PRE- AND POST-COPULATORY SEXUAL SELECTION IN TWO SPECIES OF LYGAEID SEED BUG

Liam R. Dougherty

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Pre- and post-copulatory sexual selection in two species of lygaeid seed bug

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University of
St Andrews

This thesis is submitted in partial fulfilment for the degree of
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at the
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Contents

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I, Liam Robert Dougherty, hereby certify that this thesis, which is approximately 78,000 words in length, has been written by me, and that it is the record of work carried out by me, or principally by myself in collaboration with others as acknowledged, and that it has not been submitted in any previous application for a higher degree.

I was admitted as a research student in September 2011 and as a candidate for the degree of PhD in August 2012; the higher study for which this is a record was carried out in the University of St Andrews between 2011 and 2015.

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Co-authorship statement

All studies presented in this thesis were performed and analysed solely by me, with the exception of the CT scans reported in Chapter 6. Additionally, Ginny Greenway also helped observe bugs during mating trials on several occasions. The thesis was written by me, with feedback on writing throughout from David Shuker. David Shuker provided guidance throughout on all aspects of the thesis.

The work presented in Chapter 3 has been published in *Animal Behaviour* as Dougherty & Shuker (2014). The work presented in Chapter 4 has been published in *Behavioral Ecology* as Dougherty & Shuker (2015). Part of the work presented in Chapter 6 has been published in *Proceedings of The Royal Society B: Biological Sciences* as Dougherty *et al*. (2015).

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I would like to use this opportunity to thank everyone who has helped me throughout my PhD. Additionally; I include more specific acknowledgements regarding the work presented at the start of each relevant chapter.

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Abstract

Sexual selection arises via competition for access to mates, and is thus intimately tied to the social environment. For example, individual mating success may depend strongly on how many rivals or mating partners are available. Studies of mate choice and sexual selection may vary the number of mates a subject is presented with during mating experiments, yet it is not clear how this influences the strength and shape of sexual selection acting on traits in either sex. In this thesis I investigate the effect of social environment on sexual selection acting in two closely-related species of lygaeid seed bug: *Lygaeus equestris* and *Lygaeus simulans*. Males in both species possess an extremely elongate intromittent organ, which is over two-thirds average male body length. I show that the strength of pre-copulatory selection acting on male processus length in *Lygaeus equestris* and genital clasper shape in *Lygaeus simulans* is significantly influenced by the social context. However, selection on male and female body size in *Lygaeus equestris* is not. Additionally, I use a meta-analysis of 38 published studies to show that mating preferences are significantly stronger when more than one mate option is available, compared to when only a single option is available. I also investigate the functional morphology of male genital traits in *Lygaeus simulans*, and use formal selection analysis to quantify the strength of selection acting on these traits before, during and after mating. Finally, I use experimental manipulations in *Lygaeus simulans* to confirm that male processus length directly influences sperm transfer, and that intact genital claspers are required for successful intromission. Overall, my results illustrate that sexual selection in the wild may vary both spatially and temporally depending on the social environment. It is thus especially important that experiments are performed under ecologically relevant conditions.

Thesis outline

In this thesis I investigate sexual selection in two species of lygaeid bug: *Lygaeus equestris* and *Lygaeus simulans*. I provide a brief outline of the content of each chapter below.

In Chapter 1 I present a general introduction to the key research topics of my thesis. I cover the theory of sexual selection, and how this drives the evolution of genitalia in animals. I then consider how experimental methodology may influence the measurement of mate choice

In Chapter 2 I discuss my two study species: *Lygaeus equestris* and *Lygaeus simulans*, and the general experimental methods used throughout the thesis. I first consider sexual selection in the Lygaeidae family more generally. I then introduce my two study species and describe what is currently known of their ecology, mating behaviour and reproductive anatomy. I then describe the general methods used during my experiments, concentrating on general insect husbandry, mating trials and the measurement of genitalia.

In Chapter 3 I present an experiment considering pre- and post-copulatory sexual selection on male and female body size in *L. equestris*, and the effect that social context has on the strength of this selection. I performed mating trials according to four choice designs. Overall I detected significant positive, linear pre-copulatory selection on female body length and stabilizing pre-copulatory selection on an overall measure of male body size. However, I found no significant effect of choice design on the patterns of sexual selection for males or females. Female fertility was significantly associated with copulation duration and female size, but not with male size. I suggest that social context has little effect on the strength of sexual selection in *L. equestris*

due to the method of mate assessment, which appears to be primarily via contact cues, which may limit simultaneous comparison between options.

In Chapter 4 I perform a meta-analysis testing the effect of choice design on the strength of mating preferences across published studies. I obtained 38 studies in which both a no-choice and a choice design were used to test for mating preferences in the same species/trait/sex combination. After controlling for phylogenetic non-independence, I find that female, intraspecific mating preferences are significantly stronger when tested using a choice design compared to a no-choice design. I suggest that this is due to the increased cost of rejecting an option in no-choice tests.

In Chapter 5 I consider the strength of sexual selection acting on the length of the male processus in both *L. equestris* and *L. simulans*. I first measured the processus length of a subset of males used in the experiment presented in chapter 3; thus mating trials were performed in one of four different choice designs. I find significant negative pre-copulatory selection on processus length in *L. equestris*, but only for mating trials in which two males were present (in which male-male competition and/or female choice were possible). I suggest that this selection is indirect, arising from selection on a trait correlated with processus length. I also show that there is stabilising post-copulatory selection on processus length in *L. equestris*. I then repeat this experiment in *L. simulans*, but find no significant pre- or post-copulatory selection in contrast to a previous study. Finally, I perform a meta-analysis of estimates of quadratic, post-copulatory selection on processus length in *L. simulans*, and show that overall selection is significantly stabilising.

In Chapter 6 I investigate the functional morphology of male processus length in *L. simulans*. I first show that processus breakages may be common for males that are able to mate many times, though it is not clear whether breakages happen during or after mating. I then present virtual dissections of the interaction between male and female genitalia of *L. simulans* in copula using micro-CT scanning. Finally I present

three experiments in which I experimentally shorten male processus length in *L. simulans* by varying amounts, and record the effect on male reproductive success. Experimental reduction had no effect on male mating success, and did not impair sperm transfer *per se*. I find that experimental reduction does decrease male reproductive success, and that reproductive success decreases as a greater proportion of the processus is removed.

In Chapter 7 I consider the strength of sexual selection on the size and shape of the male genital claspers in *L. simulans*. I firstly show via experimental removal that claspers are necessary for successful mating to occur in this species. I then perform two experiments in which I record male mating success following no-choice and female choice mating trials. I then quantify infer the strength of sexual selection acting on clasper size and shape. If found that right claspers are significantly larger and wider than left claspers. As in Chapter 5, I found significant pre-copulatory selection on processus length in female-choice trials but not in no-choice trials. However clasper size or shape is not correlated with processus length. Finally, I found significant sexual selection on left clasper shape, but only in no-choice trials: : mated males have a tooth section that is both straighter and thinner than in unmated males. Therefore selection on clasper shape is influenced by the social environment, but is unable to explain the significant pre-copulatory selection on processus length.

I Chapter 8 I discuss the results of the previous chapters and consider how my results give insights into the study of sexual selection more generally, and the reproductive biology of *L. equestris* and *L. simulans*.

Chapter 1

General introduction

In this chapter I present a general introduction to the main research areas that are the background to my thesis. I first provide a brief overview of modern sexual selection theory, before focusing specifically on how sexual selection has driven the evolution of genitalia in animals. I then consider how experimental methodology may influence the measurement of mate choice.

1.1 Sexual selection theory

Sexual selection is selection arising through the differential acquisition of matings (Darwin, 1871; Andersson, 1994), or more strictly differential acquisition of fertilisations (Shuker, 2010; Birkhead, 2010). Originally the theory of sexual selection was formulated to help explain the evolution of elaborate traits that appeared detrimental to survival (Darwin, 1871). Darwin's theory was that such traits function to increase the number of matings an individual achieves. However, in the last few decades it has become clear that in many species females may mate with many different males in a single reproductive cycle, and this means that sexual selection can continue after copulation (Parker, 1970; Birkhead, 2010; Parker & Birkhead, 2013).

Sexual selection is thus typically separated into those processes that occur before copulation (pre-copulatory) and those that occur during or after copulation (post-copulatory). Pre-copulatory sexual selection selects for traits that increase the number of matings gained (Andersson, 1994). In his original formulation, Darwin (1871) distinguished between two types of pre-copulatory sexual selection: intra-sexual selection, in which individuals compete with members of the same sex for access to matings; and inter-sexual selection, in which individuals attempt to attract members of the opposite sex directly. Intra-sexual selection, most commonly in the form of male-male competition, drives the evolution of armaments that increase competitive abilities during aggressive contests (Emlen, 2008). Inter-sexual selection, most commonly in the form of female choice, drives the evolution of male traits that attract females (Andersson, 1994). Furthermore, both processes may interact; for example, females may observe male-male contests and then choose to mate with the winner (Wong & Candolin, 2005).

Mate choice is defined as "the process that occurs whenever the effects of traits expressed in one sex leads to non-random matings with members of the opposite sex" (Halliday, 1983; Kokko *et al*., 2003; Edward, 2015). In this sense mate choice is an

outcome that can be observed (Wiley & Poston, 1996; Jennions & Petrie, 1997). Individuals may also have mating preferences, defined as 'the sensory and behavioural properties that influence the propensity of individuals to mate with certain phenotypes' (Jennions & Petrie, 1997). Mate choice thus arises in part due to mating preferences, as well as other factors such as the degree of mate sampling performed (Janetos, 1980; Wittenberger, 1983; Benton & Evans, 1998; Jennions & Petrie, 1997), and the phenotypes and/or receptivity of the available mates (Halliday, 1983; Jennions & Petrie, 1997; Casares *et al*., 1998). These other factors will limit whether individual mating preferences can be realised. As such mate choice can be influenced by a wide range of extrinsic and intrinsic factors (**Table 1.1**).

I note here that modern sexual selection theory does not define specific sex roles as was historically the case (e.g. Darwin, 1871; Bateman, 1948). Instead, the strength of sexual selection acting in each sex depends on the relative investment in reproduction that each sex makes (Trivers, 1972; Gwynne, 1991; Kokko & Jennions, 2008; Shuker, 2010), as well as other factors such as the availability and variation in quality of potential mates (Kvarnemo & Simmons, 1999; Bonduriansky, 2001; Kokko & Jennions, 2008; Rosvall, 2011; Fitzpatrick, 2015). In many species females tend to invest more in reproductive, or benefit less from multiple mating, so that females tend to be choosier and males tend to be subject to stronger sexual selection (Andersson, 1994; Kokko & Jennions, 2008). However females may also compete for access to mates (Gwynne, 1991; Rosvall, 2011), and males may also be choosy in their mating decisions (Bonduriansky, 2001; Edward & Chapman, 2001). This can lead to mutual mate choice by both sexes within a species (Kraak & Bakker, 1998; Kokko & Johnstone, 2002; Kraaijeveld *et al*., 2007).

As already highlighted above, sexual selection may also act after mating has occurred (post-copulatory sexual selection; Eberhard, 1996; Birkhead & Pizzari, 2000; Simmons, 2001). The original formulation of sexual selection theory did not include post-

Table 1.1. A selected list of factors that have been shown to influence mating preferences in animals.

Category	Factor	Reference
Social factors	Density	Berglund, 1995
		Lehmann, 2007
	OSR	Berglund, 1994
		Jirotkul, 1999
		Kvarnemo & Simmons, 1999
	Previous experience	Bateman & Fleming, 2006
		Bailey & Zuk, 2008
		Jordan & Brooks, 2012
	Type of mate encounter	MacLaren & Rowland, 2006
		Barry *et al*., 2010
		Booksmythe *et al*., 2011
	Mate encounter rate	Shelly & Bailey, 1992
		Berglund, 1995
		Svensson *et al*., 2010
	Choices of other females	Bennett *et al*., 1998
		Witte & Ryan, 2002
		Mery *et al*., 2009
	Number of options	Beckers & Wagner, 2011
	Attractiveness of options	Callander *et al*., 2012
Environmental factors	Season	Milner *et al*., 2010
	Search costs	Milinski & Bakker, 1992
		Wong & Jennions, 2003
		Booksmythe *et al*., 2008
	Predator exposure	Hedrick & Dill, 1993
		Gong & Gibson, 1996
		Johnson & Basolo, 2003
	Signal efficacy	Seehausen *et al*., 1997
		Schwartz *et al*., 2001
		Swaddle & Page, 2007
Intrinsic factors	Parasite infection	Cordoba-Aguilar *et al*., 2003
		Mazzi, 2004
		Beckers & Wagner, 2013
	Female mated status	Kumano *et al*., 2009
		Judge *et al*., 2010
	Age	Moore & Moore, 2001
		Uetz & Norton, 2007
		Mautz & Sakaluk, 2008
	Condition	Hingle *et al*., 2001
		Hunt *et al*., 2005

copulatory effects, due to the widespread belief that female animals were strictly monogamous (Birkhead, 2010).

However observations starting in the 1960s (e.g. Parker, 1970) began to erode this view, and since then modern genetic techniques have showed that, in the large majority of species for which data have been obtained, females mate with more than one male during a given reproductive cycle (Birkhead & Møller, 1998; Arnqvist & Nilsson, 2000; Simmons, 2001; Griffith *et al*., 2002). Post-copulatory sexual selection is strongest when females mate with several different males prior to egg laying (so that there is competition among male gametes for access to female gametes), and when sperm are stored following insemination (Simmons, 2001; Wedell & Hosken, 2010; Orr & Brennan, 2015). Post-copulatory selection can be split into two processes: sperm competition (Simmons, 2001) and cryptic female choice (Eberhard, 1996). This is analogous to the Darwinian distinction between intra-sexual competition and inter-sexual choice for pre-copulatory sexual selection. Mechanisms of post-copulatory selection are discussed in more detail in relation to the evolution of genitalia below (Section 1.2.4). It is important to note however that the traditional strict definition of post-copulatory selection requires that females mate multiply between reproductive events, so that male ejaculates are simultaneously competing (Eberhard, 1996; Pizzari & Birkhead, 2000; Parker & Birkhead, 2013). This is discussed in more detail in Chapter 8.

1.2 Sexual selection and genitalia

Genitalia show an extraordinary amount of morphological variation across the animal kingdom, even amongst very closely related species (Eberhard, 1985; Deckert, 1990; Hosken & Stockley, 2004; Takami & Sota, 2007; **Figure 1.1**). The high variation in genital morphology is the primary reason why genital traits are considered to be important taxonomic characters in many clades, especially in arthropods (Tuxen, 1970; Eberhard, 1985; Scudder, 1997). Furthermore, in many taxa genitalia appear to be

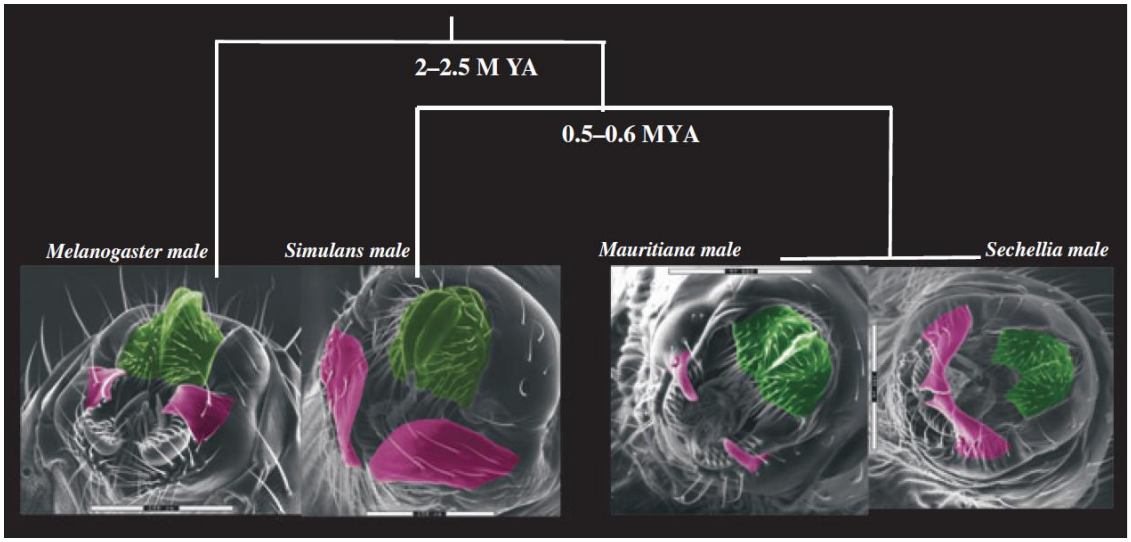

Figure 1.1. Male genitalia can be highly variable even between very closely related species. In the *Drosophila melanogaster* subgroup, the posterior processus (pink) of the male genital arch is involved in genital coupling, and is highly variable among closely related species. Adapted from Jagadeeshan & Singh, 2006.

extremely complex (Eberhard, 1985). This morphological diversity seems paradoxical if we consider that all genitalia have the same basic function: to bring male and female gametes together. Such diversity is not seen for example when comparing gut morphology in closely related species, so why do we see this striking pattern for genitalia?

Before going further I define genitalia following Eberhard (1985), with male genitalia classed as “all male structures that are inserted into the female or that hold her near her gonopore during sperm transfer”, and female genitalia classed as “parts of the female reproductive tract that make direct contact with male genitalia or male seminal products during or immediately following mating”. The traditional distinction between male and female genitalia is useful in almost all cases, though there are notable exceptions (for example see Yoshizawa *et al*., 2014). Such a distinction can also apply to hermaphroditic species, in which individuals may possess both ‘male’ and ‘female’ genitalic structures. I note that obtaining a rigorous definition of genitalia is fraught

with difficulty given the morphological diversity seen throughout nature (Eberhard, 1985).

Genitalia can be further divided into internal or external structures. I define internal genital structures as those that are contained either temporarily or permanently inside the body cavity. In this sense almost all female genitalia can be considered internal (but see Yoshizawa *et al*., 2014). This definition also includes male intromittent organs which are stored inside the male prior to mating. External genital structures are those that are contained outside of the body cavity. This includes intromittent organs that are stored permanently outside of the body, and external clasping organs that are used to maintain genital contact during mating. Structures traditionally considered to be external genitalia are traditionally considered to be located near the genital opening, however this condition is somewhat arbitrary and may be problematic in some cases (Eberhard, 1985). For example, in spiders males use secondary appendages called pedipalps to transfer sperm to females (Eberhard, 1985; Uhl *et al*., 2010; Eberhard & Huber, 2010). These structures are used to transfer sperm directly to the female, and so can be considered as genitalia. Additionally, it has been argued that there is no reason why the processes discussed below may not also apply to non-genitalic contact structures that are used during mating (Eberhard, 1985; 2010; Arnqvist & Rowe, 2005; see also Chapter 7). However the distinction between internal and external genitalia is more straightforward for my study species (see Chapter 2).

Early hypotheses to explain the morphological diversity of genitalia include the lock-and-key hypothesis, first suggested by Dufour (1844). Almost 100 years later, Mayr (1963) argued that because genitalia are generally internal structures they should be subject to little natural selection, and so will be free to diverge randomly due to pleiotropic effects caused by selection on many other genes. However, these explanations have not withstood scrutiny, and genital variation is now widely accepted to be driven primarily by sexual selection (Eberhard, 1985, 1996; Arnqvist, 1997; Hosken & Stockley, 2004). In this section I first consider the lock-and-key hypothesis

and outline why it is not well supported (at least in its most strict definition). I then present an overview of evidence suggesting that sexual selection has played a major role in the evolution of animal genitalia. I finally discuss a specific type of male genitalia seen in many insect groups: extremely long intromittent organs.

1.2.1 The lock-and-key hypothesis

The oldest theory used to explain the extreme diversity of genitalia in closely related species is known as the 'lock-and-key' hypothesis (Eberhard, 1985; Shapiro & Porter, 1989). This theory relies on the assertion that genitalia tend to be species-specific, with a tight fit observed between male and female reproductive morphology. The earliest recorded formulation of this hypothesis is by Dufour (1844) in a study of Dipteran anatomy. The hypothesis states that genitalia evolve to be species specific in order to prevent hybridisation; so that only males with the correct 'key' can fit the female 'lock' (Eberhard, 1985).

In a detailed treatment of the topic, Eberhard (1985) lists several reasons why the lock-and-key hypothesis is unlikely to be a general explanation for the evolution of genitalia. For example, in many species the fit between male and female genitalia does not appear to be species specific (Scudder, 1971; Shapiro & Porter, 1989; Mutanen *et al*., 2006). However this evidence is not conclusive, partly because the 'fit' of genitalia may be cryptic or driven for example by sensory processes (Eberhard, 1985; Masly, 2012; Simmons, 2014). Furthermore, even if genitalia are species-specific, merely showing that interspecific matings are not possible for current species does not mean that reproductive isolation was the selective factor driving divergence in the first place (Robertson & Paterson, 1982). Additionally, the close fit between male and female genitalia is also predicted by other hypotheses (Eberhard, 1985).

Other objections include the presence of species-specific genitalia in species which appear to have evolved in isolation from closely related species (Eberhard, 1985). The

most convincing evidence is probably the apparent lack of character displacement among male genitalia in zones of sympatry compared to allopatry for most species that have been tested (e.g. Ware & Opell, 1989; Holwell, 2008; Eberhard, 2010). However, there are some exceptions to this, with character displacement appearing to be stronger in sympatry for example in stag beetles (Kawano, 2003) and rhinoceros beetles (Kawano, 2002). Male genitalia that harm females may also lead to reproductive interference between closely related species (e.g. Kyogoku & Sota, 2015; Masly, 2012), which may lead to character displacement. Though potentially rare, lock-and-key mechanisms may be important in species which do not exhibit any pre-copulatory mate recognition (Wojcieszek & Simmons, 2011).

