behaviours, body size, body mass, weaponry) and post-copulatory traits (testes mass and sperm viability) showed no difference of pre- or post-copulatory traits evolving under pre-copulatory, post-copulatory or weakened sexual selection (Tukey Post-Hoc test for pre-copulatory traits: Post-P – Pre-M: estimate 0.14 ± 0.15, p=0.62; No-Sel – Pre-M: estimate 0.29 ± 0.16, p=0.15; No-Sel – Post-P: estimate 0.15 ± 0.16, p=0.6; post-copulatory traits: Post-P – Pre-M: estimate -0.23 ± 0.21, p=0.5; No-Sel – Pre-M: estimate 0.22 ± 0.23, p= 0.6; No-Sel – Post-P: estimate 0.45 ± 0.23, p=0.12, **Table S6, Figure S3**). While in the pre-copulatory traits there was a visual trend for pre-copulatory selection yielding lower trait values than weakened selection, in the post-copulatory traits a visual trend suggests that post-copulatory selection yielded lower trait values than weakened selection (**Figure S3**).

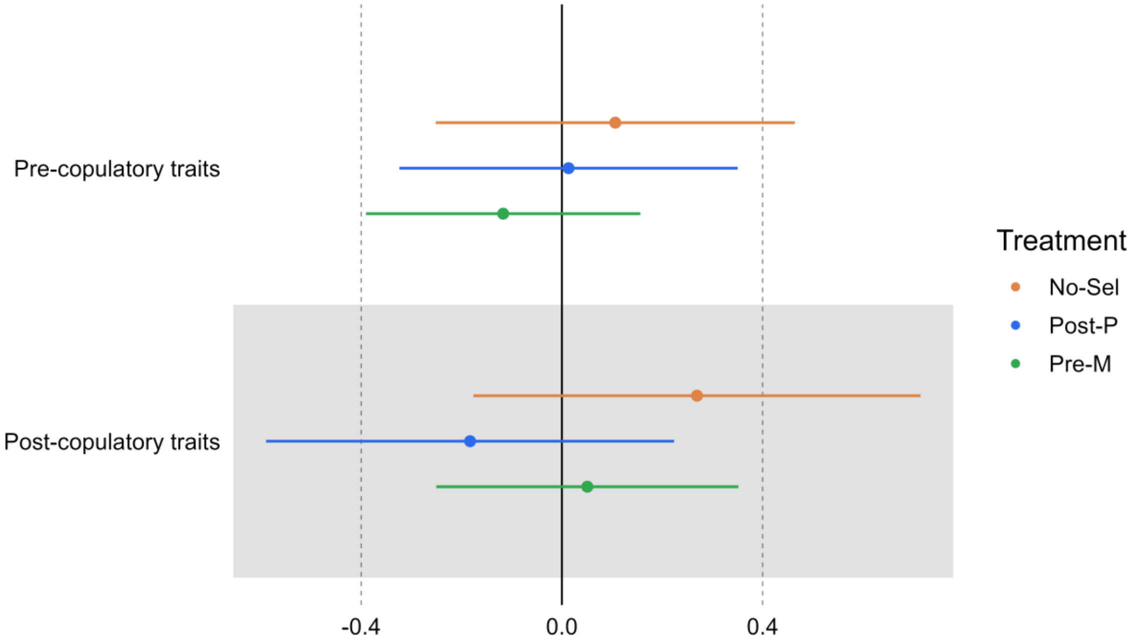

Figure S3: Meta-analysis of pre- vs. post-copulatory traits measured at F3; standardized effect sizes (± standard errors) are depicted along a vertical zero line with the horizontal position (positive or negative) indicating the direction, and the distance between effect sizes of different treatments indicating the strength of the effect; there was no difference between treatments in either pre- or post-copulatory traits.

Chapter III

Publication II: Food Limitation but Not Enhanced Rates of Ejaculate Production Imposes Reproductive and Survival Costs to Male Crickets

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Food Limitation but Not Enhanced Rates of Ejaculate Production Imposes Reproductive and Survival Costs to Male Crickets

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Abstract: Estimating costs of ejaculate production is challenging. Metabolic investment in ejacu- lates may come at the expense of other physiological functions and may negatively affect future reproduction and/or survival. These trade-offs are especially likely to occur under constrained resource pools (e.g., poor nutrition). Here, we investigated costs of ejaculate production via trade-offs in the field cricket Gryllus bimaculatus. We experimentally increased rates of ejaculate production, while keeping an unmanipulated group, in adult males kept at high and low feeding regimes and tested the effects of our treatments on (i) somatic maintenance (i.e., changes in male body mass), (ii) future reproduction (i.e., the likelihood of producing a spermatophore and the viability of its sperm), and (iii) lifetime survival and longevity. We predicted investment in ejaculates to impinge upon all measured responses, especially in low-fed individuals. Instead, we only found negative effects of food limitation, suggesting low or undetectable costs of spermatophore production. High mating rates may select for males to maximize their capacity of ejaculate production, making ejaculate traits less prone to trade-offs with other fitness-related life history traits. Nevertheless, males were impaired due to nutrient deficiency in producing viable ejaculates, suggesting condition-dependent costs for ejaculate production.

Keywords: reproductive costs; trade-offs; Gryllid; condition dependence; sperm quality; ejaculates

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1. Introduction

The view that costs of gamete production are sustained exclusively by females has been largely challenged; females do incur relatively higher costs for producing a small number of large eggs (for example, in terms of the gamete biomass production rate [1]), yet costs for ejaculate production in males are not trivial [1–5]. Ejaculates consist of multiple components that function as a unit, including sperm as well as a number of proteins and peptides in the seminal fluids [6,7]. They are favored by natural selection to ensure male fertility and by post-mating sexual selection to maximize male siring success during competitive fertilizations in polyandrous systems [8,9]. Hence, males should generally be under selection to invest greatly in each of their ejaculates, increasing rates of sperm production [10,11] and possessing fast-swimming and viable sperm to increase sperm competitiveness and siring success [8]. However, quantifying the costs of ejaculate production remains challenging. A few studies have addressed the physiological costs of ejaculate production through estimates of the basal metabolic rate [12,13] and/or energy expenditure (i.e., reserves of glycogen, lipids, protein [14], caloric analyses [15]), while most failed to disentangle these costs from the costs of mating [16]. Trade-offs are generally indicators of costs, where the high energetic demand of reproduction is expected to negatively impact future reproduction and survival [17–20]. Our understanding of reproductive trade-offs is rooted in the idea that, given individuals' limited resource budget, any investment in one function (e.g., sperm production) comes at the expense of investment in other functions (e.g., soma maintenance) [18,19]. Energetic investments in ejaculate production have, in fact, been reported to trade against a number of physiological functions. For example, males undergoing sperm production are known to quickly lose body mass [21,22], to suffer from weakened immunity [23] and reduced survival [5], and to lower their investments in other aspects of reproduction such as secondary sexual traits [24]. Intensified ejaculate production may also reduce males' ability to produce viable ejaculates in subsequent mating events [25–27], as frequent mating can deplete sperm and seminal fluids, the latter being important in ensuring sperm survival [6,28]. Importantly, since the quantity of metabolic resources available for reproduction is largely determined by variation in nutrient intake [29], energetic limitations may mediate individual resource allocation trade-offs between ejaculate production and other functions or traits [30–33]. Not surprisingly, trade-offs are most likely to appear under constrained resource availability (e.g., poor feeding conditions) [34–36].

Insects are a particularly valuable taxonomic group for advancing our understanding of the costs of ejaculate production [37–40]. Here, we use the common two-spotted field cricket, *Gryllus bimaculatus,* to investigate the costs of ejaculate production on male investment into soma maintenance, future reproduction, and survival and ask whether such costs are mediated by variation in food availability. Ejaculates of field crickets are packed into discrete spermatophores (i.e., protein capsules filled with sperm and accessory fluids), which are transferred to females upon genital coupling during copulation [41,42]. Spermatophores are located in a pouch on the tip of the male's abdomen and can be easily sampled from males without major disruptions and, most importantly, without the need for a mating interaction with a female [43]. This allows disentangling ejaculate production from the mating event. In our study, we experimentally increased rates of spermatophore production by repeatedly removing spermatophores from males—while keeping an unmanipulated control group—from individuals that were reared under either high or low feeding regimes as adults. We then measured (i) changes in male body mass, the likelihood of producing a spermatophore and its quality (i.e., sperm viability), and longevity and lifetime survival in males, in order to understand whether increased spermatophore production trades against investment in somatic maintenance, future fer- tilization, and lifespan, respectively. Sperm viability, defined as the proportion of live cells within an ejaculate, is a well-justified metric for ejaculate quality [44] and is the main predictor for the outcome of competitive fertilizations in insects [45,46]. Sperm viability is also known to co-vary with the intensity of post-mating selection, with polyandrous species possessing higher sperm viability than their monogamous relatives (see [47] for in- sects, [48] for mammals). This suggests that under an intense sperm demand due to sperm competition or enhanced mating rates to ensure female sperm supply, males may evolve mechanisms to preserve sperm viability, for example, through higher resistance to stressors that could impact sperm integrity and function [49]. This may apply to our study species, known to be polygynandrous [50]. On the other hand, increased metabolism needed to fulfil an intensified ejaculate demand could reduce the efficiency of sperm production and maturation (spermatogenesis), resulting in a higher occurrence of cell defects, as reported for DNA sperm damage in rodent species with the highest levels of sperm competition [51]. Sperm viability may therefore decrease significantly across consecutive mating events, resulting in a decline in male fertility (e.g., the cockroach *Nauphoeta cinerea* [52]). Food restrictions may further exacerbate the negative effects of enhanced ejaculate production on sperm viability as energetic restrictions may reduce the range of seminal fluid proteins synthesized [53].