Overall then, evidence suggests that the key prediction of the lock-and-key hypothesis, that selection on genital morphology acts to enforce species isolation so as to reduce hybridization, has not been conclusively demonstrated in most empirical tests (Masly, 2012). However this prediction follows from considering the lock-and-key hypothesis in the strict sense. An alternative situation would be one in which sexual selection initially leads to divergent morphology between closely related species, and these differences are then in turn reinforced due to selection against hybrids (Masly, 2012). In this case lock-and-key may arise as a by-product of other evolutionary processes. In this way lock-and-key can be seen as one end of a continuum, with selection targeting isolation between populations at one end and selection targeting variation in male quality within populations (i.e. sexual selection) at the other end (Simmons, 2014).

1.2.2 Evidence that sexual selection drives genital evolution

Empirical studies have adopted several approaches to investigate the role of sexual selection in genital evolution (Simmons, 2014). The first is a comparative approach, comparing genital morphology across species with some indicator of the strength of sexual selection, such as the level of polyandry (Arnqvist, 1998; Rowe & Arnqvist, 2012). Despite the difficulties in comparing complex genital morphology between

species, these comparative analyses have in many cases produced a clear relationship between genital morphology and the strength of sexual selection (e.g. Eberhard, 1985; Takami & Sota, 2007; Vahed *et al*., 2011; Rowe & Arnqvist, 2012, but see e.g. Hosken *et al*., 2001). For example, Arnqvist (1998) used a phylogenetically-controlled analysis to show that polyandrous clades exhibit genital divergence that is twice as strong as monandrous clades in four insect orders.

The second approach is to correlate variation in reproductive success with genital morphology in a single species (Arnqvist, 1997). Using this approach, male genital morphology has been shown to significantly influence male mating success (e.g. Blanckenhorn *et al*., 2004; Bertin & Fairbairn, 2005, Simmons *et al*., 2009; Xu & Wang, 2010), copulation duration (e.g. van Lieshout & Elgar, 2011), sperm transfer (e.g. Tadler, 1999; Holwell *et al*., 2010) and paternity (Otronen, 1998; Arnqvist & Danielsson, 1999; House & Simmons, 2003) in a range of species.

Third, the role of specific genital traits in influencing reproductive success can also be investigated by using experimental manipulation of the trait in question (Simmons, 2014). For example, Arnqvist & Rowe (1995) experimentally lengthened the female abdominal spines of the water strider *Gerris incognitus* using resin glue, and showed that females with larger spines had fewer matings, suggesting that spines function to prevent forced matings by males. More commonly, genital structures are experimentally removed or damaged (Rodriguez *et al*., 2004; Moreno-García & Cordero, 2008; Tsuchiya & Hayashi, 2008; Hotzy *et al*., 2012). These studies are known as genital ablation studies. This approach is discussed in more detail in Chapters 6 & 7.

Finally, in recent years studies have begun to use experimental evolution, either to artificially select for differences in genital morphology directly or to assess how genital morphology evolves in populations exposed to artificially-elevated mating rates (Simmons, 2014). For example, elevated mating rates have been show to lead to changes in the shape of the aedeagus in the dung beetle *Onthophagus taurus*

(Simmons *et al*., 2009), and the thickness of the baculum in the house mouse *Mus domesticus* (Simmons & Firman, 2013). Cayetano *et al*., (2011) performed such an experiment in reverse, by imposing monogamy on the typically polygamous seed beetle *Callosobruchus maculatus*. After around 20 generations male genital spine length had significantly reduced in size (Cayetano *et al*., 2011). Other studies have also artificially selected for specific genital traits, and detected a significant change in male reproductive success (e.g. Hotzy *et al*., 2012). These studies illustrate that such artificial selection can read to rapid evolutionary change, with significant changes in morphology detected after less than 20 generations of selection in some cases (Simmons *et al*., 2009; Cayetano *et al*., 2011).

1.2.3 Mechanisms of selection

The evidence for the role of sexual selection in the evolution of genitalia is now very strong. Sexual selection on genital morphology can act both prior to copulation by influencing mating success, or post-copulation by influencing sperm transfer and fertilisation success (Hosken & Stockley, 2004; Simmons, 2014). One important consequence of this is that pre-copulatory sexual selection on genital morphology may occur in monandrous species, for example through selection on male traits that prevent genital contact during takeover attempts by other males during mating (Thornhill & Alcock, 1983; see Chapter 8 for a discussion of post-copulatory sexual selection in monandrous species). Pre-copulatory selection acts primarily on external genital traits that influence mating success, such as genital claspers which are needed for genital coupling (e.g. Yang & Wang, 2004; Moreno-García & Cordero, 2008; Grieshop & Polak, 2012). However a few studies have also detected pre-copulatory selection on male intromittent organ morphology (Simmons *et al*., 2009; Xu & Wang, 2010). Additionally, in species in which male intromittent organs are held externally, penis size may even be selected directly by female choice (e.g. Langerhans *et al*., 2005; Kahn *et al*., 2009; Mautz *et al*., 2013), though this is probably rare.

Though post-copulatory sexual selection on genitalia is likely very common, distinguishing between the specific mechanisms of sexual selection has been difficult (Hosken & Stockley 2004; Simmons 2001; 2014), in part because post-copulatory interactions occur inside the female and so cannot be observed (Eberhard, 1996). Post-copulatory sexual selection has been hypothesised to evolve via three main processes: a) male adaptations in response to sperm competition (Simmons, 2001), b) selection via cryptic female choice/copulatory courtship (Eberhard, 1996), and c) sexually-antagonistic coevolution due to sexual conflict (Arnqvist & Rowe, 2005).

Sperm competition occurs when females mate multiply so that the ejaculates of multiple males interact simultaneously within the female tract (Parker, 1970; Simmons, 2001). Such competition drives the evolution of male genital traits that increase insemination success relative to other males. For example, the intromittent organs of male damselflies possess complex structures which function to remove the sperm of rivals prior to ejaculation (Waage, 1979; Tsuchiya & Hayashi, 2008).

Cryptic female choice is the term used for any process leading to variation in male post-copulatory reproductive success that is caused by a female trait (Thornhill & Alcock, 1983; Eberhard, 1996; Arnqvist, 2014). In his original monograph, Eberhard (1996) emphasised the idea that cryptic female choice could impose a form of runaway selection on male genital morphology through stimulatory effects (in the same way that pre-copulatory mate choice may lead to such selection if preferences are open-ended), and that this could lead to the divergent genitalia seen in many species groups. Demonstrating that females actively select male sperm based on a given male trait may be very difficult though (Birkhead, 1998; Pitnick & Brown, 2000). However, recent definitions are much broader, suggesting that any process that biases fertilisation success towards some males more than others be considered a form of cryptic female choice, as such a process is always influenced by female anatomy and physiology to some extent (Arnqvist, 2014).

Finally, genital morphology may also evolve through sexually-antagonistic coevolution driven by sexual conflict. Sexual conflict arises when a trait that increases the fitness of one sex leads to reduced fitness in the opposite sex (Arnqvist & Rowe, 2005). Such conflict of interest drives the evolution of adaptations in either sex that attempt to drive such behaviours back towards their own fitness optima. For example, the optimal number of matings in a single reproductive cycle is usually larger for males than for females. In some species males attempt to force matings with females using grasping genital traits (e.g. Bertin & Fairbairn, 2005), and this leads to the co-evolution of traits in females to resist such attempts (Arnqvist & Rowe, 1995). In *Callosobruchus* seed beetles, the male intromittent organ may be covered in genital spines which damage the female reproductive tract during mating (Crudginton & Siva-Jothy, 2000), with males that inflict more damage on females having higher fertilisation success (Hotzy *et al*., 2012). However genital damage reduced female longevity, and there is a strong correlation between penis spine length and female tract thickness across species which is likely driven by this conflict (Rönn *et al*., 2007; **Figure 1.2**).

These different processes have proven especially difficult to separate (Eberhard, 1996; 2004; Arnqvist & Rowe, 2005). However, they are not mutually exclusive, and it may be possible that a combination of any of them may act in a given species, or even on a single trait, at any one time (Simmons, 2001; Eberhard, 2010). It has been suggested that a much more fruitful approach will be to try to determine exactly which female traits impose selection on male genital morphology, rather than trying to isolate the effect of specific mechanisms that may not work in isolation (Simmons, 2014).

1.2.4 Elongate genitalia

In many insects the male intromittent organ ends in an extremely thin, elongate tube, commonly referred to as a flagellum or processus. This trait is seen for example in Coleoptera (Rodriguez, 1995; Gack & Peschke, 2005; Matsumura & Yoshizawa, 2010), Hemiptera (Ashlock, 1957; Deckert, 1990), Dermaptera (Kamimura, 2005; van Lieshout

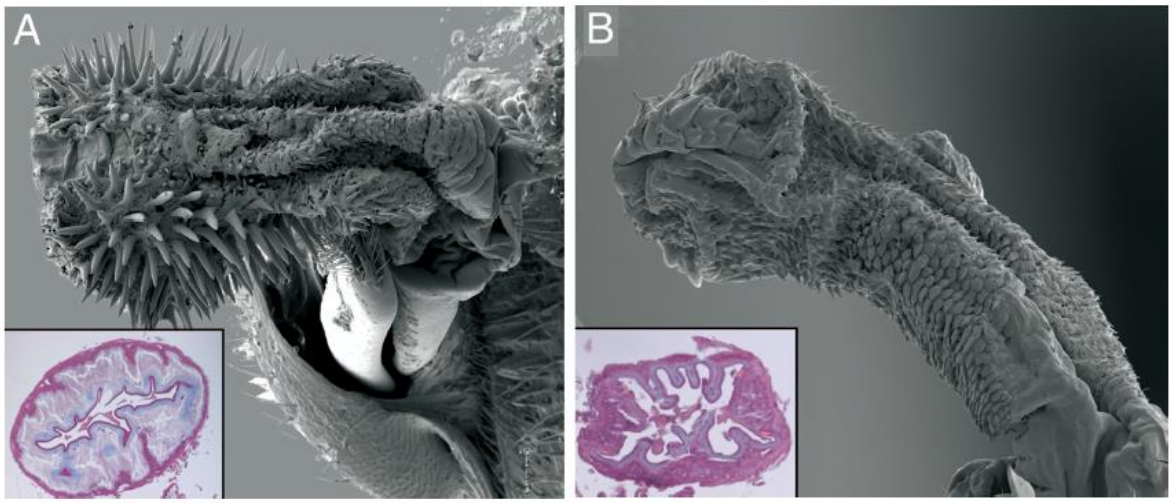

Figure 1.2. Coevolution of male and female genitalia in two species of *Callosobruchus* beetles: A) *C. analis*, and B) *C. phaseoli*. The thickness of the female reproductive tract (stained light blue) correlates with the degree of elaboration of genital spines across species. Adapted from Rönn *et al*., 2007.

& Elgar, 2011), Diptera (Ilango & Lane, 2000), Siphonaptera (Eberhard, 1985) and Neuroptera (Sziráki, 2002; Aspöck & Aspöck, 2008). In some species this structure is hollow and involved directly in sperm transfer (e.g. in Hemiptera: Deckert, 1990; **Figure 1.3**). However in others it may be used in conjunction with a spermatophore (Gack & Peschke, 2005; Matsumura *et al*., 2014). In the rove beetle *Aleochara tristis* the flagellum is more than twice the length of the males' body, and is tightly coiled inside the aedeagus when not in use (Gack & Peschke, 2005). The flagellum is so long that in order to prevent entanglement after copulating with the female, the male holds it taut between the mesothorax and the pronotum whilst withdrawing it into the body, a behaviour known as 'shouldering' (Gack & Peschke, 2005). The flagellum does not appear to be involved directly in sperm transfer; instead it is used as a guide for a tube that grows out of the spermatophore (Gack & Peschke, 1994).

In spiders, males transfer sperm to females using appendages known as pedipalps (Eberhard & Huber, 2010). The terminal section of the pedipalps ends in a structure known as the embolus, which enters the female to transfer sperm. In some species the

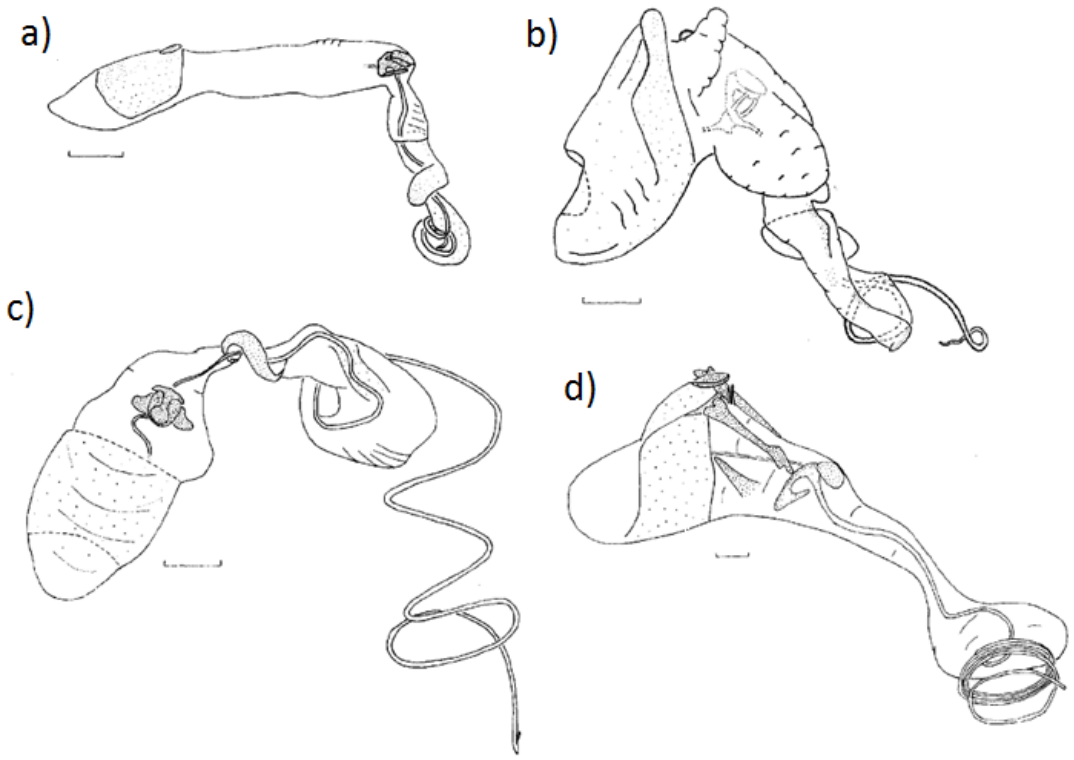

Figure 1.3. Male intromittent organs of four species of Lygaeinae (Heteroptera; Lygaeidae), showing the thin, coiled processus (Deckert, 1990). Species shown are **a)** *Orsillacis product,* **b)** *Aulocopeltus minor*, **c)** *Emphanisis cuprea,* and **d)** *Caenocoris nerii*.

embolus can be incredibly long and thin and resembles the insect flagellum (Jager, 2005; Hormiga & Scharff, 2005; Snow *et al*., 2006; Agnarsson *et al*., 2007; Eberhard & Huber, 2010). In species with extreme lengthening of the embolus the female spermathecal duct is correspondingly long and coiled (Eberhard, 1996; Jager, 2005; Eberhard & Huber, 2010).

Elongate genitalia are also seen in waterfowl (Anatidae), which are one of the few bird families in which males possess an intromittent organ (Briskie & Montgomerie, 1997), and in some species the penis may reach great lengths (McCracken *et al*., 2001; Brennan *et al*., 2010; **Figure 1.4**). A comparative study of the Anatidae shows that penis length and testis size correlate with the frequency of forced copulations across species, suggesting a role for sexual conflict in the evolution of long penises in this

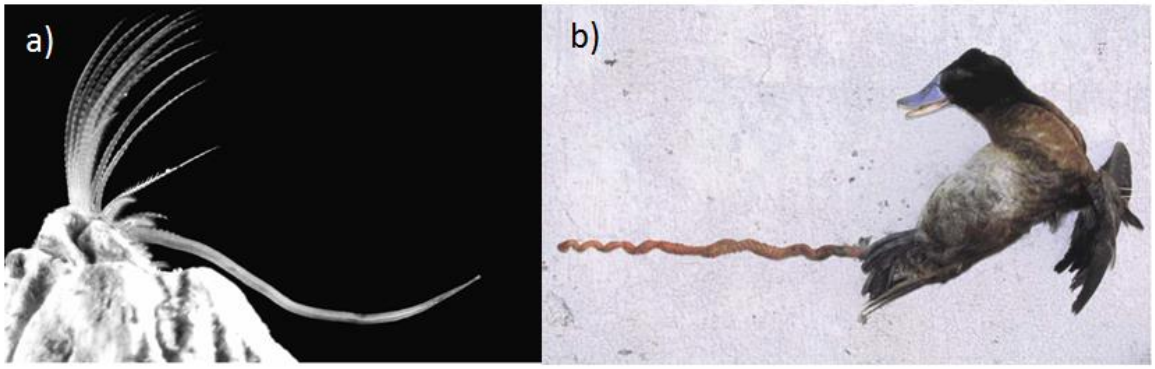

Figure 1.4. Elongate genitalia in other animals. A) penis of the barnacle *Balanus glandula*, from Neufeld & Palmer, 2008. B) penis of the male Argentine lake duck *Oxyura vittata*, from McCracken *et al*., 2001.

group (Coker *et al*., 2002). Furthermore, the female reproductive tract in waterfowl is very elaborate in comparison to most bird species, and in some species exhibits anatomical novelties such as blind-ended pouches that may function to prevent male intromission without the complicity of the female (Brennan *et al*., 2007).

In some animals elongate genitalia may arise due to natural selection. For example, barnacles are one of the few sessile organisms to use internal fertilisation (Neufeld & Palmer, 2008). Because both males and females secrete very large calcareous shells the intromittent organ needs to be very long (relative to the animal's actual body size) in order to be able to reach adjacent individuals (Darwin, 1854; Murata *et al*., 2001; Neufeld & Palmer, 2008). As such, barnacles have the longest penises in relation to body length of any animals (Neufeld & Palmer, 2008; **Figure 1.4**). The length of the intromittent organ is this case has arisen due to the large shell size, which presumably increases survival. As such this can be considered a form of natural selection: long intromittent organs are needed to contact adjacent individuals or reproduction cannot take place. Note that longer organs could also be sexually selected if they allow an individual to mate with more individuals that are positioned further away (effectively increasing the mate searching ability), however this is probably not the primary reason for their extreme size.

However in most cases the evolution of extreme intromittent organ length has likely been driven by sexual selection. However, few studies have actually investigated how selection acts on such traits (but see Rodriguez, 1995; Tadler, 1999; Tadler *et al*., 1999; Rodriguez *et al*., 2004). This is discussed in more detail in Chapter 5. One problem is that the functional morphology of such traits needs to be investigated before inferences about the sources of selection can be made (Simmons, 2014; Chapter 6). For species in which the flagellum is directly involved in sperm transfer, sexual selection probably plays an important role in the maintenance of extreme length. However, the exact mechanisms will depend on other morphological factors. For example, in the earwig *Euborellia plebeja*, the long intromittent organ (or virga) enters the spermatheca and is able to remove rival males' sperm, and has thus likely evolved in response to the risk of sperm competition in this species (Kamimura, 2005).

The morphology of the female reproductive tract may also be informative when considering the functional morphology of elongate intromittent organs. For example, in some species the female tract is as approximately as long as, or slightly longer than, the male intromittent organ (e.g. Gack & Peschke, 2005; Matsumura & Akimoto, 2009; Eberhard & Huber, 2010). This suggests that the male and female genital morphology has coevolved, and this could suggest a role for sexual conflict (as in waterfowl: Brennan *et al*., 2007) or cryptic female choice (Eberhard, 1996). In some species the flagellum may enter the spermatheca directly (Rodriguez *et al*., 2004; Kamimura, 2005), whereas in other species the female reproductive tract may contain structures that prevent it from ever reaching the spermatheca (e.g. Gschwenter & Tadler, 2000). Finally, in several taxa the male flagellum appears to be significantly longer than the female spermathecal duct (Gschwenter & Tadler, 2000; Ilango & Lane, 2000; Rodriguez *et al*., 2004). In these cases it is not clear why the male intromittent organ is apparently so excessively long, but it suggests that the fit between the flagellum and the spermathecal duct is not the only important factor for male reproductive success.

1.3 Experimental design and mate choice

The nature of behavioural ecology means that mate choice is studied in an incredibly wide range of study species testing a wide range of traits; this means that each species has its own idiosyncrasies in regards to how experiments can actually be carried out. This leads to a wide diversity of experimental designs in the mate choice literature. As seen above, it is well known that mate choice in animals can be influenced by a wide range of external factors (**Table 1.1**). However, much less attention has been paid to the potential influence of experimental design on the strength and patterns of mate choice seen in empirical studies. These effects are important if, for example, we want to use the results of these experiments to infer the strength of sexual selection in natural populations. In this section I describe different aspects of experimental design that commonly vary in the mate choice literature, and attempt to assess how these factors may influence the measurement of mating preferences.

1.3.1 Proxy measures vs choice measures

Mate choice can be measured using two approaches. The first approach is to measure actual mate choice by allowing males and females to interact and mate. The second approach is to measure a behavioural outcome that is assumed to correlate with a mating preference, which I refer to as a 'proxy measure' of preference. For example, female phonotaxis towards a speaker is the most common measure of preference for studies of courtship song (Wagner, 1998). Each approach has benefits but also potential problems that researchers should be aware of.