By applying an experimental treatment that increases rates of spermatophore production, we hypothesized intensified ejaculate production to carry reproductive and survival costs for males. We expected these costs to be revealed in experimentally manipulated males through (i) a drop in male body mass, (ii) a lower likelihood of spermatophore production and lower sperm viability within the spermatophore, and (iii) reduced longevity and survival probabilities. These costs should be more pronounced in energetically constrained males from the low feeding treatment. We found, instead, that restricted feeding conditions, but not intensified spermatophore production, imposed reproductive and survival costs on males.

2. Materials and Methods

2.1. Animal Rearing

Crickets of the species *Gryllus bimaculatus* used in our study were part of a large, outbred laboratory population, originated from wild-caught animals (approximately 200) collected in Tuscany (Italy) during summer 2018. Crickets were kept in multiple tanks (20 × 37 × 30 cm) in a climate room at a constant humidity (65%) and temperature (28 °C), with a 14:10 h light/dark cycle at the Ludwig Maximilians University of Munich (Germany). Each tank hosted approximately 30–40 crickets and was equipped with an egg carton to pro- vide shelter and ad libitum access to dry cat food (Ja! Knusper-Mix Rind & Gemüse), fish flakes (sera® Pond flakes), and water (using water vials with cotton stoppers). Tanks were kept at equal sex ratios and provided with small cups (diameter × height: 7 × 4.5 cm^3) with moist soil for females to lay their eggs upon reaching adulthood. The offspring were raised communally as described above. After three generations, randomly chosen females were mated either monogamously (one female mated to one male for 3 times; *n* = 120) or polyandrously (one female mated to three consecutive males, *n* = 80) as part of a separate experiment. Animals were paired inside open arenas (15 × 15 × 6 cm^3), and mating events were observed. Once mated, females were placed in individual tanks (30 × 18 × 20 cm^3) provided with two successive oviposition cups (one per week) that were collected after one week, and eggs were allowed to hatch. Nymphs were raised com- munally within their mating treatment background (monogamous and polyandrous), and at their penultimate nymph stages, randomly chosen males were isolated into containers (10 × 10 × 9 cm^3) equipped with food, shelter, and water. They were checked daily for emergence to adulthood.

2.2. Experimental Treatments

At their final molt, animals were randomly allocated to different life-lasting food treatments. Food treatments consisted of males with high food availability (high-fed, HF, *n* = 54) receiving 0.015 g of fish flakes, and with low food availability (low-fed, LF, *n* = 54) receiving 0.003 g of fish flakes, every three days. Such feeding regimes were chosen based on their significant effect on the male body condition reported in the field cricket *Gryllus campestris* [54]. Twelve days after the start of the feeding regimes, animals from each food treatment were then further randomly assigned to each one of the two treatments: for 7 consecutive days, spermatophores were either experimentally removed from males twice per day (sprmt-removal) to enhance spermatophore production, or spermatophores were not removed (sprmt-control). This resulted in 4 different experimental groups, LF sprmt-control (*n* = 30), LF sprmt-removal (*n* = 24), HF sprmt-control (*n* = 27), and HF sprmt-removal (*n* = 27). Removal of spermatophores was conducted by gently pressing on the male genital opening and collecting the spermatophore with soft forceps. The release of a spermatophore generally triggers the production of a new spermatophore [55]. Following spermatophore discharge, a refractory period—in which *G. bimaculatus* males start to manufacture a new spermatophore—is known to occur, and in the presence of a female, such process may start after 5 min [56]. Studies have shown that it takes 70 min for complete formation of the spermatophore [41]. We conducted a supplementary study to assess the likelihood and timing of formation of a replacement spermatophore following experimental removal and show that in the absence of a female, approximately 50% of males produce a fully formed spermatophore within 120 min from removal (Appendix A). Hence, we removed spermatophores once in the morning and once in the afternoon with a 4-h interval to allow males ample time to replace the collected spermatophore. When removing spermatophores, given that not all males possessed one upon inspection, we recorded the daily number of harvested spermatophores across LF and HF males. During the 7 days of experimental spermatophore removal, the average daily number of spermatophores removed from each male (range 0–2) did not differ significantly between HF and LF males (mean ± SE, HF 1.02 ± 0.05, n = 27; LF 1.01 ± 0.06, n = 24; two-sample t-test: t = −0.49, df = 50, p = 0.63).

The control group was not handled intentionally to avoid any form of stress that could potentially lead to autonomous spermatophore extrusion. Spermatophore auto-expulsion, where males discard a spermatophore autonomously in the absence of mating (also known as "spontaneous cycle renewal"), is common among *G. bimaculatus* [42,57] and crickets in general, occurring at rates lower than the experimental treatment imposed in our study (i.e., in the absence of a female, 81–84% males produce one spermatophore per day in *Teleogryllus commodus* [58] and 87.5% once every 2.6 days for *Acheta domesticus* [59]). To validate these assumptions, we investigated auto-extrusion in a small number of *G. bimaculatus* males (n = 10) from our laboratory population. We inspected the genital opening of two-week- old adult males (raised as described above and isolated individually upon emergence to adulthood), and when a spermatophore was present, it was marked using either acrylic paint (IDENA) or a black permanent marker (edding® 3000). We then inspected these males on the following day to assess whether males retained the marked spermatophore or not in their pouch for 24 h. We found that 3 males did not retain the marked spermatophore (1 male produced a new one, and 2 did not have a spermatophore), suggesting low rates of auto-expulsion in the absence of a female or mating event.

2.3. *Body Mass Measures*

Male body mass was measured using a KERN PKT (KERN & SOHN GmbH, Balingen, Germany) digital scale at three time points: (i) at adulthood before animals were randomly allocated to different life-lasting food treatments (measure 1), (ii) twelve days from the start of the feeding regimes before being randomly assigned to one of the two spermatophore removal treatments (measure 2), and (iii) at the end of the 7 days of spermatophore removal treatments (measure 3).

2.4. *Spermatophore Production and Sperm Viability*

On the day following the end of the spermatophore removal treatments, all individuals, from both treatments, were inspected for spermatophore production, and spermatophores were sampled for sperm viability assays following established procedures [60,61]. Spermatophore age was standardized by removing the spermatophore from all males on the day before the assay. Spermatophores were removed and placed into a 0.5 mL Eppendorf tube with Beadle saline (200 µL) for 10 min to allow sperm to exit the protein capsule. The naturally occurring evacuation tube was also removed to ease release of semen. A total of 5 µL of the sperm–saline solution was pipetted onto a microscopy glass slide and stained with the LIVE/DEAD® sperm viability kit (Invitrogen, Molecular Probes Inc, Eugene, OR, USA). We used 5 µL SYBR (1:50) and 2 µL of propidium iodide (PI), incubating the sample for 5 min in darkness after each addition. A cover slip was added, and the solution was then viewed under a fluorescent microscope (Olympus BX61; Olympus, Tokyo, Japan) with live sperm displaying as green (due to SYBR) and dead sperm as red (due to PI). Live and dead cells were counted in a total amount of 300 cells. On five occasions, we did not reach a total count of 300 cells. We excluded 4 data points (1 LF sprmt-control, 1 LF sprmt-removal, and 2 HF sprmt-control) as the number of total cells present in the sample ranged between 14 and 62, suggesting potential methodological issues in sample collection.

2.5. *Longevity and Lifetime Survival*

At the end of the sperm assay, males were returned to their individual housing boxes and inspected every 3 days to score mortality, until no surviving males remained. Mortality rates were also noted during the food and spermatophore removal treatments.

2.6. Statistical Analysis

All analyses were conducted using R version 4.0.1 [62].

Body mass. A t-test was used to test differences in male body mass measured before random allocation to the high and low feeding regimes (measure 1) to ensure lack of initial bias. To analyze whether our experimental procedure (food and spermatophore removal treatments) affected the change in male body mass (difference between the body mass before and after the spermatophore removal treatment, i.e., measure 2–measure 3), we ran a generalized linear mixed model (GLMM) using food treatment (HF and LF), spermatophore removal treatment (sprmt-control and sprmt-removal), and their interaction, as well as the measure (before and after spermatophore removal), as fixed factors in the model. To account for repeated measures, an individual male ID was included as a random effect. F and *p*-values were obtained using the univariate Anova function (car package). Here, and below, we also included the mating background of the animals' mothers (offspring of females mated polyandrously or monogamously) as a factor in the model to account for potential biases resulting from this approach (Table S1). If the term was nonsignificant, we removed it and compared the simplified model using the Akaike information criterion (AIC) [63] (Table S2).

Spermatophore production and sperm viability. To test the effects of food treatment (HF and LF), experimental spermatophore removal (sprmt-control and sprmt-removal), and their interaction on the likelihood of producing a spermatophore at the end of the experimental manipulation (proportion of males with a spermatophore), we ran a GLM using a binomial distribution (GLM-b) and a logit link function. We analyzed the proportion of live cells (number of live cells out of the total number of cells counted) in the spermatophore, using the same model structure but including an individual male ID as a random effect to account for overdispersion (therefore running a GLMM) [64].