The advantage of measuring mate choice directly is that mating success is a measure of fitness, and so can be used to determine the strength and shape of (pre-copulatory) sexual selection acting on a trait. Thus if we are concerned with how choice leads to evolution in natural populations then this should be the preferred method. However,

mating success depends on an interaction between males and females. In some species forced matings are common, so that for example mating success may reflect a male's ability to overcome female resistance rather than a female preference (Arnqvist & Rowe, 2005). Thus accurately measuring female mating preferences may be difficult in species which show a sexual conflict over mating (e.g. Shuker & Day, 2001). More generally, experiments in which individuals are able to interact are unsuitable if we wish to attribute choice to either party (Halliday, 1983; Martel & Boivin, 2011).

Using a proxy measure of mating preference means that attributing choice to either sex is no longer a problem. Additionally such measures allow the researchers to control exactly which stimuli are presented to subjects. Researchers can also present subjects with synthetic stimuli that may be simplified or exaggerated versions of a natural trait, as long as subjects do respond to such stimuli (e.g. Rowland, 1982; Uetz & Norton, 2007). Therefore such behavioural measures are commonly used in studies that are interested more in the proximate mechanisms of mating preferences; for example studies considering the neurology of mate choice (e.g. Doherty, 1985; Kostarakos *et al*., 2008), or studies that investigate which components of a complex courtship song are preferred by females (e.g. Ryan, 1985; Wagner *et al*., 1995). Finally, proxy measures may give more statistical power compared to choice outcomes, as with the former responses can be continuous, compared to the binary outcome of the latter (Wagner, 1998).

One problem with using such proxy measures however is that different measures may lead to different conclusions about the direction and strength of sexual selection acting on secondary sexual traits. For example, Bailey (2008) examined female preference for an aspect of male calling song, the proportion of long chirps in each call, in the field cricket *Teleogryllus oceanicus* using no-choice trials. When female preference was derived from dichotomous scoring of whether a female reached the speaker or not after a given time, females showed a positive linear preference for increased proportion of long chirps. However, when preference was derived from

response effort (the response time, with shorter responses indicated stronger preference) females showed a unimodal preference for intermediate proportion of long chirps. Therefore depending on the proxy measure of preference, selection on male long chirps appears either directional or stabilizing (Bailey, 2008). It is not clear how representative this study is and thus how general this problem may be.

1.3.2 Validating proxy measures

It is important to pick a proxy behavioural measure of preference that accurately reflects choice in natural populations. This can be determined by validating the assumption that the proxy measure does correspond to an actual mating preference. However, for some behavioural measures, this assumption has been shown to be false. For example, mating preferences in fishes are commonly tested using the proxy of association time (Wagner, 1998). The setup typically involves a variation on the same basic design: the focal fish is placed in a central tank and subject fish are placed in adjacent tanks, and thus separated by glass partitions (**Figure 1.5**). A region next to each glass partition is defined as an 'association zone'. The experimenter then records the time the focal subject spends in each zone in proximity with the fish in each adjacent tank, and this is used as a measure of mating preference (Wagner, 1998).

Gabor (1999) tested whether association time by male and female sailfin mollies (*Poecilia latipinna*) was a good measure of mate choice by testing association preferences with both members of the opposite sex, and members of the same sex. Both male and female mollies preferred to associate with large fish, irrespective of sex. Furthermore, when given a choice between a large female and a small male, females preferred to associate with large females. This study suggests that, at least in this species, association with members of the opposite sex may not reflect just mating preferences, but also general "social" preferences. Though possible explanations for this preference for large fish were not tested, many fish species show size-assortative shoaling which may reduce individual predation risk (Hoare *et al*., 2000). Such

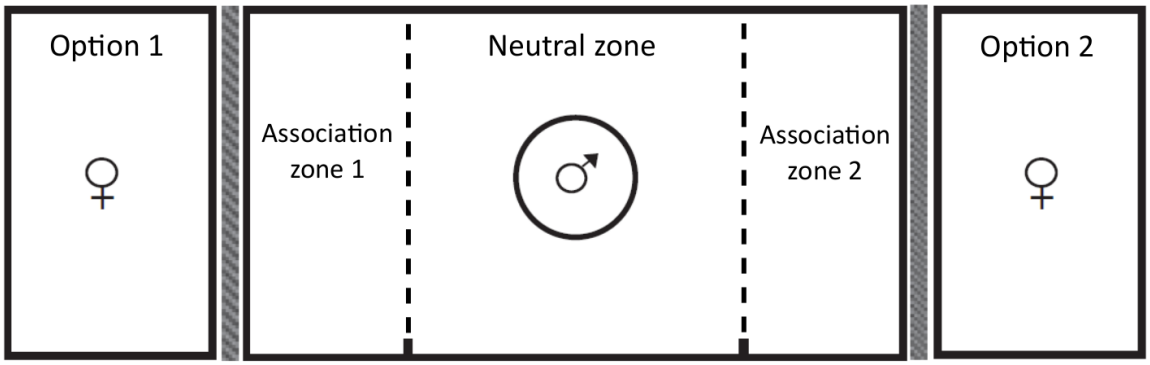

Figure 1.5. Typical experimental setup for association tests in fish. The focal subject (in this case a male) is placed in a central tank, with two tanks placed on either side separated by clear glass walls. An option (in this case a female) is placed in either of the side tanks, and the time the subject spends in each association zone is recorded. Adapted from Jeswiet & Godin, 2011.

preferences could also arise due to a sensory bias for larger individuals (Ryan & Keddy-Hector, 1992). Additionally, it has been shown that association time in the California mouse *Peromyscus californicus* is not a reliable indicator of mating preferences when the sexes are allowed to fully interact (Gubernick, & Addington, 1994).

Other studies have validated the association time method in a range of fish species, by correlating preferences observed in association tests with free-swimming choice tests and/or measures of reproductive success (e.g. Kodric-Brown, 1993; Plath *et al*., 2006; Walling *et al*., 2010; Jeswiet & Godin, 2011). Association time has also been validated in studies of birds, for example in Japanese quail *Coturnix japonica* (White & Galef, 1999) and zebra finches *Taeniopygia guttata* (Witte, 2006; Holveck & Riebel, 2007). In the black field cricket, *Teleogryllus commodus*, female latency to mate with a given male in a no-choice test was shown to successfully predict a male's long-term mating success (Shackleton *et al*., 2005).

1.3.3 Preference functions and repeatability

Another important methodological issue is how many times each subject is used in mating trials. This will depend primarily on what type of preference an experimenter intends to measure. Based on the number of times each subject is tested we can measure two types of preference: population-level preferences and individual preference functions. In a population-level test, many individuals are tested once using each stimulus and a population-level preference is obtained by taking the average response across all subjects. However, such designs may mask inter-individual variation in preferences which may significantly influence the shape of selection on traits (Boake, 1989; Wagner, 1998; Fowler-Finn & Rodríguez, 2013). An alternative is to test the same subject multiple times using a range of stimuli (Wagner, 1998). This has two advantages. First, it allows the repeatability of choice to be estimated (Boake, 1989; Møller, 1994; Jennions *et al*., 1995; Godin & Dugatkin, 1995; Godin & Auld, 2013; Fowler-Finn & Rodríguez, 2013). Second, it allows the creation of individual preference functions (Wagner, 1998). Such preference functions may be complex, and can be visualised using non-parametric curves (Schluter, 1988; Ritchie, 1996; Bentsen *et al*., 2006; Fowler-Finn & Rodríguez, 2012; 2013). By testing multiple subjects in this way the variability in preferences between individual subjects can be estimated. However the wide range of potential shapes that such preference functions can take means that defining aspects of choice can be difficult (Edwards, 2015).

1.3.4 Choice designs

An important way in which experiments testing mate preferences can vary is in the number of options the subject is presented with during the test, which I refer to as the 'choice design'. Tests can use either no-choice or choice designs (Wagner 1998; **Figure 1.6**). In a no-choice test each subject is presented with a single stimulus. In contrast, in a choice test each subject is given a choice between multiple (usually two) stimuli presented simultaneously.

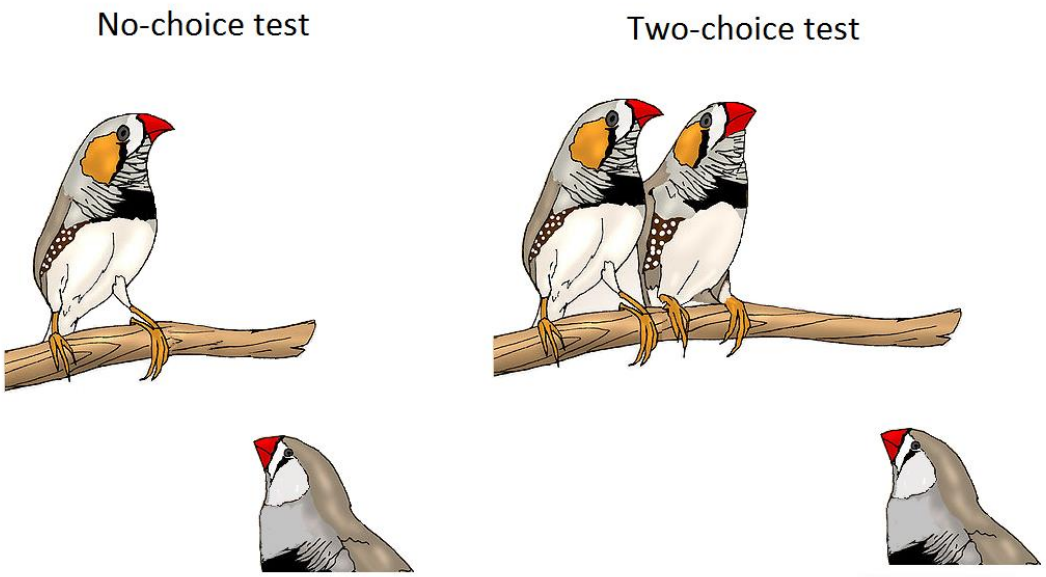

Figure 1.6. No-choice and two-choice tests differ in whether options can be compared or not. Image credit: Wikihow.

These two designs appear to be used interchangeably in the mate choice literature, with experiments performed using different designs being compared as if equal. In a recent sample 71% of empirical studies citing Wagner (1998), an influential study discussing experimental design and mate choice, performed a choice test (Owen *et al*., 2012). The two designs differ most importantly in whether options can be directly compared or not, and previous authors have suggested that mating preferences may vary according to which experimental design is used (Doherty, 1985; Wagner, 1998). However in most cases it is not clear how comparable mating preferences found using the two designs are. Is there any evidence that different choice designs influence the measurement of mate choice?

There are many cases of both no-choice and choice designs being used to test for mating preferences in the same species. Several have shown little effect of choice design on the strength of mating preference (e.g. Gabor *et al*., 2000; Jang & Gerhardt, 2006; Gershman & Sakaluk, 2009; Jordan & Brooks, 2011). For example, male Pacific Blue-eye fish *Pseudomugil signifier* prefer larger females in both simultaneous (Wong

& Jennions, 2003) and sequential choice tests (Wong *et al*., 2004). However several studies have found that mating preferences are significantly stronger in choice tests compared to no-choice tests (e.g. Barry *et al*., 2010; Booksmythe *et al*., 2011; Owen *et al*., 2012). For example, MacLaren & Rowland (2006) assessed female preference for male body size in the sailfin molly *Poecilia latipinna*, by presenting the same individuals dummy males using both no-choice (sequential choice tests; see below) and two-choice tests. The female preference for larger males was significantly stronger in simultaneous choice tests compared to sequential no-choice tests (**Figure 1.7**).

Therefore there appears to be some evidence that choice design can influence the measurement of mating preferences. However there has as yet been no attempt to synthesize these results or quantitatively assess the impact of experimental choice design on the measurement of mate choice. I consider this question in detail in Chapter 4.

Some experiments employ a no-choice design in which each subject is tested multiple times. This is referred to as a sequential choice design (Wagner, 1998). When designing such experiments there are additional confounding factors that should be considered. For example, the interval between each presentation may influence the strength of choice: in many species choosiness has been shown to decrease as the interval between presentations increases (Bakker & Milinski, 1991; Shelly & Bailey, 1992; Berglund, 1995; Lehmann, 2007; Svensson *et al*., 2010). This is because the perceived mate density decreases as this interval gets longer (Barry & Kokko, 2010; Booksmythe *et al*., 2011). In addition, sequential presentations mean that subjects have prior experience of options in later trials (Wagner, 1998). This may affect choice in later presentations, for example if females become progressively choosier with each successive mating opportunity (Pitcher *et al*., 2003). In the guppy *Poecilia reticulata*, males show a preference for large females in sequential choice tests, but only after encountering females of variable size (Jordan & Brooks, 2012). The perceived mate density will also be higher in later trials, and this could also lead to increased

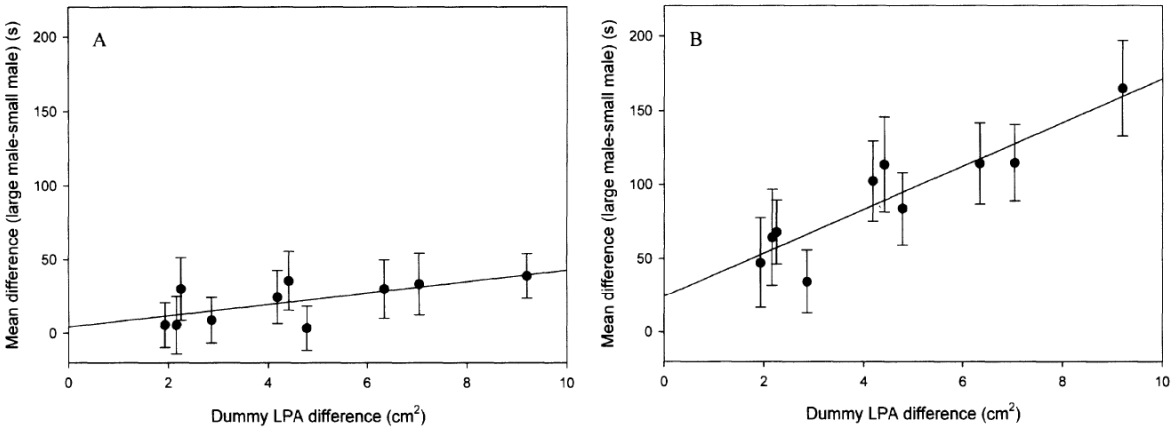

Figure 1.7. Female preference for male body size in sailfin mollies is significantly stronger in choice tests (Right panel) compared to no-choice tests (Left panel). The x axis shows the difference in size between two model males presented either sequentially or simultaneously. The y axis shows the average preference for the larger male, with preference derived from the amount of time spent in the association zone of each male. From MacLaren & Rowland, 2006.

choosiness. Therefore prior experience may strongly affect mate choice, and this should be controlled for when performing choice experiments, either by fully randomising the order that options are presented in, or by ensuring that all subjects are presented with the same stimuli when possible.

When subjects are given a choice of mate options (and mating is possible with either option) a key assumption is that all individuals are sexually receptive. Any difference in receptivity between mate options may lead to 'apparent choice' which may not reflect mating preferences. This may be a problem in studies testing for sexual isolation between species or populations if these species or populations have different mating propensities (Casares *et al*., 1998). For example, *D. melanogaster* females are known to be more active than *D. simulans* females (Wood & Ringo, 1980), and non-random patterns of mating may potentially be explained by differences in mating propensity between species (Casares *et al*., 1998). Ideally, mating propensities for any populations used in an experiment should be measured before choice tests are performed, though this rarely happens (Casares *et al*., 1998).

1.3.5 Number of options

Multiple choice tests may also vary in the number of options subjects are presented with. Though much less common than two-choice tests, some studies, such as those considering acoustic communication, may perform three-choice (Beckers & Wagner, 2011) or even four-choice tests (Swaddle & Cuthill, 1994; Bishop *et al*., 1995; Jennions, 1998). Such designs may be more realistic than two-choice designs if they more closely resemble demographic conditions during the mating season, for example in species in which high-density male choruses are common (Beckers & Wagner, 2011). However increasing the number of stimuli increases the time needed to perform experiments, and may also reduce statistical power if fewer trials are performed for each option (Hutchinson, 2005).

Studies of acoustic communication in animals frequently suggest that more options may reduce the effectiveness of choice (Hutchinson, 2005). For example, in the frog *Hyperolius marmoratus*, the ability of a female to locate the loudest speaker was reduced when four speakers were broadcasting compared to two (Bishop *et al*., 1995). This effect could be because multiple signals interfere with each other (Forrest, 1994; Schwartz *et al*., 2001; Greenfield, 2015). This is supported by the observation that choosiness decreases when white noise is broadcast over calling males in some species (Schwartz *et al*., 2001; Swaddle & Page, 2007; Bee & Schwartz, 2009). Other species appear to overcome this problem by only comparing a limited number of stimuli at any one time. For example, in the bushcricket *Tettigonia viridissima*, females may be in the vicinity of many calling males, however the auditory neurons appear sensitive only to the loudest signal in each ear (Römer & Krusch, 2000). Therefore only two options are compared at any one time, and this may commonly be those closest to the female (Hutchinson, 2005).

However the effect of the number of options on the choice of non-acoustic stimuli is not clear. Studies on human mate choice suggest that subjects find it harder to choose when presented with a large number of options, both when choosing dating partners (Lenton *et al*., 2009; Lenton & Francesconi, 2011) and food options (Scheibehenne *et al*., 2010; Iyengar & Lepper, 2010). This effect probably arises due to the cognitive difficulties of assessing multiple options (Hutchinson, 2005). These studies are able to present human subjects with a large number of options (e.g. over 30: Lenton *et al*., 2009), as humans have an obvious ability to compare and remember multiple options. However this may be much harder for animals in which this ability does not exist. I can find no examples of studies testing for this effect using non-acoustic communication in animals. This is important because acoustic signals can interfere with each other whereas visual or olfactory signals cannot. Across lekking species there is some evidence for a female preference for larger leks (Hutchinson, 2005; Isvaran & Ponkshe, 2013; Alem *et al*., 2015). This has been suggested as a mechanism by which females are able to more accurately assess males (Höglund & Alatalo, 2005; Hutchinson, 2005; Isvaran & Ponkshe, 2013), however it is hard to rule out alternative explanations. I suggest that studies investigating the effect of the number of mate options on the strength of mate choice in species that use visual or olfactory assessment would be very informative.

Chapter 2

Study species and general methods

In the first part of this chapter I present a general introduction to my two study species: *Lygaeus equestris* and *Lygaeus simulans*. To do this I first provide a brief overview of studies of sexual selection performed in the Lygaeidae more generally. I then summarise the ecology of *L. equestris* and *L. simulans*, giving detailed descriptions of male and female reproductive anatomy and reproductive behaviour. In the second part, I describe the general experimental methods used when working with these species, focusing on general husbandry methods used for maintaining study populations and experimental animals, behavioural matings trials, and the measurement of male genitalia.

2.1 The Lygaeidae

L. equestris and *L. simulans* are hemipteran bugs belonging to the family Lygaeidae (*sensu lato*: Hemiptera; Heteroptera; Pentatomomorpha), known commonly as 'seed bugs' (Schuh & Slater, 1995). It should be noted that the common name of seed bug can also apply to other bug species, and not all lygaeids feed on seeds (Schuh & Slater, 1995; Burdfield-Steel & Shuker, 2014). Also, the traditional Lygaeidae family is probably paraphyletic, and the phylogeny of the Heteroptera is still poorly resolved (Weirauch & Schuh, 2011). As the name suggests, many lygaeid species feed on seeds, and many are of economic importance as crop pests (Sweet, 2000; Yang & Wang, 2004). Many lygaeid species feed on toxic food plants, and are able to sequester toxins such as cardiac glycosides (Von Euw *et al*., 1971; Duffey & Scudder, 1972). Because of this toxicity, red-and-black warning colouration is widespread, especially within the Lygaeinae subfamily (Burdfield-Steel & Shuker, 2014), in which the defensive compounds used have been well described (Aldrich *et al*., 1997). Probably because of such chemical defences, the metathoracic scent gland is less well developed than in other Pentatomomorpha, such as the Pentatomidae, or 'stink bugs' (Aldrich, 1988). However, in some lygaeid species, for example the milkweed bug *Oncopeltus fasciatus*, this gland is used to produce attractant pheromones, which may attract both males and females to suitable host plants (Aldrich, 1995, Aldrich *et al*., 1999).

Most lygaeid species studied in the wild exhibit a promiscuous mating system, with both males and females mating multiply (e.g. Economopoulos & Gordon, 1972; McLain, 1989; Wang & Shi, 2004). Importantly, females may commonly mate several times between oviposition events so that sperm competition is predicted to be strong (Simmons, 2001). Very long copulations are seen in many lygaeid species (Burdfield-Steel & Shuker, 2014), and this may be a male mate-guarding strategy to ensure paternity (Alcock, 1994); this is supported by the fact that copulation duration increases when the sex ratio is male-biased (Sillén-Tullberg, 1981; McLain, 1989; García-Gonzaléz & Gomendio, 2004; Wang *et al*., 2008; Himuro & Fujisaki, 2012).

Table 2.1. Table summarising studies to date considering pre- and post-copulatory sexual selection on morphological traits in the Lygaeidae.

Study	Species	Fitness measure	Evidence for selection on	No selection on
McLain, 1992	*Neacoryphus bicrucis*	Number of matings	Male body size	*n/a*
Tadler, 1999	*Lygaeus simulans*	Insemination success	Male processus length	*n/a*
Tadler *et al.,* 1999	*Lygaeus simulans*	Fertilisation success	Male asymmetry	Processus length
Yang & Wang, 2004	*Nysius huttoni*	Mating success	Female abdomen width, ovipositor length Male genital capsule width, phallus length, clasper length, antennal length	Male and female body length

Males may also attempt to reduce female remating rates between oviposition events; for example male *Togo hemipterus* have been shown to transfer accessory gland products to the female during mating which inhibit female remating (Himuro & Fujisaki, 2008). Female lygaeids are generally bigger than males (Burdfield-Steel & Shuker, 2014; but see Himuro & Fujisaki, 2011), and in insects female body size tends to correlate with fecundity (Honěk, 1993), so that male preference for large females may be a common pattern. Both mating itself, and also male harassment, has been shown to be costly to females in several species (McLain & Pratt, 1999; Shuker *et al*., 2006; Himuro & Fujisaki, 2010).

Sexual selection has been investigated in only a few lygaeid species (**Table 2.1**). In *Nysius huttoni*, mating experiments have shown that females with wider abdomens and longer ovipositors gain more matings, whereas males with longer antennae and internal and external genitalia gain more matings (Yang & Wang, 2004; **Table 2.1**).

However there was no selection on male or female body length (Yang & Wang, 2004). Male mating success in this species is also influenced by the operational sex ratio (Wang *et al*., 2009). Another study found no evidence for direct or indirect benefits of

multiple mating to females (Wang & Davis, 2006). In the ragwort seed bug *Neacoryphus bicrucis*, males patrol small territories consisting of ragwort bushes, and attempt to exclude rival males and monopolise receptive females (McLain, 1984). By marking males in the wild, it was shown that males mate on average 0.8 times per day (McLain, 1989). Large males gain significantly more matings than small males (McLain, 1992; **Table 2.1**). Male mating success is also significantly influenced by the population sex ratio (McLain, 1992) and host patch quality (McLain, 1984; 1986). Finally, male-male competition has been suggested as the main function for the spiny forelegs seen in males of the lygaeid *Scolopostethus affinis* (Rodriguez, 2000).

2.2 Lygaeus equestris and Lygaeus simulans

In the next section I introduce the study species used in my thesis: *Lygaeus equestris* and *Lygaeus simulans.* I will first consider the main anatomical differences between these species, and then briefly describe the general ecology of both species in the wild. I will then focus in more detail on their reproductive behaviour and genital morphology.

2.2.1 Species differences

L. equestris and *L. simulans* are very closely related species, and thus are morphologically very similar: indeed *L. simulans* was only formally described as a separate species in 1985 (Deckert, 1985). Both species have a wide European and Central Asian distribution (Solbreck *et al.,* 1989), and co-occur across most of their range, although *L. simulans* does not appear to be present in Scandinavia or North Africa (Tadler *et al*., 1999; Rabitsch & Deckert, 2007). It has thus been suggested that *L. simulans* prefers a warmer and more continental climate (Deckert, 1985; Gusev & Tatarnikov, 1991). The fact that *L. simulans* was described so recently means that determining exactly which species was used in studies before 1985 is problematic,

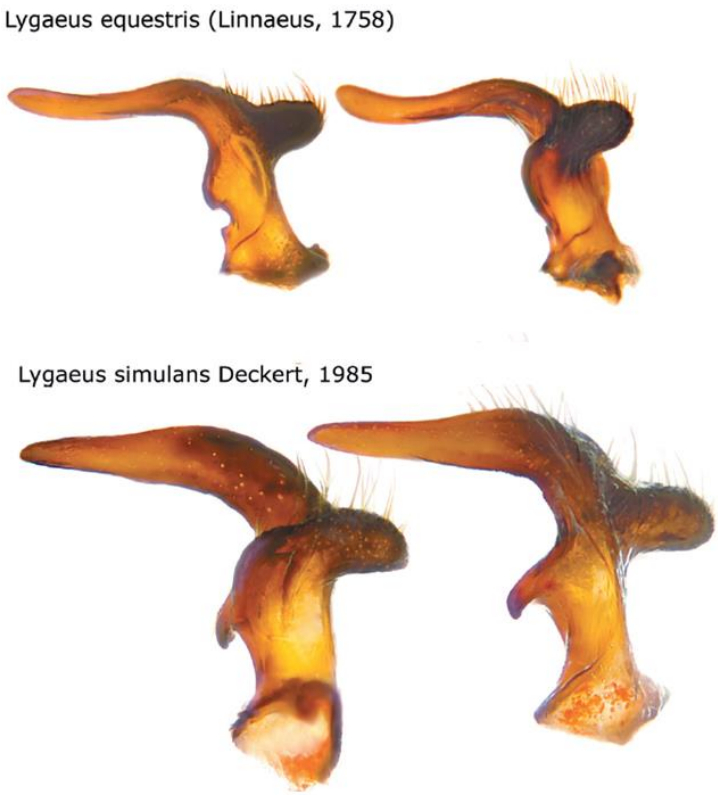

Figure 2.1. Comparison of male genital clasper morphology of *L. equestris* and *L. simulans*. The two species can most clearly be distinguished by the shape of the notch on the body of the clasper. Figure adapted from Rabitsch & Deckert, 2007.

though studies from Scandinavia (specifically the large body of work by Solbreck and Sillén-Tullberg in Sweden) were likely performed using *L. equestris* (Tadler *et al*., 1999).

The approximate time since divergence between the two species is not known, though this may have been as recent as the end of the last ice age in Europe (Burdfield-Steel & Shuker, 2014). The two species have been shown to produce fertile hybrids in the lab; however pre-mating isolation appears strong, so that obtaining crosses is difficult (Evans, 2011). However there seems to be little post-mating isolation, with hybrid crosses producing normal numbers of offspring (Evans, 2011). It is unclear if hybrids are produced in the wild, though this may be rare due to such strong pre-mating barriers.

As suggested by the only very recent classification as separate species, *L. equestris* and *L. simulans* look and behave almost identically. It is very difficult to tell the species apart by the naked eye, though Deckert (1985) describes small difference in the antennae, the size of the red colouration on the head and hairs on the back of the scutellum. However the species are most easily distinguished based on the external genitalia (Deckert, 1985; Gusev & Tatarnikov, 1991): in *L. simulans* the notch on the base section of the clasper is much larger (**Figure 2.1**). The rest of the reproductive anatomy is almost identical however (*pers. obs*.), as is the mating behaviour, and so the remaining descriptions below apply to both species.

2.2.2 Life cycle and general ecology

Nymphs are born in the summer at feeding sites, and mature over several weeks. Development in the Hemiptera is hemimetabolous, with several nymph stages of increasing size separated by successive moults (Snodgrass, 1935). *L. equestris* and *L. simulans* show five nymph stages and an adult stage, and adults are around 1 cm in length. Development occurs in late summer, and immature adults then enter reproductive diapause, which is triggered by short day lengths (Solbreck & Sillén-Tullberg, 1981). Adults overwinter in large aggregations (Solbreck & Sillén-Tullberg, 1990a; **Figure 2.2a**). Maturation occurs the following spring, which is accompanied by migration back to feeding sites, where mating and egg-laying also occur (Sillén-Tullberg, 1981). In the wild adults may thus live for several months (Solbreck *et al.,* 1989). It is not clear if males attract females to suitable host plants as in other lygaeids (Burdfield-Steel & Shuker, 2014). Clutch size is around 20 eggs, and females may lay over a thousand during their lifetime (Solbreck *et al.,* 1989). Eggs are laid in the soil at the base of host plants. In Northern Europe both species are monovoltine, whereas in lower latitudes there may be more than one generation per year (Solbreck & Sillén-Tullberg, 1981).

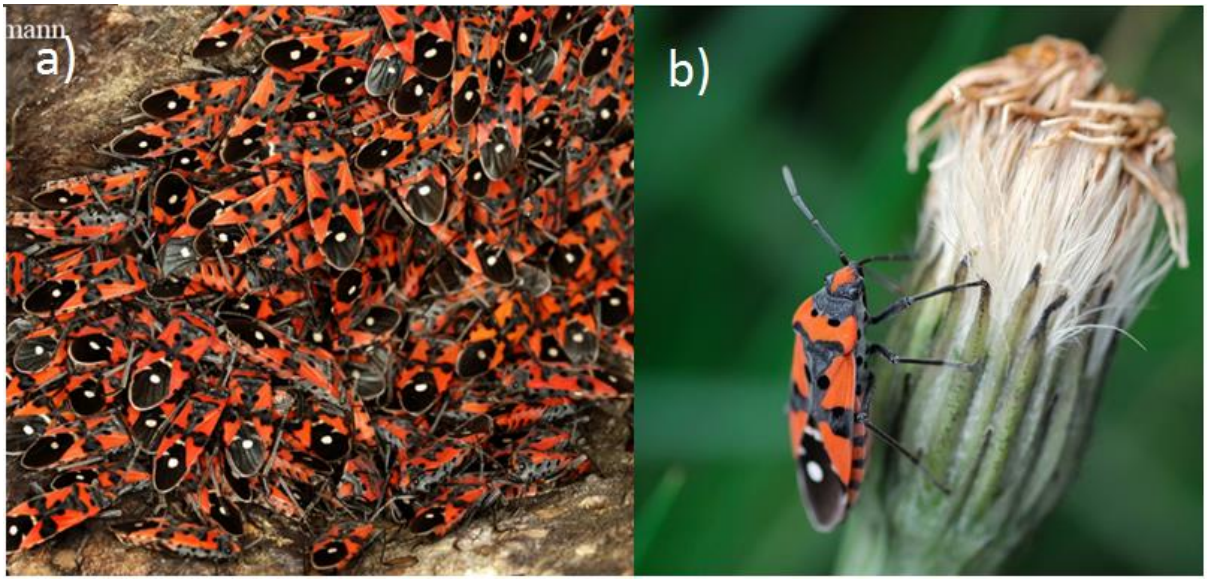

Figure 2.2: Photographs of *L*ygaeus *equestris* in the wild, showing **a)** an overwintering aggregation, and **b)** an individual feeding. Credits: E. Wachmann and Stefano Trucco.

The preferred host plant of *L. equestris* and *L. simulans* is *Vincetoxicum hirudinaria* (Apocynaceae) (Solbreck *et al.,* 1989), also referred to as *Cynanchum vincetoxicum*, and commonly known as white swallow-wort. Adult bugs feed on the seeds as they develop on the flower (**Figure 2.2b**), and early-instar nymphs also feed on seeds on the ground (Solbreck & Kugelberg, 1972). Bugs are reliant on the seed production of bushes, and thus population density is highly variable both spatially, as plants tend to have a patchy distribution (Solbreck & Sillén-Tullberg, 1990a), and also temporally, as seed production varies seasonally (Solbreck & Kugelberg, 1972; Solbreck & Sillén-Tullberg, 1990b). For example, in Sweden the population size of *L. equestris* is highly variable across years (Solbreck & Sillén-Tullberg, 1990b). As predation is low (see below), the biggest cause of mortality is likely failing to find host plants, especially in early instar nymphs (Kugelberg, 1997). In the lab bugs can be raised on a variety of different seeds (Kugelberg, 1973), though *Vincetoxicum* is preferred if given a choice (Kugelberg, 1974). Though not well investigated, *L. equestris* and *L. simulans* are likely able to feed generally in the wild when the preferred host plant is unavailable, as suggested by the fact that *L. equestris* is occasionally reported as a crop pest (e.g. Horvath & Frank, 2002).

Adults of both species (and to a lesser extent nymphs) show the striking red and black colouration seen in many lygaeid bugs (Aldrich, 1997; **Figure 2.2**). This colouration is aposematic: individuals sequester toxic cardenolides from the host plant and so have a bitter taste (Sillén-Tullberg *et al.,* 1982). As such, *L. equestris* has been used as a model organism for the study of aposematism (Sillén-Tullberg, 1985; Sillén-Tullberg *et al.,* 1982; 2000; Hotová Svádová *et al*., 2013). Such defences mean that predation is low compared to undefended insect species, and they appear to be avoided by small passerines (Sillén-Tullberg, 1985). Experiments have shown that wild blackbirds (*Turdus merula*) and yellowhammers (*Emberiza citrinella*) in the Czech Republic will attack and kill *L. equestris*, but individual birds appear to avoid the bugs after a single encounter (Hotová Svádová *et al*., 2010). Parasites may be a greater threat to survival in the southern range, with populations from Sicily being parasitized by tachinid flies (Solbreck *et al*., 1989).

2.2.3 Mating system and reproductive behaviour

Mating rarely occurs at hibernation sites prior to spring migration, and instead occurs mainly at the feeding sites throughout the summer (Solbreck, 1972; Sillén-Tullberg, 1981). At both hibernation and feeding sites the bugs are gregarious (Solbreck & Kugelberg, 1972; Solbreck & Sillén-Tullberg, 1990a). At feeding sites, the mating system appears to be a form of resource-based polygyny, with males and females aggregating at food patches (Burdfield-Steel & Shuker, 2014), though there is no evidence that males defend territories as in other lygaeid species (e.g. McLain, 1984). It appears that both males and females may mate multiply during the breeding season, though direct estimates of individual mating rates are not available. In Sweden approximately 60% of adults were observed in copula at the peak of the breeding season in June and July (Solbreck, 1972). This suggests that individuals mate multiple times during the season, notwithstanding the fact that copulation duration is so long (so that mating pairs are much more likely to be seen). In the lab, females may mate

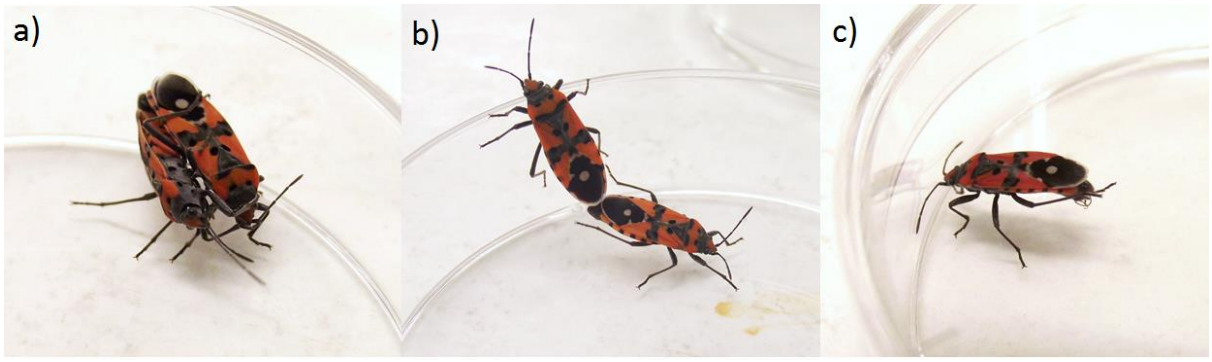

Figure 2.3. Three examples of *L. equestris*/*L. simulans* mating behaviour. Panels show **a)** a male (left) attempting to mate with a female, **b)** a pair in copula, and **c)** a male performing the 'handstand' behaviour, attempting to re-coil the processus into the genital capsule with his back legs following mating.

over 40 times during their lifetime (Kugelberg, 1973). Because of such multiple mating, both the risk and intensity of sperm competition (Simmons, 2001; Kelly & Jennions, 2011) is likely high.

Mating behaviour is relatively simple. Bugs do not appear to be aware of each other until physical contact is made. Once a male locates the female he will frequently attempt to copulate. There is no obvious courtship: copulation appears to depend on whether a male can overpower a female or not. He may proceed slowly, tapping the head and back of the female with his antennae, and then slowly climb onto her back. She can resist by slowly walking away, especially as the male will sometimes be facing the wrong direction. Usually however the male makes a sudden dash for the female from a few centimetres away and attempts to climb onto her back. Once he is on her back, he must line up so that he is facing the same way, and then move to the side to attempt to line up his genitalia, so that the claspers can open the female ovipositor (**Figure 2.3a**). Males also attempt to hook all three pairs of legs around the female's abdomen to stop her struggling. The female may have to resist these attempts quite vigorously if she does not want to mate. She resists by running away, 'bucking' her abdomen using her back legs, or kicking with her back legs.

Once the male intromittent organ has been properly inserted the pair will move round after a few minutes into the characteristic end-to-end mating position (**Figure 2.3b**). The pair usually remains still during mating, though the female may walk, forcing the male to follow backwards. Females are able to feed whilst in copula. The female frequently kicks the male with her back legs, and the male occasionally kicks back, though the function for kicking by either sex is unclear. The female may also buck her abdomen and rock side to side, and there is some evidence that the female may use this to shorten copulation (Sillén-Tullberg, 1985).

Copulation duration is extremely variable, and can be as short as 30 minutes, to reportedly up to 24 hours (Sillén-Tullberg, 1981; Shuker *et al*., 2006). However, most copulations last less than 8 hours (Shuker *et al.,* 2006), and duration within this range is generally bimodal, with peaks in frequency at around 2 hours and 6 hours observed for *L. equestris* (Shuker *et al.,* 2006). Very long copulations have been theorized to be a form of post-copulatory mate guarding (for review see Alcock, 1994), as in *L. equestris* there is no difference in the number of eggs fertilized between long and short copulations, and fertilization rate appears to be greatest in the first hour of copulation (Sillén-Tullberg, 1981). There is also no difference in receptivity in females after long or short copulations (Sillén-Tullberg, 1981). Long copulation may therefore be a male tactic to prevent rival inseminations, with the male acting as a 'living mating plug' (Sillén-Tullberg, 1981). This may be favoured as multiple mating by females between oviposition events seems high, and there is strong last-male sperm precedence (Sillén-Tullberg, 1981). Alternatively, during long matings males may be additionally transferring non-sperm ejaculate components to the female, such as accessory gland proteins (e.g. Chapman, 2001; Perry *et al*., 2013). It is unknown whether such accessory gland products are transferred in either species, or whether they are used to manipulate the female in any way (e.g. Himuro & Fujisaki, 2008).

The end of copulation does not seem to be preceded by any stereotypical male or female behaviour, and the final detaching and removal of the male aedeagus happens

very quickly. Once the genitalia uncouple, the male can be seen 'rolling up' his aedeagus using his back legs (**Figure 2.3c**). This can take several hours in some cases. There is no evidence of a female refractory period: females have been observed remating after as little as 15 minutes, and often with the same male.

Several males may attempt to copulate with a female at once; this can lead to 'mating balls' with up to four or five males climbing on a single female. Males will also continuously attempt to copulate with a female even if she is already mating. However, it seems males have no way of separating a pair once in copula. I have seen no evidence of direct male-male aggression, apart from the occasional misdirected copulation attempt. Therefore the main competition is indirect, caused when multiple males attempt to mate with a female at once.

To date there have been no studies on pre-copulatory sexual selection or mate choice in *L. equestris* or *L. simulans*. This is the subject of Chapter 3. It is unclear if male or females exhibit active mate choice, or if sexual selection arises primarily via sexual conflict, with selection favouring traits that allow either sex to control copulation (Arnqvist & Rowe, 2005). There appear to be significant costs of mating to females in *L. equestris*, as multiple mating has been shown to reduce both female longevity and fecundity (Shuker *et al*., 2006). However, post-copulatory processes have been studied in more detail in these species (Tadler, 1999; Tadler *et al*., 1999). Specifically, stabilising post-copulatory selection on male intromittent organ length (see below for anatomical details) has been detected in *L. simulans* (Tadler, 1999; **Table 2.1**). Sexual selection on male intromittent organ length is discussed in more detail in Chapter 5.

2.2.4 Reproductive anatomy

In this section I describe the reproductive anatomy of both males and females in detail, beginning first with the male external genitalia.

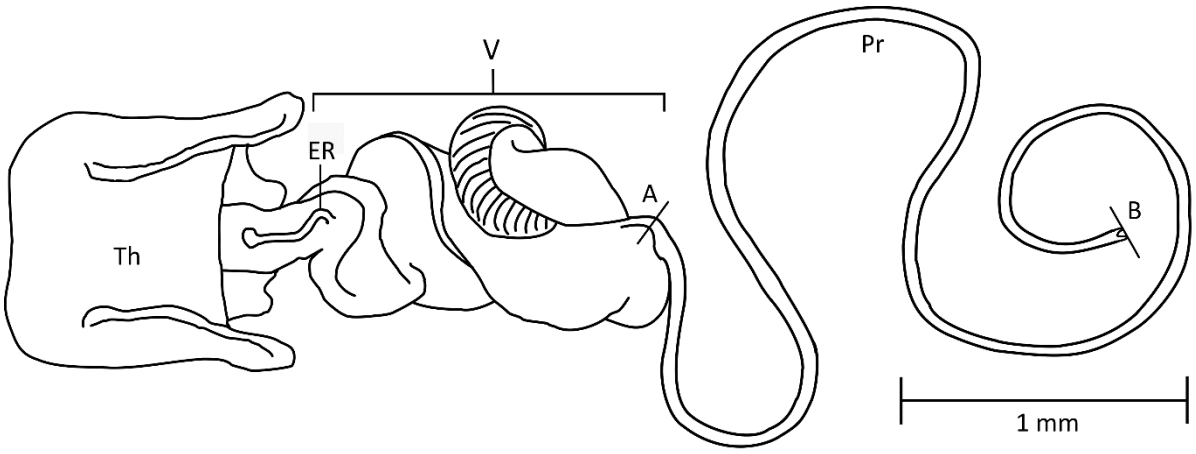

Figure 2.4. Male genitalia of *L. equestris* and *L. simulans*. Processus length was measured from the 'turning point' (Point A) to the tip (Point B). Abbreviations: Th: Theca, V: Vesica, ER: Ejaculatory reservoir, Pr: Processus.

At rest the male intromittent organ is stored inside the male genital capsule, which in the Heteroptera is a modification of the last three abdominal segments (Bonhag & Wick, 1953). At the dorsal surface of the genital capsule are the paired external genital claspers (or *gonostyli*; Bonhag & Wick, 1953). The claspers are folded at rest but are under musculature control with a wide range of movement. Clasper morphology is the easiest way to distinguish between the species (**Figure 2.1**).