Longevity and lifetime survival. We ran two GLMs to test the effects of food treatment (HF and LF), experimental spermatophore removal (sprmt-control and sprmt-removal), and their interaction on (i) the proportion of males that survived the experimental treatments (GLM-b), and (ii) male lifespan (i.e., the total number of days an individual survived, log-transformed). To analyze if lifetime survival probabilities were affected by our experimental treatments, we carried out a Kaplan–Meier survival analysis to create survival curves and tested significance using a multivariate cox regression analysis on our lifetime data using the *survminer* package. Time (in days) was defined as the response variable with food treatment (HF and LF), spermatophore manipulation (sprmt-control and sprmt-removal), and their interaction as the independent variables. This is a non-parametric test to estimate the probability of survival at any given time interval in the data. Mating background was included in all models.

3. Results

3.1. Male Body Mass

Males allocated to the two food treatments (HF and LF) did not differ in their mean body mass prior to the start of the feeding regimes (measure 1, t-test, t = 0.75, df = 106, $p = 0.46$; Figure 1). Hereafter, the changes in sample size are driven by male mortality (see below) and two missing data points (LF sprmt-control). After 12 days of differential feeding regimes, males from the HF treatment had a significantly higher body mass than those from the LF treatment (measure 2, Figure 1; Table 1). Once the spermatophore removal treatment started, male body mass generally decreased with time and was significantly affected by the feeding treatment, with HF males possessing a higher body mass than LF males, but not by the spermatophore removal treatment, nor their interaction (Figure 1; Table 1). Estimated effect sizes and 95% CIs around the mean of predictors are reported in Table S3.

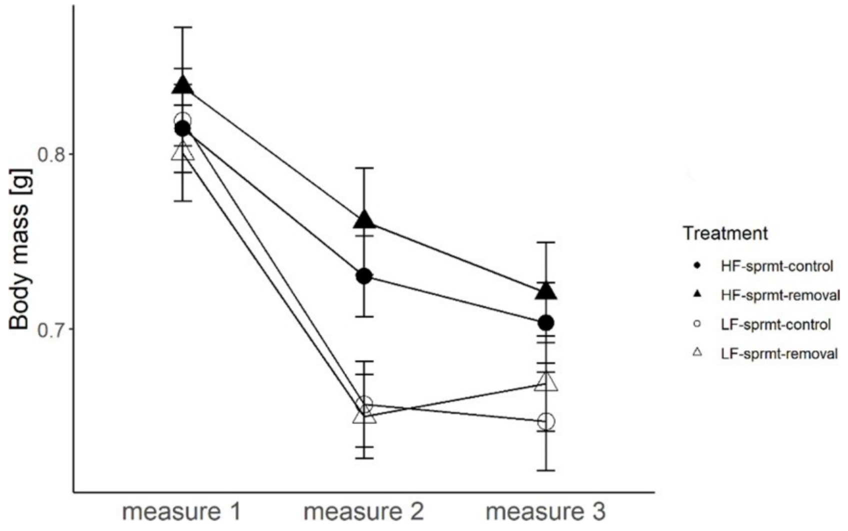

Figure 1. Change in body mass of males exposed to high and low feeding treatments (HF and LF), with and without experimental spermatophore removal (respectively, sprmt-removal and sprmt-control) measured at three time points (measure 1, before the start of the experimental food treatments, measure 2 before the start of the spermatophore removal treatment, measure 3 at the end of the spermatophore removal treatment) before and after the spermatophore removal treatment.

Table 1. Results of statistical models (GLMs unless specified) showing the effect of food treatment (high-fed and low-fed), experimental spermatophore removal (removal and control), their interaction, and the time points for body mass measures (measures 2 and 3) on male responses indicating investment in (i) soma maintenance (change in body mass), (ii) future reproduction (spermatophore production and sperm viability), and (iii) survival (% males surviving at the end of the experimental treatment) and longevity (number of days alive). Significant effects are shown in italics.

Response variable		**Effect (Wald X^2 or F; df; P)**			
	N	**Food Treatment**	**Spermatophore Removal treatment**	**Food x Spermatophore Treatment**	**Timepoint of Measure**
Body mass[1]	188				*41.53; 1; <.0001*
Spermatophore production (% males)[2]	84	*20.6; 1; <.0001*	0.72; 1; 0.4	1.12; 1; 0.29	
Sperm viability (% live sperm)[1,2]	73	3.97; 1; 0.046	0.08; 1; 0.77	*3.53; 1; 0.06*	
Survival post-spermatophore removal (% males)[2]	109	*3.99; 1; 0.046*	3.87; 1; 0.049	0.0; 1; 1	
Longevity (N days alive)	104	*104.1; 1; <.0001*	0.0001; 1; 0.99	3.68; 1; 0.06	

[1] GLMM. [2] binomial.

3.2. Spermatophore Production and Sperm Viability

The likelihood that a male produced a spermatophore at the end of the experimental treatments was affected by the food treatment, with a higher proportion of LF males having a spermatophore compared to HF males (Figure 2; Table 1).

Sperm viability was higher in HF males compared to LF males and was not affected by the spermatophore removal treatment (Figure 3; Table 1).

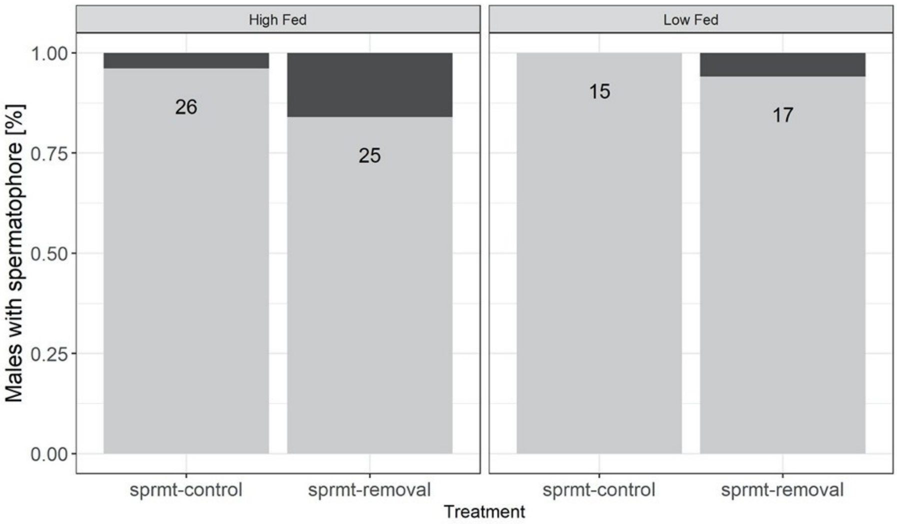

Figure 2. Proportion of males possessing a spermatophore at the end of the experimental treatment. Males were exposed to high and low feeding treatments (HF and LF), with and without experimental spermatophore removal (respectively, sprmt-removal and sprmt-control). Numbers inside bars are total sample sizes.

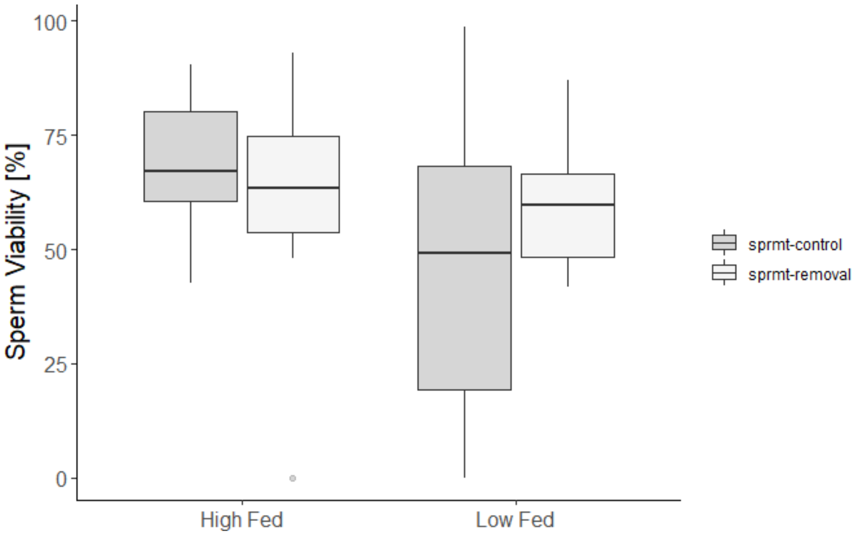

Figure 3. Proportion of live cells in the ejaculate of males exposed to high and low feeding treatments (high-fed and low-fed), with and without experimental spermatophore removal (respectively, sprmt-control and sprmt-removal).

3.3. Longevity and Lifetime Survival

After 12 days of differential feeding regimes, two males from the LF treatment died. The proportion of males that survived the 7-day spermatophore removal treatment was significantly affected by the feeding treatment (94.4% HF and 59.3% LF) and by the spermatophore removal treatment (sprmt-control 77.4% and sprmt-removal 80.8%) (Figure 4; Table 1).