The male intromittent organ (in insects this is also referred to as the phallus or aedeagus; Deckert, 1990) consists of two distinct parts (**Figure 2.4**): a soft proximal region which I refer to as the vesica (following Deckert, 1990), and a much longer distal processus (or processus gonopori: Ludwig 1926; Ashlock, 1957; Deckert, 1990). The processus is a long, thin sclerotized tube, with no vascularisation or musculature (Ludwig, 1926). At rest the processus is coiled around the vesica, and the entire aedeagus is stored inside the male genital capsule. Average processus length is also consistently different between *L. equestris* and *L. simulans* (approximately 7.2 mm and 6.8 mm respectively; see Chapters 3 & 5). In both cases however this is over two-thirds male average body length. The male processus is negatively allometric (the regression

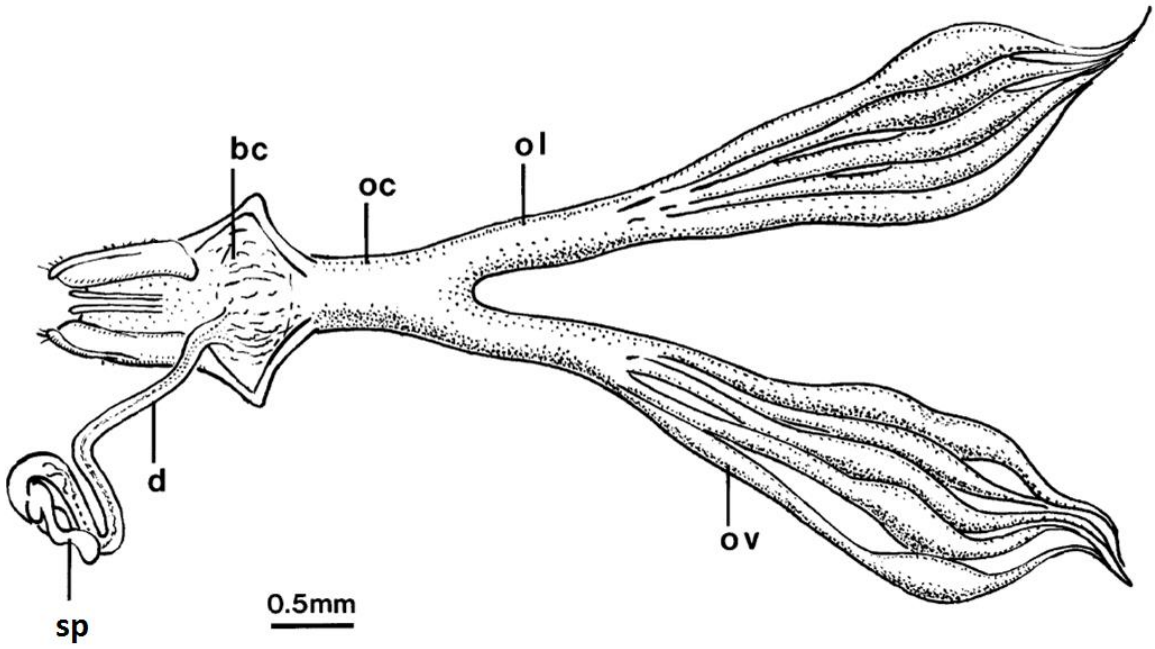

Figure 2.5. Female reproductive anatomy of *L. equestris* and *L. simulans*. Abbreviations: sp: spermatheca, bc: bursa, d: spermathecal duct, oc: common oviduct, ol: lateral oviduct, ov: ovary. Figure adapted from Gschwentner & Tadler, 2000.

of processus length against body length has a slope that is less than one) despite its great length (Higgins *et al*., 2009); though note that this is typical for genital traits (Bonduriansky, 2007; Eberhard, 2009).

The female reproductive tract has been described in detail for *L. simulans* (Gschwentner & Tadler, 2000; **Figure 2.5**), and the morphology is identical in *L. equestris* (*pers. obs*.). The ovipositor opens into a cavity known as the bursa (*bursa copulatrix*; Gschwentner & Tadler, 2000). The bursa opens proximally into the paired oviducts, from which mature eggs are released. The dorsal wall of the bursa opens into a very narrow spermathecal duct, at the end of which is the sperm storage organ or spermatheca (Gschwentner & Tadler, 2000). The spermathecal duct ends first in a valve, and beyond that a tightly coiled corkscrew region at the entrance to the spermatheca that the aedaegus is unable to pass (Gschwentner & Tadler, 2000; **Figure 2.6**). The valve at the entrance to the spermatheca may be to prevent backflow of

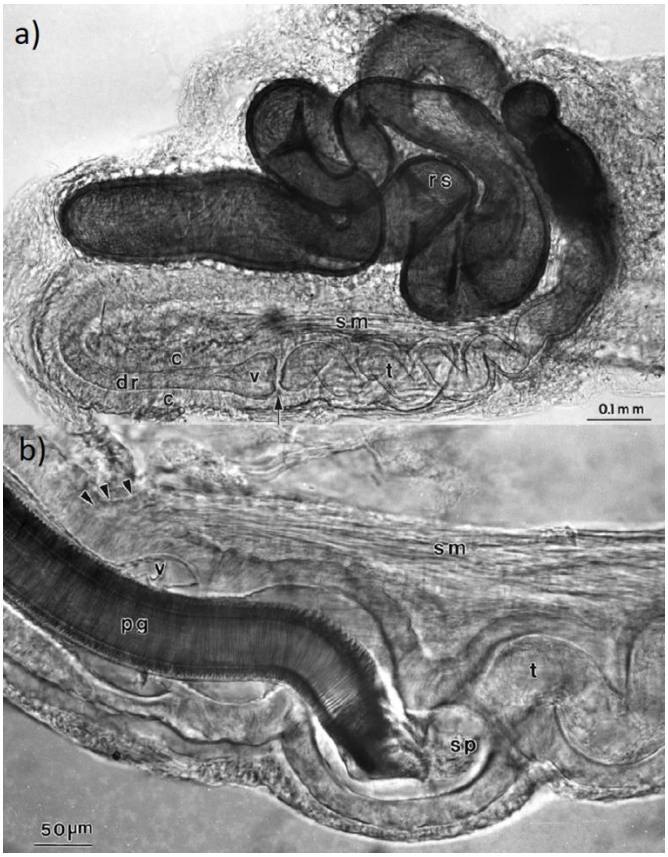

Figure 2.6. Female reproductive tract anatomy in *L. simulans*. **a)** the female spermathecal duct and spermatheca; **b)** the male processus at the entrance to the spermatheca. Abbreviations: rs: spermatheca, dr: spermathecal duct, c: thick wall of spermathecal duct, v: valve at entrance to the spermatheca, sm: spermathecal muscle, t: corkscrew section at entrance to the spermatheca, pg: male processus, sp: male ejaculate. Adapted from Gschwentner & Tadler, 2000.

sperm into the spermathecal duct. However the walls around the valve appear to be muscularized, suggesting the female may be able to control its action, though this has not been shown experimentally (Gschwentner & Tadler, 2000). The spermatheca is irregularly coiled and blind-ended; sperm are stored and then released back along the spermathecal duct to fertilise eggs during oviposition (Snodgrass, 1935, but see Chiang, 2010). The spermatheca also appears sclerotized and cannot be compressed; therefore it likely does not expand when filled with sperm.

During mating the male and female genitalia interact in several ways. First, the male claspers are used to open the female ovipositor, and also to hold the female in place

during copulation. Once coupling is achieved, the aedeagus is expanded via fluid pressure from the ejaculatory reservoir and forced out of the genital capsule and into the female bursa. The processus must then find the entrance to the spermathecal duct on the roof of the bursa and enter. The processus then moves along the spermathecal duct. Note that the spermathecal duct is approximately 1.9 mm long in *L. simulans*, which is much shorter than the average length of the processus (Gschwentner & Tadler, 2000). According to Gschwentner & Tadler (2000), the spermathecal duct expands significantly during mating, up to around 6 mm in length (but see Chapter 6). The processus needs to reach past the valve at the entrance to the spermatheca in order for insemination to occur successfully (Gschwentner & Tadler, 2000). This takes at least 30 minutes, and thus places a lower bound on the time needed for successful insemination during mating (Micholitsch *et al*., 2000).

Sperm are motile and released free (i.e. not packaged in a spermatophore) as in other lygaeid species (Dallai & Afzelius, 1980; Tadler, 1999; Werner & Simmons, 2008a). Previous work has suggested that sperm transfer is fastest during the first few hours of copulation, which supports the post-copulatory mate guarding hypothesis (Sillén-Tullberg, 1981). Sillén-Tullberg (1981) also showed that there is strong last-male sperm precedence in *L. equestris*, with an average P2 (proportion of eggs fertilised by the second male) of 90%.

2.3 General methods

In this section I describe the general experimental methods used throughout my thesis. I focus on methods used in more than one chapter, with the intention being to consider these methods here so as not to repeat myself in later chapters. However note that I do provide a brief description of the experimental methods used in each chapter in their respective methods sections. I also describe more specific methods applicable to single chapters (for example clasper measurements; Chapter 7) in detail in the relevant chapters. In this section I first consider the general husbandry methods

for maintaining the study populations in the lab, before describing the general methodology for behavioural mating trials.

2.3.1 Insect husbandry

All experiments presented herein were performed using individuals taken from lab populations in continuous culture. The *L. equestris* population originates from individuals collected in the Dolomites region of Northern Italy in 2004 (Shuker *et al*., 2006). The *L. simulans* population originates from individuals collected in 2008 and 2009 from the Pratomagno region of Tuscany in Central Italy (Evans, 2011). Thus the individuals used in all experiments were derived from populations that had been in the lab for at least six years for *L. equestris* and at least three years for *L. simulans*. This corresponds to between 150 and 300 generations in the lab (at 29°C the generation time of both species is four to five weeks: Shuker et al, 2006). This has likely resulted in significant adaptation to life in the laboratory.

All populations are kept in large incubators at a constant temperature of 29 °C (**Figure 2.7a**), which serves to reduce the generation time significantly, though this is naturally much higher than both species would normally encounter in the wild. Humidity is maintained at ambient levels. Incubators are set to a 22:2 hour light/dark cycle. This is to prevent the induction of reproductive diapause: an 18:6 hour light/dark cycle in the lab leads to more than 85% of individuals entering diapause (Solbreck & Sillén-Tullberg, 1981). In the lab, egg laying to adult eclosion takes around 20 days at 29 °C, and adults are reproductively mature after a further seven days (Evans, 2011).

Stock populations are kept in large plastic containers (16.5 x 23 x 11 cm) with two large holes cut in the top and covered by a fine mesh to allow air flow. These containers contain sunflower seeds, two water tubes and a layer of cotton wool to provide shelter and substrate for oviposition (**Figure 2.7b**). These stock containers usually contain several hundred individuals at any one time, though population size can fluctuate.

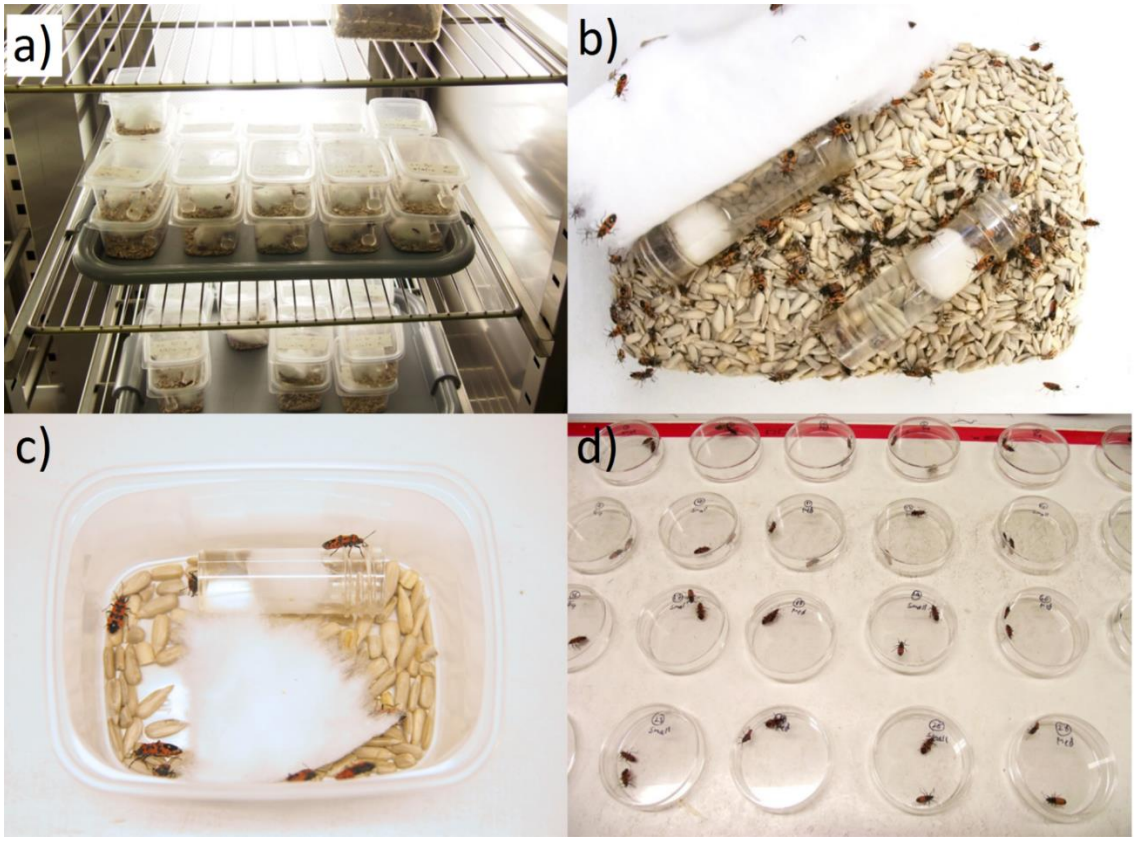

Figure 2.7. Experimental setup used for rearing bugs and performing experiments. Panels show **a)** small tubs stored on trays in an incubator, **b)** a population of *L. equestris* in a newly-created stock container, **c)** close-up of bugs in experimental tubs, and **d)** example of several dishes being observed during a mating trial.

Individuals of all developmental stages and both sexes are present. If maintained these containers provide a constant supply of new nymphs. At any one time there are at least four replicate containers of the two species in the lab. Populations are moved to a new container, with fresh seeds and water, every six to eight weeks. During this process, a random sample of around 100 individuals are taken from the existing container and moved to the new. Individuals of all ages are taken in order to maintain overlapping generations. Individuals are also taken randomly from at least one other replicate stock container every time a container is changed over, so that all replicates are essentially part of a single, large population containing upwards of 500 individuals at any one time. Bugs are moved between containers either using a large pooter or stork-bill forceps.

All stock and experimental individuals are maintained on a diet of de-husked, organic sunflower seeds (Goodness Direct, UK). De-husking is required as early-instar nymphs are unable to penetrate the thick outer coating with their proboscis (Kugelberg, 1973). Food is provided *ad libitum* as a substrate on the base of the container. In the stock cages seeds are provided in a layer 1-2 cm thick, whereas in individual tubs a single layer of seeds is provided. For experimental tubs a layer of seeds is provided and this is usually not changed for the duration of the experiment (which may be several weeks). Though it has not been quantified directly, individual bugs appear to consume very little food throughout their lifetime. For example, 1g of seeds (approx. 20 seeds) per five nymphs did not significantly reduce adult size or body mass during development (Dougherty, unpublished data). Therefore I am confident that food is never a limiting factor in stock or experimental individuals.

Though sunflower seeds are not the natural diet of *L. equestris* and *L. simulans*, a diet of sunflower seeds does not appear to affect development time or adult size in comparison to milkweed seeds (Kugelberg, 1973). Agricultural sunflower seeds are highly nutritious, and this probably explains why *L. equestris* may be found as a pest species of sunflowers (Horvath & Frank, 2002). However, this means that lab-reared bugs do not have any chemical defences, unlike bugs in the wild. It is not clear if toxicity influences behaviour in any way, though in *L. equestris* mating frequency was significantly lower for males fed milkweed seeds compared to males fed sunflower seeds (Burdfield-Steel *et al*., 2013).

The main limiting factor on bug development is the amount of drinking water available. This is because bugs are unable to feed unless they can produce sufficient amounts of saliva (Schuh & Slater, 1995). As a result, adults may starve after several days without water. All bugs are provided with distilled water in plastic tubes stopped with cotton wool, with the size of the tube varying depending on the number of individuals in the container. Bugs drink with their proboscis via the cotton wool which

remains damp as long as water is in the tube. In all containers water tubes are replaced once a week. For small experimental tubs this assures that no water tubes are less than one-half full.

Prior to experiments, individuals are usually taken as nymphs (usually 4th or 5th instar) from the stock containers, and moved into smaller 'nymph boxes' (20 x 10 x 8 cm) without sexing (this is difficult to do by eye before the final moult). These tubs are then checked every two days for newly-eclosed adults, which are then sexed and moved to same-sex tubs to ensure virginity. For experiments, individuals are kept in small plastic deli tubs (11 x 8 x 5.5 cm) with several air holes punched into the lid (**Figure 2.7c**). These tubs again contain a layer of sunflower seeds, a single small water tube, and a piece of cotton wool substrate. Prior to mating trials tubs usually contain eight to ten individuals. The majority of males and females are reproductively mature after seven days (Evans, 2011), and are used in mating trials at between 7 and 14 days old. Following trials, mated females are also kept in such tubs in order to assess offspring production. If large numbers of nymphs are present, tubs need to be changed once a week to prevent mould building up. Bugs are moved between tubs using stork-bill forceps and are handled as little as possible.

2.3.2 Mating trials

I present experiments involving mating trials in Chapters 3, 5, 6 and 7. All mating trials are performed in small plastic vented petri dishes (55 mm diameter). Dishes are placed on the lab bench and at room temperature and under ambient lighting (**Figure 2.7d**). Room temperature is usually between 22 and 25 °C. Dishes contain different numbers of males and females depending on the choice design used. Trials were usually started in the morning (between 9 and 10 am), primarily in order to give pairs enough time for very long copulations. No trials were performed for longer than ten hours. In order to achieve the appropriate sample sizes many dishes were usually observed at once. Depending on the availability of subjects this ranged from a minimum of 5 dishes to a

maximum of 40 dishes, although usually around 20-30 dishes were observed at a time. The exact length of the trials, observation period and number of dishes observed varies depending on the experiment.

I performed two types of observations. Usually trials start with a period of continuous observation, which usually lasts for two hours, during which time I do not leave the bench. This is used to assess the general activity of the experimental subjects. During these observations I record whether pairs are in copula, which I use to determine copulation duration. A pair is classed as in copula once they are seen in the end-to-end mating position, and intromission has been achieved. In some cases the pair forms the characteristic mating position despite aedeagus intromission not being achieved (the aedeagus can clearly be seen outside the body at the point of genital contact). This can be seen without disturbing the pair, and is classed as an 'improper coupling'. This usually leads to the copulation being broken off after less than an hour (though I have observed one such coupling last over seven hours, with the male aedaegus visible throughout). In almost all cases females were restricted to a single mating in order to gain an accurate measure of male insemination success. I classed copulation as 'sufficient' if it was at least 20 minutes long (or two consecutive ten-minute checks; see below). This is probably slightly conservative, as it reportedly takes at least 30 minutes to achieve successful insemination in *L. simulans* (Gschwentner & Tadler, 2000). However I chose this in order to be sure that females were restricted to a single, potentially successful copulation. Anything less than 20 minutes was thus considered 'short', and as sperm transfer is not possible these were not considered viable copulations. Therefore when considering female fertility a pair was allowed several 'short' copulations, but only a single 'sufficient' copulation.

During continuous observations I also recorded the number of male mating attempts a female received. A mating attempt is characterised by the male mounting or grabbing the female, and curling the underside of his abdomen toward the female. Such attempts may be brief or may last several minutes; however unless the male stops or

lets go of the female this is classed as a single attempt. Attempts are also frequently aimed at the wrong place (such as the head instead of the female genitalia) or the wrong individual (such as nymphs or other males). For most chapters the abdomen curling was the main criteria for a mating attempt (though note a stricter criterion was used in Chapter 7).

When more than one individual of a sex is present in a dish (such as in Chapters 3 and 7), each individual was marked prior to the trial using enamel paint (Plasti-Kote® 'Projekt paint' Fast Dry Enamel). This was done by first sedating bugs by placing in a -13 °C freezer for three minutes. Individuals were then marked on either the right or left side of the pronotum with paint using a fine paintbrush (see **Figure 2.8**). Individuals with alternately-marked sides were then used in a given dish, and this allows clear identification of individuals during mating trials.

After the initial observation period there usually followed an extended period of intermittent checks on dishes, during which I recorded whether pairs were in copula or not (and that the aedeagus is properly inserted), to determine copulation duration. For Chapter 3 these checks were performed every 30 minutes. For later experiments this was reduced to a check every ten minutes, in order to reduce the likelihood of by chance observing two short matings 30 minutes apart.

At the end of the mating trials any mating pairs were separated manually by brushing with a fine paintbrush, which stimulates the male and female to detach (but does not damage the genitalia in any way). Males and females may then be kept for use in further experiments or euthanized as needed.

2.3.3 Measuring genitalia

I discuss sexual selection on male processus length in Chapters 5, 6 & 7. The protocol for obtaining processus measurements is as follows. After mating trials, all males were

Figure 2.8. Two *L. simulans* males marked on opposite sides of the pronotum with enamel white paint.

euthanized and the male genitalia were measured in the following way (following Higgins *et al*., 2009). The male genital capsule was opened with fine forceps, the aedeagus was uncurled and then the processus was cut away from the vesica above the 'turning point' (Tadler, 1999). The processus was then carefully placed onto a microscope slide covered in a small square of double-sided sticky tape. Care needs to be taken to not break the processus at this stage. A cover slip was then carefully placed over the processus, and pressure applied to stick it down. Photos of the processus slides were taken using an Olympus SZX10 stereo microscope and attached camera (**Figure 2.9**). Length measurements were obtained using the image analysis program Cell^D (Soft Imaging System, Olympus Corp.), using the polyline tool, after measurements were calibrated using a calibration image. The processus was measured from the middle of the 'turning point' (Ludwig, 1926) to the tip (point A to B in **Figure 2.4**), following (Tadler, 1999).