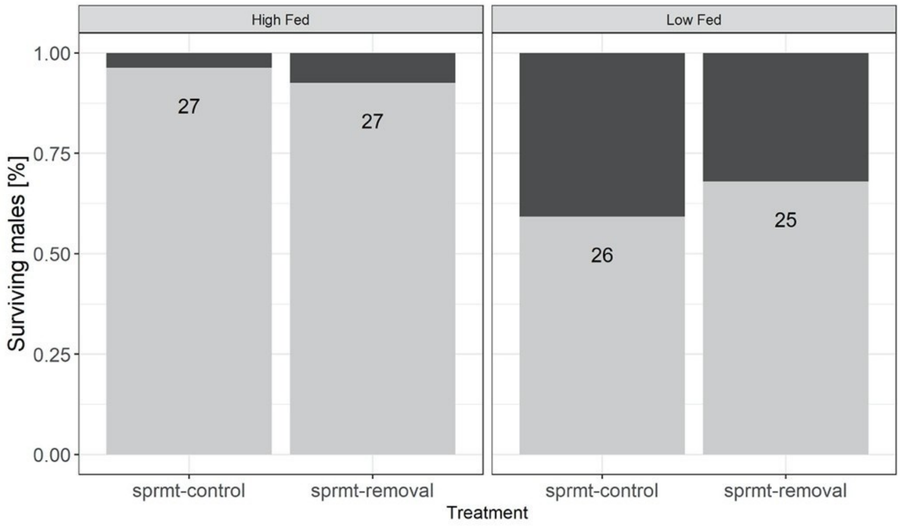

Figure 4. Proportion of males surviving the spermatophore removal treatment exposed to high and low feeding treatments (high-fed and low-fed), with and without experimental spermatophore removal (respectively, sprmt-control and sprmt-removal). Numbers inside bars are total sample sizes.

Survival probabilities were significantly higher for HF males compared to LF males (HR = 9.77, df = 1, $p < 0.0001$), but did not differ between males with and without spermatophore removal (HR = 1.26, df = 1, $p = 0.440$), or in the interaction between food treatment and spermatophore removal treatment (HR = 0.59, df = 1, $p = 0.203$) (Figure 5).

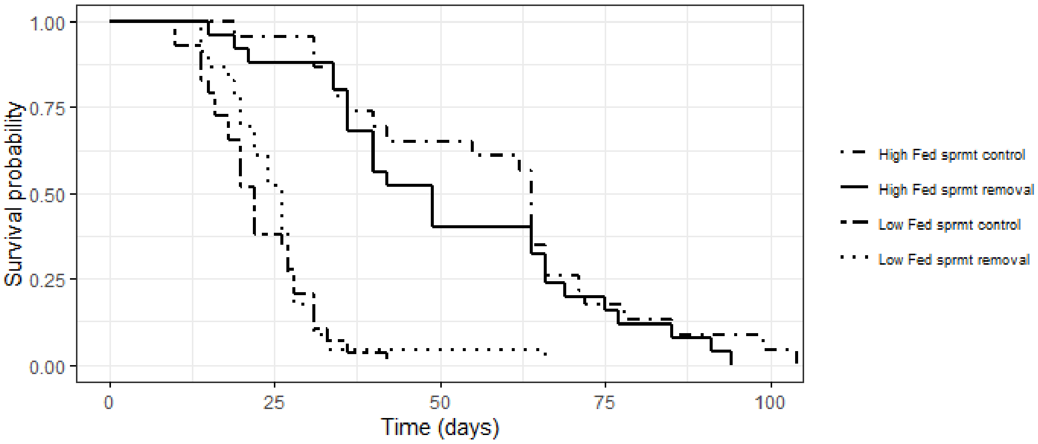

Figure 5. Lifetime survival probabilities of males exposed to high and low feeding treatments (high-fed and low-fed), with and without experimental spermatophore removal (respectively, sprmt-removal and sprmt-control).

Overall, we found a significant effect of the food treatment on male longevity (mean number of days alive (±) SE; HF 53.92 ± 3.16, $n = 50$; LF 24.12 ± 1.23, $n = 54$), while there was no effect of spermatophore removal (mean number of days alive (±) SE; sprmt-control 37.96 ± 3.21, $n = 54$; sprmt-removal 38.98 ± 3.02, $n = 50$) (Table 1).

4. Discussion

Organisms must allocate limited resources among competing life history functions and traits. When the reproductive effort increases, individuals' feeding state may influence how the allocation of resources is partitioned [29], with allocation trade-offs being more pronounced in low-condition individuals. Despite the hypothesized costs associated with ejaculate production, our study did not unveil a direct physical trade-off between the energy allocated to enhanced rates of spermatophore production and that which is allocated to other organismal functions, such as somatic maintenance, future fertilization, and survival.

This is in net contrast to studies reporting current vs. future reproductive trade-offs and/or reproductive vs. survival trade-offs in males [3,16,65,66].

We instead found that restricted feeding conditions imposed the highest costs on male crickets, leading to reduced body mass, lower viability of sperm, and impaired lifetime survival. The negative effects of low feeding regimes are not surprising, as resource availability plays a central role in individuals' investment in life history traits, such as growth, survival, and reproduction [18,19,33]. The feeding regimes applied led to divergence in male body mass, with low-fed males losing more weight, especially in the first 12 days of differential food treatment. High-fed males had higher survival probabilities and lived twice as long as low-fed males. Interestingly, our results show condition-dependent differences in sperm viability, as the proportion of live sperm encapsulated in the spermatophore was, although not strongly, positively affected by access to food. The negative nutrient-dependent effects uncovered in our study suggest that dietary restrictions may strongly limit male mate acquisition and competitive fertilizations. Female crickets are indeed known to select mating partners based on variation in song parameters (i.e., higher chirp rates) which depend on male nutrient intake [67–69], and to prefer larger males [70], which are also more successful at defeating other males during aggressive agonistic interactions over the control of breeding territories [71]. Limited access to food may, however, also compromise male fertilization success when competing against ejaculates of rivals by enhancing sperm mortality [45]. Whereas it is well established that males acquiring more energetic resources are better at investing in costly secondary traits such as weapons (e.g., antlers, horns) or ornaments (e.g., long and colorful plumages) [72,73], condition dependence of ejaculates has been long debated. There is, in fact, contrasting evidence from empirical studies showing either positive [4,74,75], negative [76], or no [77] dietary effects on ejaculate traits. A recent meta-analysis showed that, despite the fact that the condition dependence of ejaculate traits is taxonomically widespread, traits differ in their response, with seminal fluids being strongly condition-dependent, while sperm traits are only moderately (i.e., sperm numbers) and less consistently reduced (i.e., sperm length, movement, viability) under nutrient limitation [53]. Studies on insects show consensus on the small or lack of effects of food availability and diet on sperm viability when testing for pollen restriction in the honey bee [77], protein restriction in male ants [78], protein and carbohydrate intake in the cockroach [79], poor nutrition in the leaf-footed cactus bug [80], or diet type in the milkweed bug [81]. Studies on field crickets have, instead, been suggestive of positive effects of body resources on sperm viability by reporting higher proportions of live sperm in the ejaculate for heavier males (*Gryllus bimaculatus* [60]). Apart from our study which establishes a causal relationship through experimental manipulation of food availability, manipulation of macro- and micronutrients is also known to affect trait expression in the species *Teleogryllus oceanicus* [82], with males producing more viable sperm under higher consumption of micronutrients, but the lowest amounts on high-protein diets.

A possible explanation for the apparent low or negligible costs of enhanced spermatophore production reported in our study may reside in the fact that high mating rates, known to occur in natural populations of *G. bimaculatus* [50], may select for males to maximize fertilization rates through an increased capacity of spermatophore produc- tion [83]. *G. bimaculatus* males are also known to mate repeatedly throughout their adult life [84], further suggesting they can bear an elevated lifetime reproductive potential. Our findings may also point to potentially low energetic demands of multiple-spermatophore manufacturing. Interestingly, to this end, we found that despite the decreased body mass, low-fed males were able to maintain rates of daily spermatophore production similar to those of well-fed males, and, at the end of the experimental manipulation, the likelihood of low-fed males possessing a spermatophore was even higher than for high-fed males. We also show from an auxiliary study conducted without diet manipulation that investigates the timing and likelihood of spermatophore formation that male body mass does not positively correlate with the probability of producing a replacement spermatophore after discharge. This finding is also known for other cricket species, such as *Gryllus veletis* and

Gryllus pensilvanicus [85], and suggests a lack of energetic limitations for spermatophore production. Spermatophores of field crickets are relatively small [86], hardened, sack-like, sperm-containing ampullae. In *Gryllus bimaculatus*, they constitute 0.18% of the male's body weight [84]. Males can initiate spermatophore production 5 min after discharge [41] and complete its syntheses within 1 h (our study and [41]). In many other species of Orthopterans, spermatophores instead include the spermatophylax, a large gelatinous non-sperm component rich in proteins [87,88] which surrounds the ampulla, and that is eaten by the female at mating. These spermatophores may represent a large percentage of the male body mass with reports of up to 26% of the male's weight in certain bushcrickets (i.e., genus *Poecilimon* [89,90]). Despite being rare in field crickets, the spermatophylax is present in the decorated cricket *Gryllodes sigillatus*, where costs of production are supported by long refractory periods (i.e., it takes 3 h to synthesize a new spermatophore [91]) and by trade-offs between increasing rates of spermatophore production and immunity, revealed by applying a very similar manipulation to that of our study (i.e., 5 consecutive days of spermatophore removal) [38].

Considering spermatophore production, an entirely cost-free physiological process may, however, be unlikely. We show that despite the fact that nutritionally restricted males can afford to produce spermatophores at high rates, they are impaired in producing high-quality ejaculates, suggesting that, to some extent, certain aspects of ejaculate production are costly. It is plausible that sperm viability is modulated by seminal fluid proteins that serve to nourish sperm cells [6,49,92], with seminal fluid production itself being largely affected by diet [93]. We cannot exclude that a more stringent experimental treatment (i.e., higher rates of spermatophore removal) could have revealed measurable costs in our target traits and functions, or that, alternatively, trade-offs occur between functions other than those addressed in our study. For example, if males hold their investment constant by maintaining high spermatophore production rates, they may need longer refractory periods between mating events that may, overall, lead to reduced lifetime reproductive success [84]. How quickly males are able to produce sperm and replenish sperm reserves strongly affects their fertilization advantage [94], as possessing a ready-formed spermatophore would allow promptly courting females upon an encounter and, if accepted, readily transferring sperm. Males investing in spermatophore production may otherwise reduce their investment in other fundamental and costly secondary sexual traits, such as fighting [24]. Male *G. bimaculatus* that win fights against rivals are shown to produce lower-quality ejaculates (less viable sperm), suggesting a trade-off in pre-mating and post-mating competitiveness [60]. Unknowingly, sperm characteristics other than the one measured may have been impaired by our experimental treatment. The most common negative effect of repeated mating events across a wide range of species is sperm depletion [95–97]. In field crickets, the number of sperm encapsulated in the spermatophore during the second and third mating events contains 50–60% of the sperm transferred during the first mating event [98]. A decline in ejaculate mass with an increasing number of mating events is also documented in various species of seed beetles [25] and in lepidopterans, where spermatophores, following a previous mating experience, are smaller [26]. Over consecutive mating events, males may also become depleted of other important components of their ejaculates. For example, with repeated mating events, male *Drosophila melanogaster* recover their ability to manufacture and transfer seminal fluid proteins only after 3 days of sexual inactivity [99]. Male accessory glands, responsible for secretion of seminal fluids, are known to reduce in size after mating in several species [100,101]. Finally, sperm traits are also known to correlate negatively with each other [102], potentially masking the occurrence of trade-offs if only one trait is measured. Our findings may also stem from methodological differences with other studies addressing costs of reproduction for males. In order to exclusively target ejaculate production costs, we adopted a design that excludes mating. On the contrary, many of the studies reporting a decline in ejaculate quality, growth, and survival involved a female presence and/or allowed mating to take place [3,16]. These studies may not be able to fully distinguish between the effects of behavioral exhaustion derived from performing energetically demanding courtship [103] and/or those of copulation [104] from ejaculate production alone [5] in limiting the male fertilization potential. In addition, males may face strategic allocation decisions in the presence of varying mating opportunities, investing in each ejaculate in a way that maximizes their fitness return [105]. Males may, for example, partition their resource investment among multiple mating events, reducing sperm allocation per mating event [106,107], hence hindering interpretations of the exact constraints of ejaculate production.

Interestingly, crickets that were challenged by reduced access to food were able to maintain higher sperm viability under intensified spermatophore production. We here interpret these findings with caution. On the one hand, these may indicate that under harsh environmental conditions, such as nutrient restrictions, males that are exposed to enhanced reproductive effort may invest more in reproduction (e.g., keeping vital sperm cells). If an individual's perception of the increased mortality risk increases, evolutionary theory indeed predicts an increased investment in current reproduction (namely, the terminal investment hypothesis) [34,108,109]. It is, however, also possible that if males in low feeding regimes were energetically impaired in their rates of spermatophore discharge (auto-extrusion) [110], they may have spent longer periods without active sperm production. Ejaculate quality after periods of abstinence from mating is known to only increase in subsequent mating events or ejaculations [111]. This is most likely due to sperm storage mechanisms and sperm aging lowering sperm performance, as sperm stored by males before mating may incur post-meiotic sperm senescence, leading to a decline in the number of viable sperm, and sperm motility and velocity [112,113]. In our study, we aimed to experimentally control differences in sperm age by removing the spermatophore from all males on the day prior to the sperm assays. However, older sperm may remain in the male reproductive tract prior to being loaded in the newly formed spermatophore [59]. Hence, increased rates of spermatophore production may have proved beneficial in maintaining viable sperm cells.

5. Conclusions

In conclusion, dietary restrictions may strongly limit male reproductive success through profound negative effects on important physiological functions (e.g., soma maintenance, sperm production, and survival), stressing the importance of individual resource availability. Yet, investment towards ejaculate production may not necessarily occur at the expense of such functions, even when resources are scarce [29]. Our findings suggest that ejaculate traits may be less prone to trade-offs with other fitness-related life history traits while unveiling condition-dependent costs. Indeed, although male field crickets appeared to bear the costs of producing multiple spermatophores, they were impaired from nutrient deficiency in producing high-quality ejaculates. This extends our understanding of the condition dependence of ejaculate quality, as a direct relationship between energy intake and sperm viability is seldom reported in insects.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/cells10061498/s1, Table S1: Results of statistical models including male family background (polyandrous or monogamous mothers). Table S2: Akaike information criterion value (AIC) for models including or not including male family background (polyandrous or monogamous mothers). Table S3: Estimated effect sizes and 95% CIs around the mean of predictors of body mass measured at two time points (measures 2 and 3), before and after the spermatophore removal treatment, including (+) or not including (-) male family background (polyandrous or monogamous mothers). Table S4: Full dataset.

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Appendix A

A total number of 38 males were used to assess the likelihood and timing of production of a replacement spermatophore following experimental removal, in the absence of a female. Animals belonged to a large outbred population originated from wild-caught individuals collected in Tuscany, Italy, during summer 2015. They were raised in the laboratory following standardized conditions, as described above. Males were isolated individually during their penultimate instar and used 3–4 weeks after adult eclosion. On the day of the test, after measuring male body mass using a KERN PKT (KERN & SOHN GmbH, Balingen, Germany) digital scale, the male's spermatophore was experimentally removed with soft forceps. The male was then returned to its housing container, and his genital opening was inspected regularly after 10, 20, 30, 40, 50, 60, and 120 min. On the following day (24 h later), we inspected males that did not produce a spermatophore 120 min from removal. During each inspection, we noted whether males initiated spermatophore production. The process is visible, as the genital pouch at first contains white and soft material (at 10 and 20 min) to then become clear (at 30 and 40 min) and finally hardens (at 50 and 60 min) into a fully formed spermatophore. This process was observable in 47% of the males (n = 18), which possessed a fully formed spermatophore in their pouch 60 min from removal. Of the remaining 53% (n = 20) that did not initiate spermatophore production within the first 10 min, five had spermatophores after 120 min and the remaining never did on that day. However, they all possessed a spermatophore on the following day. We ran GLM binomial testing for the effect of male age and body mass on the likelihood of producing a replacement spermatophore within 120 min from removal. Interestingly, we found no effect of body mass on the likelihood to produce a replacement spermatophore, but older males were more likely to produce one (GLM-b, male age $\chi^2 = 7.21$, df = 1, $p = 0.0073$*; male body mass $\chi^2 = 0.68$, df = 1, $p = 0.41$). These results suggest an increase in reproductive investment with a decreasing reproductive value [34].

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Article

Food limitation but not enhanced rates of ejaculate production impose reproductive and survival costs to male crickets

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Supplementary Materials

Table S1. Results of statistical models (GLMs unless specified) showing the effect of food treatment (high fed and low fed), experimental spermatophore removal (removal and control), their interaction, the time points for body mass measures and male family background (polyandrous or monogamous mothers) on male responses indicating investment in i) soma maintenance (change in body mass), ii) future reproduction (spermatophore production and sperm viability) and iii) survival (% males surviving at the end of the experimental treatment) and longevity (number of days alive). Significant effects are shown in italics.

Response variable		**Effect (Wald X^2 or F; df; P)**				
	N	**Food treatment**	**Spermatophore removal treatment**	**Food x Spermt**	**Family background**	**Measure (1 and 2)**
Body mass[1]	188	*19.89; 1; <.0001*	0.002; 1; 0.96	0.15; 1; 0.7	*10.74; 1; 0.001*	*40.91; 1; <.0001*
Spermatophore production (% males)[2]	84	*20.52; 1; <.0001*	0.65; 1; 0.42	0.99; 1; 0.32	0.26; 1; 0.61	
Sperm viability (% live sperm)[1,2]	73	3.68; 1; 0.055	0.09; 1; 0.76	*4.23; 1; 0.04*	1.81; 1; 0.18	
Survival (% males)[1]	109	*4.14; 1; 0.042*	4.0; 1; 0.045	0.0; 1; 1	0.24; 1; 0.63	
Longevity (N days alive)	104	*103.1; 1; <.0001*	0.0001; 1; 0.99	0.0007; 1; 0.98	3.6; 1; 0.06	

[1] GLMM

[2] binomial

Table S2. Akaike Information Criterion value (AIC) for models including (+) or not (-) male family background (polyandrous or monogamous mothers).

Models	**+ Family background**	**- Family background**
Body mass[1]	-397.4	-389.2
Spermatophore production (% males)[2]	104.1	102.3
Sperm viability (% live sperm)[1,2]	834.3	834.1
Survival (% males)[1]	28.8	27.1
Longevity (N days alive)	110.3	108.3

[1] GLMM

[2] binomial

Table S3. Estimated effect sizes and 95% CI around the mean of predictors of body mass measured at two time points (1 and 2), respectively before and after the spermatophore removal treatment, including (+) or not (-) male family background (polyandrous or monogamous mothers).