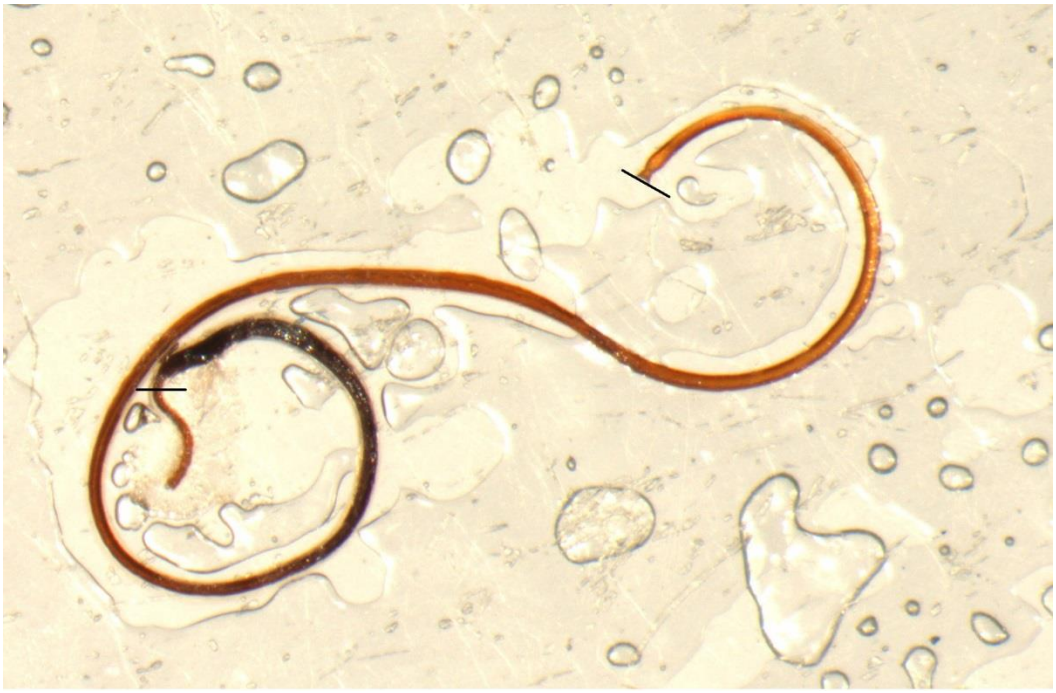

Figure 2.9. An example of a male processus removed and placed onto a microscope slide. The shape of the tip can be seen clearly. The length was measured between the two lines shown.

2.3.4 A note on the species used in each chapter

At some point in early 2013 the *L. equestris* populations used in the lab appear to have been accidentally mixed up with the *L. simulans* populations. This was first identified in late 2014. As noted above, *L. equestris* and *L. simulans* cannot be identified to the species level with the naked eye. By checking the species identity of samples kept in storage (by comparing male clasper shape) I was able to confirm that all experiments were performed using a single species; in other words a mix of species was not used in either experiment. The experiments using *L. equestris* (Chapters 3 and 5) were performed in early 2012, and the experiments using *L. simulans* (Chapters 5, 6 & 7) were performed in 2013 and 2014.

Chapter 3

Pre- and post- copulatory sexual selection on male and female size in *Lygaeus equestris*

Abstract

Here I quantify the effect the social environment has on the strength of sexual selection on male and female morphology in *L. equestris*. I performed mating trials in which I varied the amount of choice presented to each sex, giving four choice treatments: no-choice, male choice, female choice and mutual choice. Overall I find evidence for significant positive directional selection on female body length and stabilising selection on an overall measure of male body size. However, there was no significant effect of choice design on the patterns of sexual selection for males or females. This may be due to the method of mate assessment in *L. equestris,* which appears to be primarily via contact cues, which may limit simultaneous comparison between options.

Acknowledgments

I thank Darren Parker for help with the statistical analysis performed in this chapter.

3.1 Introduction

Sexual selection arises via competition for access to mates, and this can take the form of intra-sexual competition, in which males compete for access to females, and inter-sexual competition, in which females choose to mate with the most attractive males (Darwin, 1871; Andersson, 1994). Selection is thus intimately tied to the social environment: individual mating success (and ultimately population-level sexual selection) depends on how many rivals or mating partners are available. This social environment may vary in both space and time, and thus sexual selection may change over these dimensions (Miller & Svensson, 2014).

In the mate choice literature the number of options presented to the subject during a choice experiment is referred to as the 'choice design' (or choice paradigm). Tests can use either no-choice or choice designs (Wagner, 1998). In a no-choice test each subject is presented with a single stimulus. In contrast, in a choice test each subject is given a choice between multiple (usually two) stimuli presented simultaneously. Such choice designs differ in the number of individuals of each sex present, and this may influence the strength of sexual selection in several ways. First, it may influence the degree of competition between individuals for access to the opposite sex. If sexual selection primarily arises by intrasexual competition, for example in the case of selection on weapons used in contests for access to mates (Emlen, 2008), then this selection will be absent when no rivals are present (a no-choice design) but should be detectable when rivals are present (choice design).

However, choice tests do not only allow intrasexual competition: they may also influence the strength of mate choice, as the two designs differ in whether mates can be directly compared or not (Wagner, 1998). This influences the ability of the subject to compare options: simultaneous comparison may be easier than sequential comparison for some species, or vice-versa. It may also influence the type of preferences observed, as no-choice designs measure absolute preferences, whereas

choice designs measure relative preferences (Wagner, 1998). No-choice tests also differ from choice tests in that the perceived mate encounter rate is lower, and mate availability may influence choosiness (Werner & Lotem 2006; Barry & Kokko 2010; Booksmythe *et al*., 2011). It seems likely that one or all of these factors may lead to differences in the strength of sexual selection observed in each choice design.

Several studies have investigated how social environment influences the intensity or shape of sexual selection (Coyne *et al*., 2005; Head *et al*., 2007; Kasumovic & Andrade, 2008; Miller & Svensson, 2014). However, in none of these studies was the effect of choice design explicitly tested statistically, for instance by testing for an interaction between choice design and a trait presumed to be the target of sexual selection. Studies in natural populations have shown that, as predicted, sexual selection on males is higher at lower population densities, in which monopolisation of females is possible (e.g. Conner, 1989; McLain, 1992; Arnqvist, 1992b; Blanckenhorn *et al*., 2004). But even differences in the presence or absence of members of the opposite sex can be important. For example, Procter *et al*. (2012) compared the form and strength of sexual selection arising from male contests in the coreid bug *Narnia femorata*, and showed that male overall size and leg area was much more important in deciding contests when a single female was present compared to when no females were present. In the cricket *Teleogryllus commodus*, males harass females in order to delay the removal of the spermatophore, as removal leads to fewer sperm being transferred to the female (Hall *et al*., 2008). When a single male was kept with a female following mating, spermatophore attachment time was longer, and this led to a reduction in the opportunity for sexual selection (a measure of the potential for sexual selection derived from the variance in mating success; for review see Klug *et al*., 2010) and weaker selection on male courtship song and body size, compared to when the male was absent (Hall *et al*., 2008).

In this chapter I test how sexual selection varies with social context using *Lygaeus equestris* as the study species. To date there have been no studies on pre-copulatory

sexual selection or mate choice in *L. equestris* or *L. simulans*. However I can make some predictions of what traits may be important for male and female mating success based on observations of mating behaviour. For example, females appear to have to put up strong resistance from males in order to prevent mating. Both male harassment and also mating itself has been shown to be costly to females in *L. equestris*, with females kept in isolation having significantly higher longevity and lifetime fecundity compared to females kept with males (Shuker *et al*., 2006). This raises the possibility that sexual selection in both males females in *L. equestris* and *L. simulans* may arise through sexual conflict, with females evolving traits to resist mating attempts and males evolving traits to overcome female resistance (Arnqvist & Rowe, 2005). For example, male body size may be important in copulatory struggles (e.g. as seen in water striders, e.g. Arnqvist, 1992a; Sih & Krupa, 1992; Danielsson, 2001) as females are generally larger than males. Males use all three pairs of legs to grab hold of females during such struggles (Chapter 2), and thus male leg length may also be important. Conversely, female body size and leg lengths may also be important in resisting such attempts. Furthermore, in insects female body size typically correlates strongly with fecundity (Honěk, 1993), so that males may exhibit a mating preference for larger females. I therefore predict linear pre-copulatory selection on both male and female body size, and male leg length. Finally, if antennae are important for mate assessment, there may be selection on antennae morphology in both sexes.

In this study I: 1) quantify the strength and shape of pre- and post-copulatory sexual selection on male and female morphology in *L. equestris*, and 2) explicitly test how this selection varies depending on experimental choice design. I performed mating trials in which I varied the amount of choice available to both males and females, by presenting individuals with either one or two individuals of the other sex. This gave four experimental treatments: 1) no-choice for either males or females (1 male and 1 female per dish); 2) female choice only (2 males and 1 female per dish); 3) male choice only (1 male and 2 females per dish); and 4) mutual mate choice (2 males and 2 females per dish). I then recorded male and female body size, leg length (all three

pairs) and antennae length. I used mating, and also subsequent female fertility, as proxies for fitness. If the experimental choice design influences sexual selection, my key prediction is that there should be a significant interaction between experimental choice design and selection on morphological traits. Specifically, I predict that sexual selection on both males and females will be stronger in the choice treatments compared to the no-choice treatment. Comparison of sexual selection resulting from all four experimental choice designs coupled with behavioural observations should allow me to start to disentangle the effects of choice, competition and conflict on mating success in this species.

3.2 Methods

3.2.1 Mating trials

All adults were marked with paint at least one day before mating trials. Individuals were marked on either the right or left side of the pronotum, so that when there was more than one individual of a sex in a dish individuals marked on alternate sides were used. The side that each individual was marked on can be clearly seen during mating trials. In addition, a subset of no-choice trials (treatment 1) was also performed using unmarked males and females, to check for behavioural differences between marked and unmarked individuals.

All adults used in mating trials were seven days old. Trials were performed as described in Chapter 2. On the morning of a trial individual males and females were assigned randomly to one of four experimental treatments (**Figure 3.1**), either: 1) no choice, 2) female choice, 3) male choice, or 4) mutual choice. For the mating trials all dishes were watched continuously for two hours. For each dish the number of mating attempts performed by each male was scored, and towards which female the attempts

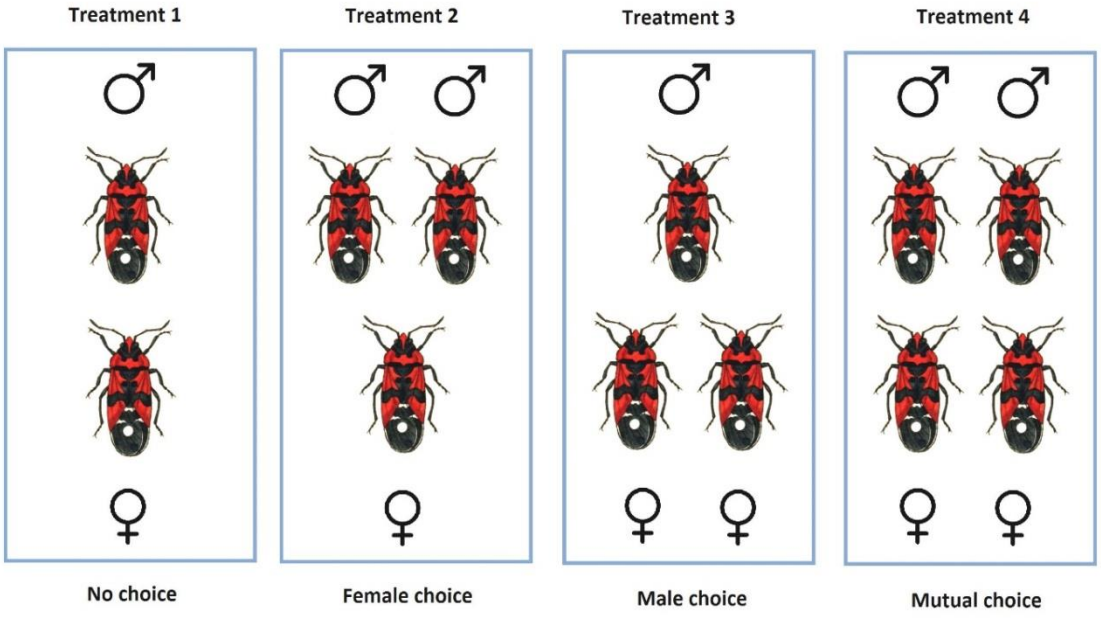

Figure 3.1. The experimental choice designs used in the experiment. Individuals were randomly placed in small plastic dishes in one of four experimental treatments: 1) no-choice treatment, 2) female choice treatment, 3) male choice treatment, and 4) mutual choice treatment.

were directed. I also recorded the latency to mate as time elapsed since the start of the trial. Finally, I recorded which pairs were in copula. After two hours any non-copulating individuals were removed from the dishes and frozen. Copulating pairs were left in their dish and checked every 30 minutes until they separated naturally, or up to a maximum of six hours (eight hours since the start of the trial), when they were separated manually with a fine paintbrush (these were classed as 'long copulations', see below). I classed copulation as 'sufficient' if a pair was observed in copula for longer than 20 minutes, or for two consecutive 30 minute checks.

All mated males were immediately frozen following trials. Mated females were placed into individual tubs for two weeks to allow oviposition. These female tubs were then checked every day for the presence of nymphs or fertile eggs to ensure insemination was successful. All mated females and nymphs were frozen after two weeks.

3.2.2 Morphometric measurements

After all trials were finished, the following morphometric traits were measured for all individuals used in mating trials: total body length, antennae length, and tibia and femur lengths for all three legs (prothoracic, mesothoracic and metathoracic) on the left hand side (when viewed dorsally). All lengths were measured using a dissecting microscope with a measuring graticule. Total body length was measured as the tip of the snout to the tip of the underside of the abdomen. Legs and antennae were removed from the body and laid flat before measuring. Sample size for morphological measurements was 613, comprising 303 males and 310 females, except for antennae length measures (and measures of 'overall size', see below), for which sample size was slightly smaller (605 in total, 300 males and 303 females).

To check the repeatability of the morphological measurements, the body length of 30 individuals was measured a second time, blind to the original measure. Repeatability was assessed using analysis of variance following Lessells & Boag (1987). Body length measures were found to be highly repeatable (r= 0.99).

3.2.3 Statistical analysis

Data on the observed number of mating attempts per male did not follow a Normal distribution (including after transformation), and so were analysed using non-parametric statistics. To control for the different numbers of individuals present in each treatment, one individual of each sex was randomly allocated as the 'focal' individual, so that each dish contained a single focal male and female. I used these focal individuals when analysing mating frequency and mating attempts data.

For treatments 2, 3 & 4 each dish contains two individuals of at least one sex. For these dishes the formation of a mating pair could influence the likelihood of mating for the unpaired individual, thus individuals of the same sex cannot be considered

independent of each other. In order to control for this in the analysis a mixed-model approach was used, fitting dish as a random effect (i.e. generalised linear mixed effects models, GLMM). I note that all models gave very small or negligible variance components associated with the random effect.

I tested for the effect of morphology on mating success using two main approaches. Given the strong correlations between morphological traits (see below), I first analysed the data in terms of sexual selection on male and female body length only. I used a generalised linear mixed model with a binary logistic response, with mating success as the response variable. To examine the effect of choice design, treatment was fitted as a main effect. Significant treatment-morphology interactions would suggest that the choice design influences the pattern of sexual selection.

Second, all morphological measurements were highly correlated with each other (**Table S26**). I therefore performed a principal component (PC) analysis in order to combine these correlated variables into fewer variables that can be analysed without the problem of collinearity. Principal components were extracted from all five morphological traits measured (body length, antenna length, prothoracic leg length, mesothoracic leg length, metathoracic leg length) for males and females separately. Principal component (covariance matrix) scores were extracted using the Anderson-Rubin method. For both males and females only one principal component had an Eigenvalue greater than one. For males, the first principal component explained 70.3% of the variance observed (Eigenvalue= 3.51). For females, the first principal component explained 71.5% of the variance observed (Eigenvalue= 3.57). For both males and females the principal component loaded heavily on all five morphological traits (all factor loadings above 0.7; **Table S27**), with body length loading highest (Loading= 0.95 for females and 0.91 for males). These components can therefore be seen as a measure of 'overall size'. I then repeated the mating success analysis as before, but using the principal component of overall size in place of body length for each sex. To

facilitate testing quadratic terms for overall size, PC values were made positive by arbitrarily adding 4 to all values.

To visualise the shape of selection I produced fitness surfaces using cubic-splines, which is a non-parametric method that can be used to visualise complex shapes (Schluter, 1988; Schluter & Nychka, 1994). Splines were derived from general additive models performed for each trait in isolation, with mating or insemination success as a binary logistic response. The smoothing parameter for the splines was obtained by minimizing the GCV score.

As copulation had to be interrupted for many pairs, I used a categorical measure of copulation duration depending on whether pairs ended copulation naturally or not: long copulations were those that had to be broken up manually at the end of the day, and short copulations were those that finished naturally. Matings shorter than 15 minutes were excluded from the analysis (sperm transfer takes at least 30 minutes; Micholitsch *et al*., 2000). There were no matings between 15 and 45 minutes in duration. I tested for determinants of copulation duration using a generalised linear model (GLM) with copulation class (short or long) as a binary logistic response variable, for all mated pairs, using male and female overall size as the only morphological traits in the model. I tested for determinants of female fertility in the same way, using a general linear model with female fertility (presence or absence of fertile eggs) as a binary logistic response variable.

I used a modified model simplification rationale in an attempt to balance the problem of multiple testing associated with model simplification (Whittingham *et al*., 2006; Mundry & Nunn, 2009) with the problem of over-parameterising models, especially when testing several interaction terms. As such, models were first fitted with main effects and any relevant interactions terms (including quadratic terms for the morphological characters of interest, to test for evidence of non-linear selection). Non-significant interactions and quadratic terms were then removed in a stepwise fashion,

with all main effects left in the final model regardless of significance, with the significance of remaining terms tested using type III sums of squares.

All statistical analyses were performed in SPSS Statistics 21 (IBM Corp., 2012), except for cubic spline plots and GLMM analyses which were performed in R version 2.15.1 (R Development Core Team, 2012). General additive models were performed using the R package MGCV (Simon Wood, 2012).

3.3 Results

Mating trials were performed using a total of 688 individuals: 344 males and 344 females. I observed 234 dishes in total: 69 dishes of the no-choice treatment and 55 dishes of the remaining three treatments.

3.3.1 Difference in marked and unmarked dishes

A subset of treatment 1 dishes contained unmarked individuals. Dishes with marked individuals had significantly more copulations (23 out of 35) than dishes with unmarked individuals (14 out of 34; Chi-squared test, χ^2_1= 4.18, *P*= 0.04). There was no significant difference between marked and unmarked males or females in body length or any of the other morphological traits measured (*t*-tests, all *P* > 0.05). There was also no significant difference in the number of attempts between marked and unmarked males (Mann-Whitney test, U= 2.3, *P*= 0.13), and no difference in the likelihood of making no attempts between marked and unmarked males (Yates' Chi-square test, χ^2_1= 0.09, *P*= 0.77).

3.3.2 Male mating attempts

There was no significant difference between treatments in the mean number of total attempts per male (Kruskal-Wallis test, H= 6.44, P= 0.09). However, the number of mating attempts females received varied with how many males and females were in each treatment (H= 50.82, P <0.001). Females received a median of 3 attempts in treatment 1 and treatment 4 (IQR= 1-6 in both cases), compared with a median of 6 in treatment 2 (IQR= 2.5-12.5) and a median of 1 in treatment 3 (IQR= 0-3).

3.3.3 Mating frequency

Mating trials resulted in 169 mated females (49% of total), with one female mating to two different males. However 54 females received no mating attempts. Therefore 58% of females that received mating attempts copulated. Focal mating frequency was similar for both males and females in the two equal sex ratio treatments (treatments 1 & 4, **Figure 3.2**). Focal male mating frequency was lower in treatment 2 (34%) compared to treatments 1, 3 and 4 (around 53%) (**Figure 3.2**), but the difference was not significant (Chi-square test, χ^2_3= 6.16, P = 0.10). Focal female mating frequency appears to reflect the differences in sex ratio between the treatments (**Figure 3.2**): mating frequency was significantly higher in treatment 2 (78%), where the sex ratio was male-biased; and lower in treatment 3 (31%), where the sex ratio was female-biased (χ^2_3= 25.13, P <0.001).

Mating latency did vary significantly among the four treatments (ANOVA: $F_{3,150}$=3.34, P = 0.021), with copulation taking longer to initiate on average in treatment 2 (approximately 39 minutes compared to 25-27 minutes in the other treatments); observations suggested that this was due to longer male struggles for access to the lone female in treatment 2.