Fixed effects	**+ Family background β (95% CI)**	**- Family background β (95% CI)**
Intercept	0.71 (0.68, 0.75)	0.75 (0.71, 0.78)
Food Treatment (LF)	-0.09 (-0.13, -0.05)	-0.09 (-0.13, -0.05)
Spermatophore removal treatment (removal)	-0.006 (-0.05, 0.03)	-0.0005 (-0.04, 0.04)
Measure (Time point 2)	-0.04 (-0.044, -0.026	-0.04 (-0.04, -0.03)
Food LF x Spermt removal	0.015 (-0.05, 0.08)	0.0003 (-0.07, 0.06)
Mating background (PP)	0.079 (0.04, 0.12)	
Random effects	**σ^2 (95% CI)**	**σ^2 (95% CI)**
Individual	0.014 (0.013, 0.016)	0.016 (0.015, 0.017)
Residual	0.001 (0.0011, 0.0015)	0.001 (0.0011, 0.0015)

General Discussion

Within the last 50 years behavioural ecologists around the world came a long way to expand our understanding on processes involved in sexual reproduction, specifically those that happen after mating. Only very recently, scientists have incorporated post-copulatory selection into the formal definition of sexual selection (Shuker & Kvarnemo, 2021), acknowledging that both processes of pre- and post-copulatory selection importantly contribute to evolutionary change. To further extend our knowledge, studies are increasingly investigating the mechanisms behind pre- and post-copulatory selection by addressing these together, instead of focusing only on one or the other (Cattelan et al., 2020; Evans & Garcia-Gonzalez, 2016; Evans et al., 2015; Filice & Dukas, 2019; Fisher et al., 2016; Gasparini et al., 2019; McDonald et al., 2017; Travers et al., 2016). Thanks to these studies we are slowly gaining insight into the relative importance of both episodes of selection and their action on trait evolution. This will further aid us in understanding the complex interplay between sexual and natural selection, hence reproduction and fitness (Cally et al., 2019; Fisher et al., 2006; Marie-Orleach et al., 2021; Power & Holman, 2014).

There are still some challenges, which make studying the relationship between pre- and post-copulatory sexual selection difficult and prevent us from formulating a general theoretical framework. First, sexual selection operates in fast changing environments, either ecologically or socially. Ecological variation in resource quality or availability may influence patterns of pre- and post-copulatory sexual selection on both sexes, as seen for example in guppies, where the expression of male pre- and post-copulatory traits is affected by diet manipulation (Devigili et al., 2013; Rahman et al., 2013, 2014), as well as in katydids, where sex-role reversal can arise when food becomes scarce (Gwynne & Simmons, 1990). Another crucial aspect affecting the strength of each episode of selection is the social environment, as for example stated in a systematic study showing that the varying intensity of male-male competition between closely related taxa can impact the covariance between pre- and post-copulatory sexual traits from strongly negative to positive (Lüpold et al., 2014). Also, social experience can effect pre- and post-copulatory mate choice, as female field crickets adjust latency to mating and spermatophore retention time according to previous experience with (un)attractive males (*Teleogryllus oceanicus*, Rebar et al., 2011). Next to environmental factors, we need to be aware of potential methodological flaws affecting our measures of sexual selection. In the case of variance-decomposition (Arnold & Wade, 1984a, b), the problem lies within the fact that the fitness components chosen to characterize each episode (pre or post) might be species specific. Thus, components assumed to be attributable exclusively to post-copulatory selection in one species, might in fact be attributable to pre-copulatory selection in another (Evans & Garcia-

Gonzalez, 2016; Marie-Orleach et al., 2016). Mating order, for example, would generally be attributed to pre-copulatory selection, as most mechanisms determining the order in which males mate with a female happen pre-copulatory. Yet, in the fruit fly *Drosophila melanogaster* 80% of the offspring can be allocated to the last male to mate. This last male sperm precedence might be due to males preventing females from remating by transferring seminal proteins that reduce female receptivity (Perry et al., 2013; Sirot et al., 2011). As a results, the strength of one episode of selection (in this example, pre-copulatory selection) might be overestimated, in case it is exclusively based on variance estimates of one fitness component (paternity shares) (Evans & Garcia-Gonzalez, 2016). Failure in accounting for the relative strength of pre- and post-copulatory selection could further lead to a miscalculation of their combined force, if, for example, post-copulatory processes are masking the effects of mating success on reproductive success (Devost & Turgeon, 2016). By focussing on pre- and post-copulatory processes in a combined experimental setup as well as taking into account the ecological and social environment, we might be able to successfully estimate the total opportunity for sexual selection (Evans & Garcia-Gonzalez, 2016).

In an effort to address all the above-mentioned aspects, my dissertation contributes an entirely novel approach. By experimentally disentangling pre- and post-copulatory sexual selection I provide a framework to test for the effects of each selective episode on the evolution of reproductive traits as well as their effects on individual fitness. By applying this framework, I first show that sexual selection, no matter if occurring before or after mating, in general increases offspring quality but imposes survival costs to females compared to those with reduced opportunity for selection. I further show that reproductive traits diverge after just few generations of either intense pre-copulatory, intense post-copulatory or weakened sexual selection, namely that pre-copulatory selection elicits positive responses in pre-copulatory traits, whereas post-copulatory selection promotes reduced post-copulatory, as well as pre-copulatory trait values. Such specific trait responses could suggest a potential positive correlation, rather than genetic trade-off between pre- and post-copulatory traits. Lastly, we show that when kept under increased ejaculate production rates, there is no trade-off between ejaculate production and life history traits, but instead food limitation leads to lower sperm quality, impaired somatic maintenance, as well as decreased survival.

In brief, the answers to my research aims are:

1) **How disentangled pre-and post-copulatory sexual selection contribute to fitness**.

   I found no differences in fitness of females (or their offspring) that experienced pre- and post-copulatory sexual selection. Yet, this study highlights the potential of sexual selection enhancing indirect female fitness: those experiencing pre- and post-copulatory sexual selection were more likely to reproduce, their offspring hatched sooner, developed faster and had higher body mass at adulthood, compared to those mated with weakened selection. These benefits occurred together with direct survival costs to females. By potentially outweighing these costs, increased offspring quality could lead to beneficial population-level consequences of sexual selection. (Chapter 1; Matzke et al., 2023, Journal of Evolutionary Biology).

2) **Whether there is correlated evolution and/or evolutionary trade-offs between multiple reproductive traits in response to pre- and post-copulatory sexual selection.**

   I show that trait divergence occurred within the evolutionary timeframe of six generations, with males under intense pre-copulatory selection evolving higher body mass, while those under intense post-copulatory selection evolving lower calling and courtship song values, as well as testes mass. Overall, pre-copulatory traits responded positively to pre-copulatory selection, while post-copulatory traits do so negatively to post-copulatory selection. This potentially indicates that polyandrous mating might weaken the strength of selection, hence when males evolved under post-copulatory selection. Our findings thus suggest an underlying positive correlation between some pre- and post-copulatory traits, instead of allocation trade-off. (Chapter 2; Matzke et al., Manuscript unpublished).

3) **The costs of sperm production: are these really negligible?**

   I found no evidence that investment in ejaculates impinges upon male somatic maintenance, future reproduction, or longevity. Instead, our study revealed only negative effects of food limitation, suggesting low or undetectable costs of spermatophore production. Nevertheless, the fact that males were impaired due to nutrient deficiency in producing viable ejaculates, suggests condition-dependent costs for ejaculate production. High mating rates may select for males to maximise their capacity of ejaculate production, making ejaculate traits less prone to trade-offs with other fitness-related life history traits. (Chapter 3; McMahon et al., 2021, CELLS).

How disentangled pre-and post-copulatory sexual selection contribute to fitness.

Natural selection favours traits which increase survival and fecundity, while sexual selection favours traits increasing mating and/or fertilisation success (Rowe & Rundle, 2021). Hence, natural and sexual selection can potentially operate in opposition, with trade-offs occurring between longevity and reproductive rates (Reznick, 1992; Roff, 2002; Stearns, 1992; Williams, 1966). This was first addressed by Darwin, when he noticed that some exaggerated dimorphic traits appeared to be counter to adaptation by natural selection (Darwin, 1859, 1871). Since then, the question whether sexual selection enhances fitness or not has fueled an active field of research. Studies have both reported positive effects, such as increased population growth rates (Fox et al., 2019) and accelerated rates of adaptation to novel environments (Gómez-Llano et al., 2020; Parrett et al., 2019; Parrett & Knell, 2018; Servedio & Boughman, 2017), as well as negative effects, suggesting higher extinction rates due to costly sexual traits or reduction of effective population size and genetic drift (Kokko & Brooks, 2003; Kokko & Jennions, 2008; Whitlock, 2000).