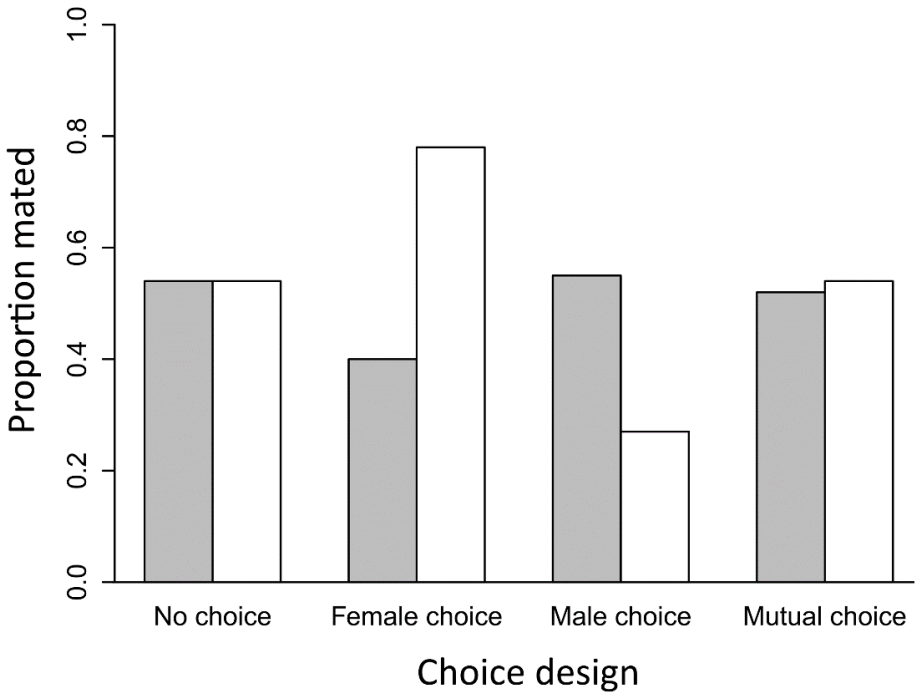

Figure 3.2. The proportion of focal males (grey bars) and focal females (white bars) that mated in the four choice designs. For both sexes, *N*= 69 for the no-choice treatment and 55 for the other three treatments.

3.3.4 Morphology and mating success

There was significant positive sexual selection on female body length ($F_{1, 305}$=7.48, P = 0.007; **Figure 3.3a**). However, choice treatment did not affect the strength of selection on female body length (Binary logistic GLMM, interaction between treatment and female body length, $F_{3, 302}$= 1.01 P = 0.39). Female mating success was significantly affected by choice treatment ($F_{3, 305}$= 12.09, P <0.001): as in our previous analysis females were more likely to mate in treatment 2 and less likely to mate in treatment 3 (see above). Male mating success was not associated with treatment ($F_{3, 298}$=1.88, p= 0.13), male body length ($F_{1, 298}$=1.55, P = 0.21), or their interaction (interaction between treatment and male body length, $F_{3, 295}$= 0.95, P = 0.42).

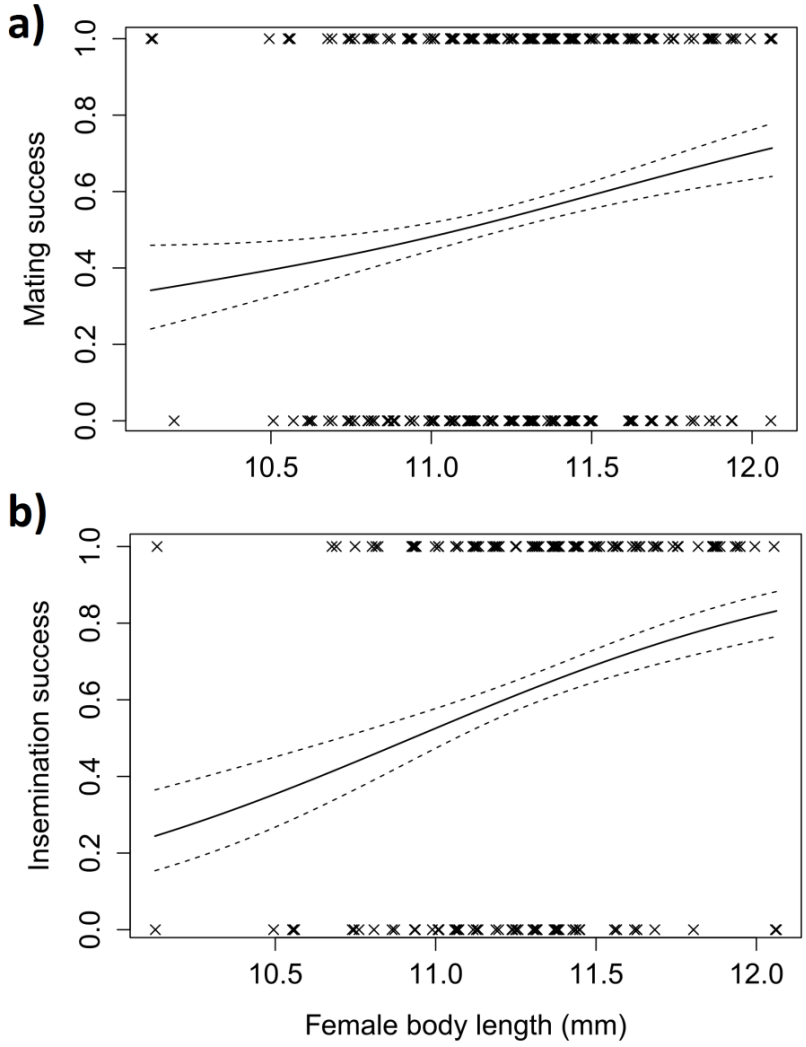

Figure 3.3. Selection on female body length (mm) across all four choice treatments in *L. equestris*. **a)** pre-copulatory selection for all females (*N*= 310), and **b)** post-copulatory selection for mated females (*N*= 167).

When 'overall size' was used in place of body length, there was no sexual selection on female overall size ($F_{1, 300}$= 1.75, *P* = 0.19). Choice treatment again did not affect the strength of selection on female size (interaction between treatment and female overall size, $F_{3, 297}$= 1.33, *P* = 0.27), but was significantly associated with female mating success ($F_{3, 300}$= 11.75, *P* <0.001). For males, there was significant non-linear selection on overall size (quadratic term: $F_{1,294}$= 4.88, *P* = 0.028; **Figure 3.4a**), and there was a marginally significant effect of choice treatment on the likelihood of mating for males ($F_{3, 294}$= 2.54, *P* = 0.057). However there was no significant effect of choice treatment

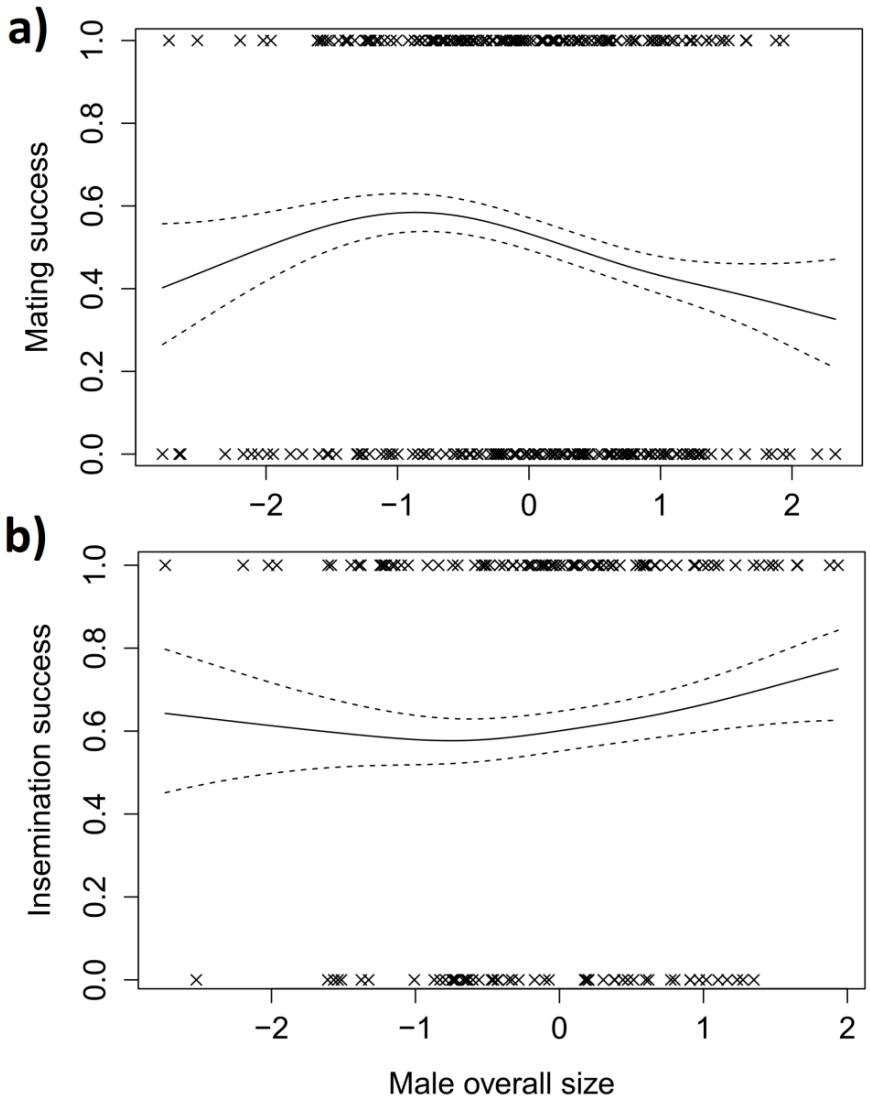

Figure 3.4. Selection on male overall size across all four choice treatments in *L. equestris*. **a)** pre-copulatory selection for all males (*N*= 300), and **b)** post-copulatory selection for mated males (*N*= 150).

on the strength of this selection (interaction between treatment and male overall size, $F_{3, 288}$= 0.85, *P* = 0.47).

3.3.5 Copulation duration

There were 170 matings overall, comprising 69 short copulations (range: 50-450 minutes, median= 290 minutes, IQR= 130-395 minutes) and 101 long copulations (range: 360-475 minutes, median= 450 minutes, IQR= 425-460 minutes). Separating pairs at the end of the day led to a large spike in frequency at around 7 hours, and gives an underestimate of the actual copulation duration. I obtained full morphological measurements for 148 mated pairs, comprising 85 long and 63 short copulations.

Females with a larger overall size were more likely to have long copulations (Binary logistic GLM; χ^2_1=6.97, P = 0.008). Male overall size on the other hand was not associated with the likelihood of having long copulations (χ^2_1= 1.75, P = 0.19). Choice treatment also had no effect on the likelihood of having long copulations (χ^2_3= 6.53, P = 0.089).

3.3.6 Female fertility

A hundred and six mated females (62% of mated females) laid fertile eggs within two weeks of mating (i.e. a 'mating failure' rate of 38%). Females that copulated for longer were more likely to subsequently lay fertile eggs (Binary logistic GLM; χ^2_1= 40.9, P < 0.001). Females that copulated for less than 400 minutes rarely laid fertile eggs (**Figure 3.5**). Larger females were also more likely to lay fertile eggs, independent of copulation duration (χ^2_1= 8.86, P= 0.003; **Figure 3.3b**). Male overall size was not associated with female fertility (χ^2_1= 1.78, P= 0.18; **Figure 3.4b**). Males do not have to copulate for as long to fertilise eggs of small females compared to those of large females (interaction between female overall size and copulation duration: χ^2_1= 12.06, P <0.001). Finally, there was no significant effect of choice treatment on the fertility of mated females (χ^2_3= 1.29, P = 0.73), but there was a significant interaction between treatment and female overall size (χ^2_3= 9.22, P = 0.03).

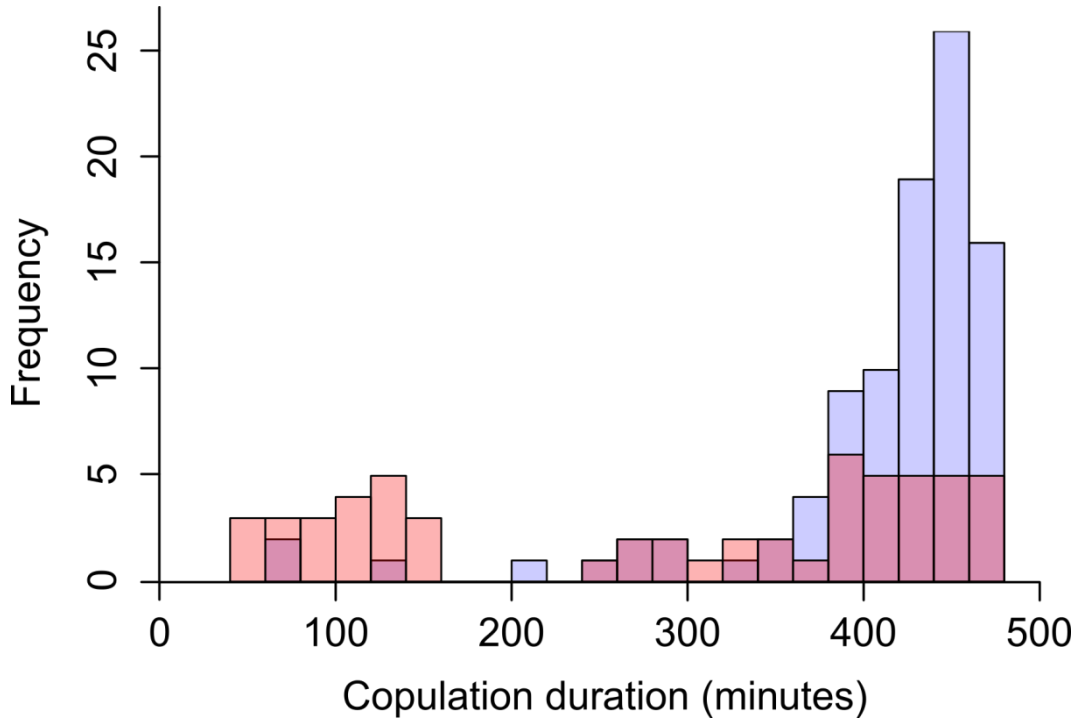

Figure 3.5. Histogram of copulation duration for pairs in which females laid fertile eggs after mating (Blue bars, *N*= 106 pairs) and pairs in which females did not lay fertile eggs (Orange bars, *N*= 63 pairs).

3.4 Discussion

The aim of this chapter was to characterise the strength and pattern of pre- and post-copulatory sexual selection on male and female morphology in *L. equestris*, and explicitly test whether the degree of choice influences the measurement of this selection. I found no significant effect of experimental choice design on the strength of either pre- or post-copulatory sexual selection on male or female morphology. This was despite finding significant selection on male overall size and female body length. Visualizing the shape of selection in terms of mating success indicated positive linear selection for larger female body length (**Figure 3.3a**) and weak non-linear selection on male overall size (**Figure 3.4**). Male preference for larger females is likely due to the increased female fecundity associated with body size (Honěk, 1993), and is commonly

seen in insects (Bonduriansky, 2001). Body length in isolation was not a significant indicator of the likelihood of mating for males. However, I predicted that larger males should be better at achieving matings because of the observed copulatory struggles in this species. This is clearly not the case, perhaps suggesting that male contributions to mating struggles may not be as important as supposed. The measure of overall size takes into account body length plus antenna and leg lengths, and so may perhaps suggest that the length of the legs is more important for males than for females (though selection is not strong enough to be detected in isolation).

Although some previous studies have considered different mate choice designs, this is the first experiment to my knowledge to attempt to explicitly *statistically* test the effect of experimental choice design on the measurement of sexual selection. A similar design was used by Coyne *et al*. (2005) to study sexual isolation due to a mating preference for conspecifics (rather than sexual selection *per se*), between two species of *Drosophila*. They measured the frequency of conspecific and heterospecific matings observed under the choice designs used in this experiment (no-choice, male choice, female choice and mutual choice). However the mutual choice design involved 30 males and 30 females of both species, and so I would suggest it is not strictly comparable with the other treatments. This mutual choice treatment was designed to mimic mating aggregations observed in other species of *Drosophila* in the wild (Coyne *et al*., 2005). The authors found that the frequency of heterospecific matings was higher in the no-choice treatment compared to all three choice treatments, leading to reduced sexual isolation between the two species. One way in which such a pattern would arise is if mating preferences were weaker in the no-choice treatment.

In the current experiment I found no significant effect of choice design on any aspects of sexual selection. This first suggests that intrasexual contest competition does not impose sexual selection on the morphological traits measured, otherwise I would expect to see a strong difference in selection due to choice design: there can be no direct intrasexual selection if rivals are not present. Accordingly, I observed no obvious

interactions between members of the same sex, such as overt aggression or contest behaviour, during trials. I did observe male struggles for access to females, which probably leads to the increased latency to mate seen in the male-biased treatment. However such encounters clearly do not influence sexual selection in a significant way, unless male-male competition only exerts a strong selection at higher male densities than those used here.

Therefore I suggest that inter-sexual selection is the most likely explanation for the patterns of selection observed in this species. One possible explanation for why choice design had no significant effect on sexual selection would be that in *L. equestris* simultaneous assessment by either males or females is simply not possible, so that the number of choices available is irrelevant to the choosing process. If this is the case all mates will be assessed sequentially, irrespective of the number of options presented. This is despite evidence that simultaneous encounters are probably common during the breeding season (Solbreck & Kugelberg, 1972; Solbreck & Sillén-Tullberg, 1990). Such an effect has been shown in studies of animal foraging behaviour: for example, starlings (*Sturnus vulgaris)* appear to assess food items sequentially, even when presented with two options (Kacelnik *et al*., 2011).

So is sequential choice the primary method of mate assessment in *L. equestris*? To answer this we first need to determine potential ways of assessing mates in this species. Possible mechanisms of signalling over short distances have not been investigated in any lygaeid bugs (though long-range attractant pheromones may be common; Aldrich, 1995; Aldrich *et al*., 1999). However my observations in this species suggest it does not play a large role. For example, individual males frequently appear unaware of the presence of females in petri dishes until the pair contacts each other. This suggests that conspecific communication is primarily through direct contact only, and that visual, olfactory and auditory stimuli are of little importance. If this is the case it would be informative to know whether females in treatments 2 and 4 frequently received mating attempts from both males (allowing comparison through direct

contact), and whether males in treatments 3 and 4 attempted to mate with both available females. If only focal individuals are considered, then 42% (46 out of 109) of focal males were observed to direct mating attempts at both females in the dish (treatments 3 & 4), and 54% (59 out of 109) of focal females were observed to receive attempts from both males present in the dish (treatments 2 & 4). Therefore if direct contact is the primary method of mate assessment then at least half of all males and females had no opportunity to assess more than one partner, and so were essentially in a no-choice situation. This could explain why the choice design had little effect on the strength of selection.

Direct contact will be especially important if individual recognition is determined using cuticular hydrocarbons, as is common in many insect species (CHCs: Howard & Blomquist, 2005). There has been little study of contact communication in the Lygaeidae or Heteroptera in general (but see Jackson, 1983; Drijfhout & Groot, 2001), though CHC composition has been shown to mediate mate recognition in a reduviid bug (Cocchiararo-Bastias *et al*., 2011). Recent work has shown that CHC profiles are sex- and species-specific in *L. equestris* and four other lygaeid bugs (Burdfield-Steel, 2014). The potential importance of chemical communication in *L. equestris* is supported by the fact that marked individuals in treatment 1 were more likely to mate than unmarked individuals. It is possible that marking the bugs interfered with CHC-mediated chemical communication associated with species discrimination and/or mate choice (Aldrich *et al*., 1999; Howard & Blomquist, 2005; Cocchiararo-Bastias *et al*., 2011), for instance if CHCs signal quality in some way (Tregenza & Wedell, 1997; Chenoweth & Blows, 2005; Harris & Moore, 2005; Ali & Tallamy, 2010). However, one might have expected disruption of such a system to lead to reduced, not increased, mating rates. I was also careful to place paint on only a small part of the pronotum (see **Figure 2.8**), so that CHC composition over the rest of the body was not disrupted. I note that marking did not appear to lead to any other systematic differences in behaviour. Nevertheless, my data need to be taken in the context that the marking

protocol may have inflated mating rates across all the treatment combinations to some extent.

I also note that the experimental design cannot be said to conclusively measure male or female mating preferences, as males and females are able to interact during trials. Therefore significant intersexual selection could arise for example due to forced copulations by males. Assigning complete agency to males or females is a common problem in insect mating systems in which pairing occurs before any obvious courtship takes place (Shuker & Day, 2002). As such, obtaining accurate estimates of mating preferences may be very difficult in this species if choice requires that individuals interact (see Martel & Boivin, 2011).

In addition to mating success, I also scored fertilisation success as a measure of post-copulatory selection. Larger females (in terms of overall size) were more likely to lay fertile eggs irrespective of copulation duration. This may be due to increased fecundity of larger females, and is probably the primary reason males show a preference for larger females. In contrast, I found no significant association between male morphology and fertilisation success. I did find a significant interaction between female overall size and copulation duration, such that males do not have to mate as long to achieve similar levels of fertilisation with small females compared to large ones. This may be for mechanical reasons, for example if it takes longer for sperm to reach the spermatheca or for the male intromittent organ to travel along the female reproductive tract in larger females.

To estimate the strength of sexual selection on traits, we must have some estimate of the difference in fitness between individuals with differing levels of each trait. Ideally, all studies of sexual selection would estimate the fitness consequences of mating through to the offspring generation (or even beyond). But even just considering the production of fertile eggs adds important resolution (see Chapter 5). This is especially important in *L. equestris* as matings frequently fail to result in the production of any

fertile eggs (including 38% of mated females in this study). In polyandrous species in general, measures of mating success alone will likely be inappropriate when estimating the total strength of sexual selection acting on male traits (García-Gonzaléz, 2004; Pischedda & Rice, 2012; Tyler & Tregenza, 2013).

3.5 Conclusion

The results of this chapter suggest that the social context need not always influence the strength of sexual selection acting on males or females, especially in species in which mate assessment or sampling methods do not require (or may even preclude) simultaneous comparison of mates. This illustrates the important point that the assumptions regarding mate assessment in a given study species need to be explicitly tested where possible. Indeed, the extent to which species compare potential mates simultaneously, even when simultaneous assessment is possible, is still unclear (Gibson, 1996; Bateson & Healy, 2005; Kacelnik *et al*., 2011).