None of the studies mentioned above attempted distinguishing between the effects of pre- and post-copulatory selection on reproductive output, highlighting a knowledge gap in our understanding of how each episode of selection contributes to fitness. With my first chapter I filled this gap by estimating fitness of females mated with exclusive pre-copulatory, exclusive post-copulatory or weakened sexual selection. The implementation of such original design required also manipulating the mating system, with the pre-copulatory and the weakened sexual selection treatment being mated monogamously, while the post-copulatory treatment was mated polyandrously. Our expectations were that direct and indirect fitness benefits should be highest in mating scenarios where sexual selection was present, but without specific expectation on which selection treatment should yield higher fitness outcomes. However, considering that our model species naturally engages into polyandrous mating behaviour (Simmons, 1986a), we did expect higher fitness benefits in the mating treatment, where polyandrous mating was enforced (Arnqvist & Nilsson, 2000; Tregenza & Wedell, 1998), hence the treatment with enabled post-copulatory sexual selection. Our results show that sexual selection, regardless of being pre- or post-copulatory enhances female fitness, as females are more likely to reproduce, their offspring hatched sooner in time, developed faster, and had higher body mass at maturation. These results can be explained by maternal resource allocation, when females invest more in their offspring if mated to preferred males ('differential-allocation hypothesis', Burley, 1986, 1988). Although the original idea of differential allocation can be applied to mate choice of either sex, meaning that either males or females can adjust their investment based on mate attractiveness (Sheldon, 2000), most studies focus on maternal investment and male attractiveness (Ratikainen &

Kokko, 2010). For example, in the variable field cricket *Gryllus lineaticeps*, females mated to males producing preferred song types are shown to live longer and produce more eggs, which have higher hatching success (Wagner & Harper, 2003). Also in *Gryllus bimaculatus*, offspring from females allowed to choose their mate develop faster and have higher survival (Simmons, 1987a), which indicates increased maternal investment when exposed to sexual choice. Yet, given the positive genetic correlations between multiple reproductive traits (Tuni et al., 2018), it could be that females in the pre- and post-copulatory selection treatment were selecting for the same overall male phenotype, as aggressiveness could only be assessed in a direct fighting contest between males (pre-copulatory selection treatment), but body mass could also be assessed when mating with males consecutively. Thus, this would lead to females in both sexual selection treatments preferentially choosing the most aggressive males, which, in our case, are also the largest ones (i.e., with highest body mass) (Tuni et al., 2018).

Nevertheless, sexual selection also entailed fitness costs, since females mated under pre- and post-copulatory selection had reduced lifetime survival compared to females mated under weakened sexual selection. This suggests that sexual conflict might be at play, reducing female lifetime reproductive success, while positively affecting offspring fitness in terms of faster and higher quality development. Although being able to exert mate choice for preferred males, the costs for females under pre- and post-copulatory selection due to vigorous courting of males (Bateman et al., 2006; Bateman, 2000) could have been more severe compared to females under relaxed selection. Females mated with pre-copulatory choice were exposed to three males simultaneously, experiencing intense male fighting, with some of the aggressive behaviour being directed to females themselves (Bateman et al., 2006; Vedenina & Shestakov, 2018). Males mated with post-copulatory choice instead were exposed to multiple mating partners sequentially, increasing the risk of disease transfer (Simmons, 2005; Thrall et al., 2000) or transfer of harmful chemicals from multiple ejaculates (Chapman et al., 1995).

Overall, this study suggests that sexual selection entails fitness benefits, aligning with current literature that fitness values are often higher in populations evolving under sexual selection, relative to populations where sexual selection is experimentally removed or weakened (Cally et al., 2019). Not only on the population level, but also on the individual level literature suggests that polyandrous matings (with sexual selection) yield higher fitness outcomes compared to monogamous, enforced matings (weakened sexual selection) (Tregenza & Wedell, 1998; Tuni, et al., 2013; Tuni & Bilde, 2010). We further uncovered that the magnitude of indirect fitness benefits to offspring development, as well as the direct costs to female survival might be comparable between pre- and post-copulatory sexual selection. Most importantly, if the increased developmental ability reported for offspring outweigh the costs of reduced female longevity, then sexual selection has the potential to contribute beneficial population-level consequences (Cally et al., 2019; Gomez-Llano et al., 2020; Parrett et al., 2019). This chapter hence highlights the relevance of indirect benefits of sexual selection in potentially maintaining population viability by providing insight into how sexual selection contributes to reproductive fitness. It thus setts the basis to explore the evolutionary consequences of such selective pressures if these are allowed to operate over multiple generations.

Trait divergence through disentangled selection

Pre- and post-copulatory sexual selection shape the evolutionary diversification of a wide range of reproductive traits. Depending on which episode of sexual selection is more relevant, males should evolve either better mate acquisition abilities (pre-copulatory traits) or better fertilisation abilities (post-copulatory traits) (Simmons et al., 2017). Yet, reproductive traits are often correlated, leading to selection acting on pre-copulatory traits potentially facilitating the evolution of post-copulatory traits (and *vice versa*) if these are positively correlated (Lande & Arnold, 1983; Stearns, 1992), or impairing their evolution if the latter are negatively correlated (Engqvist, 2011; Evans, 2010). Hence, to assess any trait response to selection, we need to understand whether correlated evolution or constraints on adaptation (genetic trade-offs) are at play. This can be achieved by gaining knowledge about the relative role that pre- and post-copulatory episodes play in sexual trait evolution, as for example by measuring the evolutionary responses of key pre- and post-copulatory reproductive traits to experimentally disentangled pre- and post-copulatory selective pressures over several generations, as done in Chapter 2. We expected that pre-copulatory traits should respond positively to intense pre-copulatory selection and post-copulatory traits to post-copulatory selection. How pre-copulatory traits respond to post-copulatory selection and *vice versa,* post-copulatory traits to pre-copulatory selection, should give insight on correlated evolution and/or evolutionary trade-offs. Our results partly concur with our predictions, as we found higher estimates of overall pre-copulatory traits, and male body mass in particular, in the pre-copulatory evolution lines. These results support the idea that males should evolve better mate acquisition abilities (pre-copulatory traits), if pre-copulatory selection is intensified. Such abilities include fighting against rivals to gain access to mates (Adamo & Hoy, 1995; Simmons, 1986b; Tachon et al., 1999), as well as successfully courting females to ensure mating success (Rantala & Kortet, 2003; Simmons, 1986a; Verburgt et al., 2011). All these activities, especially fighting and singing, are known to be highly energetically demanding (Adamo & Hoy, 1995; Hack, 1998; Mowles, 2014; Simmons, 1986b), implying that being of better body condition could be beneficial for maintaining mate acquisition trait quality. This not only agrees with the fact that males in our study evolving under pre-copulatory selection had higher body mass values, it also indicates those males might have been better at outcompeting rivals in the long term, as body mass is known to be positively correlated to aggressiveness in *G. bimaculatus* (Tuni et al., 2018).

Surprisingly, our results do not support our prediction that males should evolve better fertilisation abilities (post-copulatory traits), if post-copulatory selection is intensified, as males evolving under intense post-copulatory selection did not differ in the quality of their overall post-copulatory traits from males evolving under pre-copulatory selection. However, they did evolve lower testes mass. Testes size or mass is often used as an approximation for sperm number, with sperm number being further reported to increase due to polyandrous mating (Firman et al., 2013). Yet, studies investigating testes mass have shown conflicting evidence, with testes mass either responding to varying levels of sperm competition (Hosken et al., 2001; Hosken & Ward, 2001; Pitnick, Miller, et al., 2001; Simmons & García-González, 2008) or not (Chechi et al., 2017; Crudgington et al., 2009; Firman et al., 2018; Firman & Simmons, 2010a). Since testes mass did respond to selection in our study, although unexpectedly, we interpret these findings as potential indication that post-copulatory traits evolve at a slower evolutionary rate compared to pre-copulatory traits, as previously reported on testes mass in dung beetles (Simmons & Fitzpatrick, 2016), as well as in bovids and cervids (Reuland et al., 2021). A potential reason for that might be that polyandry weakened the strength of selection in our post-copulatory evolution lines, leading to either stabilizing, null or disruptive selection, instead of only (positive) stabilizing selection (Cramer et al., 2023).

Our study importantly shows that trait divergence can occur even after few generations of experimental evolution, reinforcing the notion that sexual selection can drive the rapid evolution of sexual traits. Specifically, we provide evidence that pre-copulatory selection elicits positive responses in pre-copulatory traits, while post-copulatory selection elicits negative responses in both pre- and post-copulatory traits. The latter responses to post-copulatory selection suggest a potential underlying positive correlation between some pre- and post-copulatory traits, song and testes mass, respectively. These results stand in contrast to current literature proposing evolutionary trade-off between pre- and post-copulatory traits (Simmons et al., 2017). Hence, my second chapter gives insight into the relative role that pre- and post-copulatory selection play in reproductive trait evolution. It further emphasizes the importance of incorporating comprehensive multi-trait approaches when aiming at understanding substantial processes of sexual selection, instead of focussing on individual traits.

Costs of sperm

Ejaculates are favoured by natural selection to ensure male fertility and by post-copulatory sexual selection to maximise male siring success (Fitzpatrick & Lüpold, 2014; Simmons & Fitzpatrick, 2012). This general selection on males to greatly invest into ejaculate production, leads to them not only producing high quality sperm but also increasing rates of sperm production to enhance sperm competitiveness (Parker, 1970, 2016; Simmons & Fitzpatrick, 2012). The costs of individual gamete cell production in males have traditionally been considered low, enabling high numbers of sperm to be produced (Scharf et al., 2013). Yet, since sexual traits which increase an individual's reproductive fitness must come with a cost counterbalancing such benefit (Kotiaho, 2001), this view has been widely challenged, with studies indicating that the costs of ejaculate production in males are not trivial (Dewsbury, 1982; Godwin et al., 2017; Hayward & Gillooly, 2011). Resources and energy allocated to sperm production will hence not be allocated to, for example, soma maintenance or other functions, which may lead to a decrease in body condition or longevity (Perry & Tse, 2013; Van Voorhies, 1992).

In my last chapter we measured resource allocation of male crickets towards life history functions and ejaculate production under restricted feeding conditions. We expected that with increasing reproductive effort (high spermatophore production rates), allocation trade-offs should be more pronounced in low-condition (restricted feeding) individuals. However, we did not find a direct physical trade-off between the energy allocated to enhanced rates of spermatophore production and that which is allocated to life history functions, such as somatic maintenance, future fertilisation, or survival. Yet, we found that males undergoing limited feeding experienced the highest costs, namely reduced body mass, lower sperm viability, and impaired longevity, independently from their spermatophore production rates. Our results support the idea that resource availability not only importantly affects how individuals invest into life history traits, such as growth, survival and reproduction (Roff, 2002; Stearns, 1992; Zera & Harshman, 2001), but that dietary restriction may also impact male mate acquisition and competitive fertilisation abilities. As female crickets are known to prefer males with specific attractive song parameters, where the production of such is condition dependent (Holzer et al., 2003; Scheuber et al., 2003; Wagner & Reiser, 2000), as well as males of larger body size, limited access to food may potentially compromise both. In addition, low condition males may also be at a disadvantage when their less viable sperm competes against rivals in a normal or high condition state. However, condition dependence of ejaculates has long been under debate, with contrasting evidence either supporting positive (Godwin et al., 2017; Perry & Rowe, 2010; Rahman et al., 2013), negative (Mehlis et al., 2015), or no (Stürup et al., 2013) dietary effects on ejaculate traits. While a recent meta-analysis shows that condition dependence of ejaculates is taxonomically widespread, with seminal fluids responding more strongly to dietary restrictions than sperm traits (Macartney et al., 2019), evidence on field crickets also suggests a general positive connection between body condition and sperm viability *(Gryllus, bimaculatus*, Tuni et al., 2016).

The specific species level of natural polyandry might affect the general costs associated with spermatophore production in that species. Since *Gryllus bimaculatus* is known to have naturally high mating rates (Bretman & Tregenza, 2005), this may lead to males' ability to bear an elevated lifetime reproductive potential (Simmons, 1988). By mating repeatedly throughout their adult life, males demonstrate an increased capacity of spermatophore production (Svärd & Wiklund, 1989). Additionally, spermatophores produced by males of our study species are relatively small compared to many other species of Orthopterans (Simmons, 1988; Sturm, 2014), with spermatophore synthesis being completed within an hour (Hall et al., 2000), suggesting relatively low energetic demands of multiple-spermatophore manufacturing. Nevertheless, costs of spermatophore production do not seem to be entirely negligible. Limited food conditions lead to males only being able to produce ejaculates of impaired quality, despite keeping up a high production rate. This implicates that certain aspects of ejaculate production are costly, such as the manufacturing of seminal fluid proteins, which could serve to nourish sperm cells (Avila et al., 2011; den Boer et al., 2009; Simmons & Beveridge, 2011), or the production of seminal fluid itself, which is largely affected by diet (Kaldun & Otti, 2016). It further emphasizes the importance of diet to sperm quality, since certain diet components, such as antioxidants (vitamin E and beta-carotene), can enhance ejaculate competitiveness in male crickets (*Teleogryllus oceanicus*, Almbro et al., 2011), and even sperm motility and morphology in humans (Zareba et al., 2013).

This study provides new insight into the relationship between energy intake and sperm viability in insects. While we uncover condition-dependent costs of life history traits (soma maintenance and survival), we find that investment towards ejaculate production rates might not necessarily be connected to condition-dependent trade-offs. Instead, sperm quality might in fact be, as sperm viability was impaired when males were subjected to dietary restrictions.

A unified divide?

This dissertation contributes to our understanding on how individual reproductive phenotypes are maintained by considering the relative role that pre- and post-copulatory selection play in sexual trait evolution (Evans & Garcia-Gonzalez, 2016). By establishing mating conditions that only allow for exclusive pre-copulatory, exclusive post-copulatory or relaxed sexual selection we were able to estimate reproductive output from one generation to another, as well as trait divergence on an evolutionary time scale. Individual effects of pre- and post-copulatory sexual selection might not be as clearly distinguishable after just one generation. Nevertheless, there is a clear indication that sexual selection entails indirect fitness benefits while also inducing direct costs. When contrasting sexual choice (pre- and post-copulatory selection) against limited sexual choice (weakened selection), males evolving under weakened sexual selection evolved higher estimates in overall post-copulatory traits, compared to those evolving under pre- and post-copulatory selection. Hence, our results suggest that removing the potential for sexual choice entails not only different costs and benefits, but also elicits different evolutionary responses compared to when removing the potential for only either pre- or post-copulatory choice. As these observed changes in post-copulatory traits are quite puzzling, I can only speculate what they mean in relation to the first chapter; it appears that evolving under relaxed sexual selection reduces offspring quality, next to increasing female longevity on a short-term level, while enhancing male post-copulatory abilities, in particular sperm quality, on a long-term level. The potential negative population-level effects due to impaired offspring development would hence be outbalanced by a long-term enhancement of sperm quality, reflecting an adaptation to anomalous conditions when monogamy is enforced in a naturally polyandrous species (Simmons, 1986a). Such remarkable adaptations to enforced monogamy were also seen in *Drosophila melanogaster*, were the removal of sexual selection (and hence, male-male competition) led to males being less harmful to females (Holland & Rice, 1999). Experimentally enforced monogamy thus leads to the evolution of sexual benevolence, making the existence of sexual conflict redundant (Hosken et al., 2001). In our case, it seems that the occurring adaptations ought to maintain population survival, as otherwise negative effects on offspring development would immensely decrease population fitness over a longer period of time.

As previously mentioned, challenges that make studying the relationship between pre- and post-copulatory sexual selection difficult, remain. One of these challenges includes environmental factors, with ecological variation in resource quality or availability potentially influencing patterns of pre- and post-copulatory sexual selection (Evans & Garcia-Gonzalez, 2016). My third chapter captures this environmental component, as it shows that limited access to food can have detrimental effects on the quality of male sperm. Although food was provided in abundance for animals in the first and second chapter, this emphasizes that sexual selection acts in fast changing environments and that ecological, as well as social factors can influence patterns of sexual selection. Specifically, the social environment might have been a potential reason, why we were unable to measure trait responses of attractiveness and aggressiveness in the second chapter. The variation of trait values increases as soon as the execution of this trait is affected by a second individual, whether this individual being another male (combats) or female (courtship). This can further even have evolutionary consequences, such as indirect genetic effects, which are the effects of genes expressed in one individual on traits in another individual (Bailey et al., 2018; Santostefano et al., 2017; Wolf et al., 1998). While in some species it might be the previous experience with other males of varying attractiveness (Rebar et al., 2011), in other species it can be the sheer presence or absence of rival males (Dougherty & Shuker, 2016) affecting patterns of pre- and/or post-copulatory selection. Even by disentangling the effects of pre- and post-copulatory selection, it is nearly unfeasible to control for all possible factors, whether environmental or methodological, potentially affecting the manifestation of reproductive traits. Nevertheless, it is of immense importance that studies continue implementing both aspects of sexual selection, enabling us to gain a broad overview of as many different scenarios in which reproductive phenotypes are shaped as possible.

General Conclusion

My dissertation contributes knowledge to the study of sexual selection and its evolutionary consequences, with an original experimental approach that is seldom applied. The obtained results aid to demonstrate the complex interplay between pre- and post-copulatory sexual selection, next to unveiling costs and benefits of sexual selection episodes for the individual, as well as potential implications for population level fitness. Overall, the main conclusion of my work is that the time of studying reproductive traits and/or episodes of sexual selection individually is over (Evans & Garcia-Gonzalez, 2016; Simmons et al., 2017). In particular, I call for more studies that unravel genetic correlations and uncover the genetic architecture of multiple pre- and post-copulatory traits, as well as applying experimental manipulations to understand trade-offs and correlated evolution. By contributing to existing evidence that evolutionary trait responses are measurable within a short evolutionary time frame, I hope to encourage future researchers to enlarge the small pool of multivariate trait studies. By doing so, we will continue shedding light on the still enigmatic evolution of reproductive phenotypes.

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Author Contributions

Publication I: Pre- and post-copulatory sexual selection increase offspring quality but impose survival costs to female field crickets

CT conceived the study, CT and MM designed the study, MM and AR collected data, MM organized and analysed data, and drafted the manuscript, CT assisted in writing and editing. All authors gave final approval for publication and declare no conflicts of interest.

Manuscript I: Evolutionary divergence in key reproductive traits under manipulated pre- and post-copulatory selective pressures: from ejaculates to behaviour

CT conceived the study, CT and MM designed the study methodology, MM, MO and KÖU collected data, MM organized, analysed and visualised the data and drafted the manuscript, CT and FGG assisted in writing and editing, MK, RDMS and BH contributed parts of the analysis (GC-MS), CT was responsible for funding acquisition. All authors declare no conflict of interest.

Publication II: Food Limitation but Not Enhanced Rates of Ejaculate Production Imposes Reproductive and Survival Costs to Male Crickets

Conceptualization, C.T.; methodology, C.T.; formal analysis, S.M. and M.M.; investigation, S.M.; resources, C.T. and M.M.; data curation, S.M.; writing—original draft preparation, C.T., S.M., and M.M.; writing—review and editing, C.T.; visualization, M.M. and S.M.; supervision, C.T.; project administration, C.T.; funding acquisition, C.T. All authors have read and agreed to the published version of the manuscript.

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Co-author Statements

All co-authors have stated that they have not and will not use one of the publications or the manuscript included in this dissertation for the purpose of their own dissertation.

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