Furthermore, comparing the strength of choice between different social contexts may be a useful way to determine the importance of choice versus intra-sexual competition, or sequential versus simultaneous assessment, in other species. Determining when social context does influence sexual selection will be informative, for instance if differences are due to the mechanisms by which different species compare and choose potential mates. In the next chapter, I attempt to determine whether *L. equestris* is unusual in this respect, or whether there are general patterns across species. I do this by performing a meta-analysis of published studies that present mating preferences using both no-choice and choice designs.

Chapter 4

The effect of experimental design on the measurement of mate choice: a meta-analysis

Abstract

In this Chapter I present the results of a meta-analysis testing for the effect of experimental choice design on the measurement of mating preferences across species. I used a sample of 38 published studies on 40 species in which mating preferences were tested using both a no-choice design and a choice design on the same species/trait/sex combination, and in the same paper. Overall I found that mating preferences were significantly stronger when tested using a choice design compared to a no-choice design. I suggest that this difference is due to the increased cost of rejecting partners in no-choice tests; if individuals perceive they are unlikely to remate in a no-choice situation, they will be more likely to mate randomly. The difference between choice designs was seen for female mate choice but not for male mate choice, and for intra-specific choice but not for inter-species or inter-population mate discrimination.

Acknowledgments

I would like to thank Rick Howard for providing summary statistics not available in his published work. I also thank Mike Ritchie and Nathan Bailey for extremely helpful suggestions on the study, especially concerning inter-species vs inter-population choice, and Shinichi Nakagawa for very helpful advice with analysis using Metafor.

4.1 Introduction

An important way in which experiments testing mate preferences can vary is in the number of options the subject is presented with during the test. This is known as the choice paradigm' or 'choice design' (Chapter 1). Tests can use a no-choice design, in which a single option is presented to each subject, or a choice design, in which more than one option is presented to each subject. Though seemingly obvious, the two designs differ in several important ways. First, the two designs differ in whether options can be directly compared or not. Because comparison is possible, choice tests detect relative, directional preferences between stimuli (Wagner, 1998; MacLaren & Rowland, 2006). In contrast, no-choice experiments test for absolute preferences, as no direct comparison is possible (Wagner, 1998). Choice tests may allow greater resolving power between options as even small differences in trait values may lead to large differences in choice outcomes (Doherty, 1985; Wagner 1998). However, this effect may amplify the strength of preferences observed if a dichotomous yes or no response is recorded (Wagner *et al*., 1995; Wagner, 1998).

No-choice tests also differ from choice tests in that the perceived mate encounter rate is lower: if a mate is rejected in a no-choice tests there may be no guarantee of a mating opportunity in the future (Werner & Lotem, 2006; Barry & Kokko, 2010; Booksmythe *et al*., 2011). Thus rejection of an option in a no-choice test may indicate a more robust preference than that seen in a choice test, because the subject has foregone mating despite this extra cost of rejection. No-choice tests may also underestimate mating preferences, as subjects that do not respond to stimuli are usually discarded from the analysis for choice tests, but not for no-choice tests (Kokko & Jennions, 2015). This means that no-choice designs are more likely to include data from non-responsive subjects, thus potentially underestimating the strength of selection.

There are several examples in the literature showing little effect of choice design on the strength of pre-copulatory sexual selection in the form of mating preferences (e.g. Gabor *et al*., 2000; Jang & Gerhardt 2006; Gershman & Sakaluk, 2009; Jordan & Brooks, 2012). Additionally, in the previous chapter I found no significant effect of choice design on the strength of sexual selection on morphology in *Lygaeus equestris*. However, other studies show very clear effects of choice design, with subjects exhibiting stronger mating preferences in choice tests compared to no-choice tests (MacLaren & Rowland, 2006; Barry *et al*., 2010; Booksmythe *et al*., 2011; Owen *et al*., 2012). Therefore empirical evidence for the effect of choice design on mating preferences is mixed. However, as yet there has been no attempt to synthesize these results or quantitatively assess the impact of experimental choice design on the measurement of mate choice.

One potential way to determine the strength of an effect across species is meta-analysis. Meta-analysis also allows us to combine results from multiple species and traits, and, sample size permitting, investigate potential moderators of effect size (Jennions *et al*., 2012). Meta-analysis is well suited to determining trends across many studies, especially when results across studies are mixed and many studies may report non-significant results due to low statistical power (Arnqvist & Wooster, 1995; Koricheva *et al*., 2013). Recent years have seen a rapid increase in the use of meta-analysis in ecology and evolution research, including in the field of sexual selection (for an overview see Jennions *et al*., 2012), and studies of mate choice (e.g. Milner *et al*., 2010; Simons & Verhulst, 2011; Ihle & Forstmeier, 2013; Jiang *et al*., 2013; Kamiya *et al*., 2014).

I performed a meta-analysis in order to quantify the effect of experimental choice design on the measurement of mating preferences. I searched the literature for published studies that passed three main criteria. First, mating preferences had to be tested using at least one no-choice experiment and one choice experiment, and the results presented in the same paper. Using a paired design in this way should reduce

confounding factors such as effects associated with individual researchers, animal stocks, and so forth. Second, the two (or more) choice tests had to be performed on the same species/trait/sex combination. Finally, the study needed to present the relevant statistics so that an effect size could be calculated. I included studies presenting both mate choice outcomes and also proxy measures of mating preference, male and female choice, as well as intra-species, inter-population and inter-species choice (see below for details).

4.2 Methods

In presenting the methods I have attempted to follow as close as possible the PRISMA standards for reporting meta-analyses (Moher *et al*., 2009; see Nakagawa & Poulin, 2012; see **Figure 4.1** for diagram showing search results and the study selection process).

4.2.1 Search protocol

I used three approaches to search the literature. First, after initial scoping searches in September and October 2012, I performed keyword searches of several online databases in June 2013. I took the first 100 results from the databases Google Scholar (Google) and Scirus (Elsevier) for the search terms "*sequential simultaneous mate choice*", on 17th June 2013. On 19th June I performed the following searches in both Web of Knowledge (Thomson Reuters) (in the TOPIC field) and Scopus (Elsevier) (in the "Article Title, Abstract, Keywords" field): "*no choice*" AND "*multiple choice*"; "*no choice*" AND "*two choice*"; "*no choice*" AND "*simultaneous*"; "*sequential*" AND "*simultaneous*"; "*sexual* isolat**" AND "*no choice*" AND "*multiple choice*".

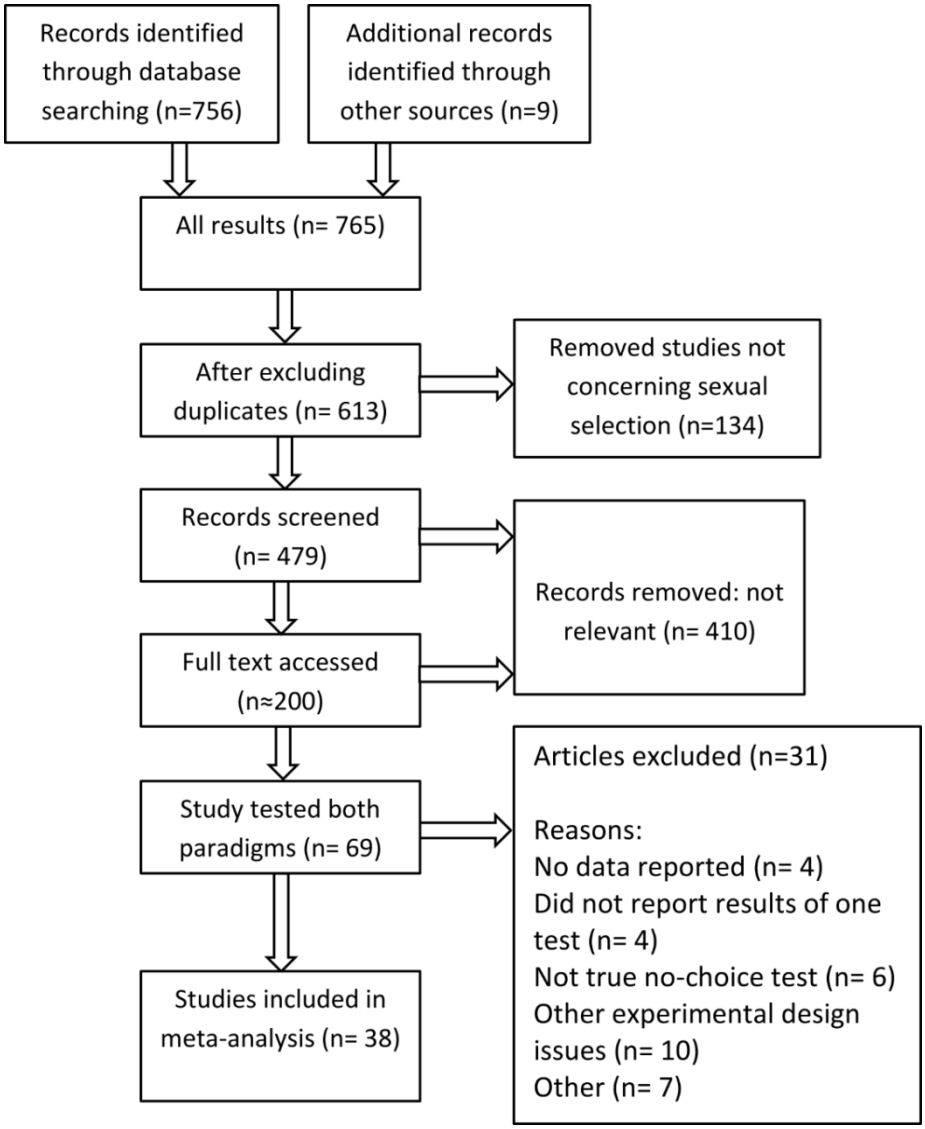

Figure 4.1. PRISMA flow chart of search results and the study selection process.

Secondly, I used Web of Knowledge to search all studies citing four papers identified as being influential in this area: the review by Wagner (1998) on measuring mating preferences and experimental design; the highly-cited study by Rowland (1982) on male choice in *Gasterosteus aculeatus*; and finally two more recent papers which explicitly tested for the effect of experimental design on mate preferences (Coyne *et al*., 2005; MacLaren & Rowland, 2006). After the online searches, I then inspected the titles and abstracts of the results in order to remove papers that were obviously not

relevant to the search. Papers that were deemed relevant were then read in detail in order to see whether the study could be included (see inclusion criteria below). Finally, I also followed papers cited in the text if my searches had not already located them.

4.2.2 Criteria for inclusion

I used several criteria to determine which studies to include in the analysis. Most importantly, each study needed to include at least one effect size corresponding to a no-choice test and one effect size corresponding to a choice test (for most studies multiple effect sizes were presented, see below). I included only studies in which each test was performed using the same species and sex, testing for a preference for the same trait. This is important as I found several cases where both no-choice and choice designs were performed but different traits were considered between tests (see **Figure 4.1** for the most common reasons for excluding papers from the analysis, and **Table S28** for more detailed information). Importantly, the analysis includes measures of mate choice in the form of both mating outcomes and proxy behavioural measures (such as association time or courtship effort; see Chapter 1). Such proxy measures are assumed to reflect actual mate choices and have been validated in many species (Chapter 1), though in other cases such validation is lacking. If proxy measures do not accurately reflect mating decisions this may lead to different preference estimates, though it is not clear whether these estimates will be larger or smaller than those derived from mate choice outcomes.

Both tests did not have to be performed using identical stimuli (indeed in most cases this would not be possible because individuals of the opposite sex were used as stimuli), however stimuli did need to be comparable. One example of an excluded study should help to illustrate this point. Basolo (1995) tested for a female preference for males with (artificial) swords in the unsworded Platyfish *Priapella olmecae*. First, the presence of a preference was tested using a choice test, in which a female chose between a normal, unsworded male and a male to which an artificial sword had been

experimentally added. Second, no-choice tests were used to test for female preference for swords of differing sizes. However, there was no corresponding no-choice test using an unsworded male. Therefore the choice design tests for a preference for a sword over no sword, whereas the no-choice design tests for a preference for sword *size*. Therefore I did not include this study in the analysis, as the stimuli used in each test were not directly comparable.

I define a no-choice test as one in which a subject is presented with a single stimulus or potential mate. This excludes designs commonly used in sexual isolation studies in which subjects are presented with several potential mates of a single *type* (e.g. Tomaru & Oguma, 2000). This definition also includes sequential choice tests, in which several no-choice tests are performed concurrently using different stimuli. I define a choice test as one in which a subject is presented with more than one stimulus simultaneously. Most studies use a two-choice test, but I also included those in which more than two options were given (e.g. three-choice test: Beckers & Wagner, 2011).

I included all stated measures of mate preference, and relied on the authors' judgments on whether the measured behaviours accurately reflect mating preferences or not. I did not impose any limitations on the degree of randomization regarding the order of presentation of stimuli, or whether presented stimuli were controlled (e.g. synthetic calls) or not. I also did not impose limitations regarding whether the same individuals were used in both no-choice and choice tests (this is rare: see below), or whether the same stimuli were presented to all individuals.

I included studies that tested for both male and female mate choice. I predict that overall female choice will be stronger than male choice, as females generally invest more in each reproductive event and so should be more discriminating in their choice of mate (Andersson, 1994). I also included studies considering both intraspecific traits ('intra-species choice') as well as interspecific mate choice; that is choice between a conspecific and a heterospecific individual ('inter-species choice'), as these tests are

commonly performed in studies considering reproductive isolation and incipient speciation. Inter-species choice can be considered an extreme form of mate choice (Ryan & Rand, 1993; Mendelson & Shaw, 2012). I also included studies considering choice between different intraspecific populations and strains (due to different larval host plants), which I classified as 'inter-population choice'. I refer to these three categories as 'trait types'. I predict that inter-species choice will be stronger than intra-species and inter-population choice, as there are higher costs associated with making the wrong choice when choosing between a conspecific and a heterospecific individual (Andersson, 1994).

Finally, I excluded studies for which I was unable to extract appropriate effect sizes (e.g. missing test statistics or sample sizes; **Figure 4.1**). For one study (Owen *et al*., 2012) I was provided with statistics not presented in the original paper after contacting the authors. I extracted data from text or tables, or indirectly from figures using the image analysis software Digitize It 2010 v4.0.2 (A. Carrascal). In several cases I re-analysed reported data (e.g. means and standard deviations, mating frequencies; see **Table S29)**.

4.2.3 Effect sizes

The studies included in the analysis used a very wide range of statistical tests when testing for mating preferences, which I converted to the effect size *r* (analogous to the correlation coefficient). This effect size can be interpreted as the degree of non-random response by the chooser with respect to the trait in question (e.g. non-random mating or mate association): the larger the test statistic the greater the departure from a random response, and so the 'stronger' the mating preference. For clarity I refer to the mean effect sizes derived from the analysis as the 'strength of preference' throughout. This measure can incorporate both linear and quadratic preferences (if statistical models account for quadratic effects), but cannot account for more complex aspects of preference shape (e.g. see Edward, 2015). For many tests the conversion to

r is simple (Koricheva *et al*., 2013), and it has the advantage of being an intuitive measure of the size of an effect. I used the effect size calculator in Metawin 2.0 (Rosenberg *et al*., 2000) to convert presented effect sizes into *r*. In several cases I had to repeat analyses in order to obtain useable test statistics (see **Table S29**). I extracted all effect sizes presented in a study. For most studies multiple effect sizes were reported (for example, effect sizes were presented for multiple measures of preference from the same individuals, or the same measures of preference for different groups of individuals or populations) and I controlled for this in the analysis by including study as a random factor in the meta-analysis models (see below). In many cases there were different numbers of effect sizes reported for each choice design.

All effect sizes were considered positive except in three studies in which the direction of preference differed within a study between tests. In these cases I defined one preference as positive and the other as negative (there were nine negative effect sizes in the model in total). In the first case (Wood & Ringo, 1980), significant mating preferences were detected for both con- and hetero-specific individuals in different tests; here conspecific preference was considered as positive and heterospecific preference was considered as negative. In a further two cases (McNamara, 2004; King *et al*., 2005) significant preferences were detected for both virgin and mated females in different tests; here preference for virgins was considered as positive and preference for mated females was considered as negative. I included the direction of preference in the analysis even when preferences were non-significant.

4.2.4 Meta-analyses

All meta-analyses were performed using Fishers' *z* transform of the correlation coefficient (*Zr*). Estimates of mean effect size estimates derived from the models using *Zr* were then converted back to *r* for presentation. Mean effect size was determined using a random-effects meta-analytic model. I considered the mean effect size

estimate to be significantly different from zero if the 95% confidence intervals around the mean did not include zero. Though I have multiple effects sizes per study I present model results without the inclusion of study as a random factor, as the basic model was a better fit for the data (see below). I used the I^2 statistic to determine the amount of heterogeneity in effect sizes across studies. This gives the percentage of variation in effect sizes due to heterogeneity rather than by chance (Higgins *et al*., 2003). I^2 is preferred over Cochran's Q as the relative amount of heterogeneity in the dataset can be determined (not just as a significance value), and it is less affected by the number of effect sizes in the analysis (Higgins *et al*., 2003).

I searched for the influence of categorical moderators on the strength of preference using meta-analytic mixed models (random-effects models with the addition of a categorical fixed-effect: see Nakagawa & Santos, 2012; Koricheva *et al*., 2013). I used the Q_M statistic to determine if effect size was significantly influenced by the different levels of each moderator (this is analogous to an analysis of variance). I considered the following four moderators: sex (male or female choice), trait type (intra-species, inter-population or inter-species choice), taxonomic group (arachnid, crustacean, insect, fish, amphibian, reptile or bird) and choice measure (matings or proxy measure).

To test for the influence of experimental design on the strength of mating preferences I first calculated mean effect sizes estimates separately for effect sizes from no-choice and choice tests. I then tested for a significant difference between effect sizes derived from the two experimental designs using a weighted least-squares regression model framework (in meta-analysis terminology this is a form of multi-level meta-regression; see Koricheva *et al*., 2013). This allows us to control for the non-independence of effect sizes taken from each study by including study as a random factor. Species was also fitted as a random factor, but without the addition of phylogenetic information as this had no effect on the meta-analysis models (see below). For these models effect size was weighted using the study weights derived from the overall random effects meta-analysis model (for a random-effects model weights are calculated by taking into

account the sample size of each study as well as the between-study variance of the dataset). Finally, I also obtained mean effect size estimates via random-effects models for no-choice and choice tests further split by the three main categorical variables (sex, trait type and taxonomic group), and tested for a difference between designs within each of these subgroups using weighted least-squares regression.

Kokko & Jennions (2015) have recently suggested that no-choice studies may be biased towards sexually non-responsive subjects, as these tend to be removed prior to analysis of choice tests. I thus also repeated the analysis after removing studies in which non-responsive subjects were discarded in choice tests but not in no-choice tests.

All statistical analyses were performed in R v3.0.1 (R Development Core Team, 2014) using the Metafor package v1.9-2 (Viechtbauer, 2010).

4.2.5 Phylogenetic analysis

Recent studies have shown that the addition of phylogenetic information can have a significant impact on the effect size estimates from meta-analysis models (Chamberlain *et al*., 2012), and yet only 17% of 100 meta-analyses published in ecology, evolution and behavioural ecology journals surveyed by Nakagawa & Santos (2012) controlled for phylogeny. I therefore attempted to control for the possible non-independence of effect sizes due to shared ancestry by performing a phylogenetically controlled meta-analysis. As there is no single tree available for all species in the analysis, I constructed a supertree by manually combining trees (both genetic and taxonomic) from several published sources, taking the most recent studies where possible.

I used the following sources to construct the supertree. For the basal node between vertebrates and arthropods I used Glenner *et al*. (2004). For the relationship among

arthropods I used Regier & Shultz (1998) and Giribet *et al*. (2001). For the relationship among insect orders I used Gaunt & Miles (2002) and Kjer *et al*. (2006). For the relationship among Orthoptera I used Huang *et al*. (2000) and Jost & Shaw (2006). For the relationship among Lepidoptera I used Regier *et al*. (2009) and Mutanen *et al*. (2010). For the relationship among Drosophila I used Lachaise *et al*. (2000), Spicer & Bell (2002) and van der Linde & Houle (2008). For the relationships among vertebrates I used Ureta-Vidal *et al*. (2003) and Xia *et al*. (2003). For the relationships among fish orders I used Miya *et al*. (2003) and Near *et al*. (2012); though note that the positions of these nodes are particularly unresolved and may be subject to change. For the relationships among the Cyprinodontiformes I used Meyer & Lydeard (1993) and Ghedotti (2000).

Accurate branch length data is lacking for trees such as this, and so branch lengths were first arbitrarily set to one (following Hadfield & Nakagawa, 2010), and then made ultrametric using the cladogram option in FigTree v1.4 (Andrew Rambaut, 2012). Branch lengths are thus transformed so that all tips are contemporaneous. The final tree can be seen in **Figure 4.2**. Note that branch lengths are necessarily distorted, and are thus underestimated for very distant lineages (such as arachnids) and overestimated for more recent lineages (such as in Drosophila). This tree was then imported into the R package ape v3.1.1 (Paradis *et al*., 2004) in Newick format, and a correlation matrix obtained using the vcv function. The correlation matrix can then be incorporated into a multivariate meta-analysis model as an additional random factor.

To incorporate phylogeny into the analysis I ran multivariate meta-analytic models (following the terminology of Nakagawa & Santos, 2012) with study, species and phylogeny as additional random factors. However, in comparison to these models, the simpler models (random-effects models as described above) gave a much better fit to the data: in all cases, adding these random factors increased the 95% confidence intervals associated with the mean effect size estimates, as well as greatly increasing the model AIC scores, but they did not change the significance of the results (**Table**

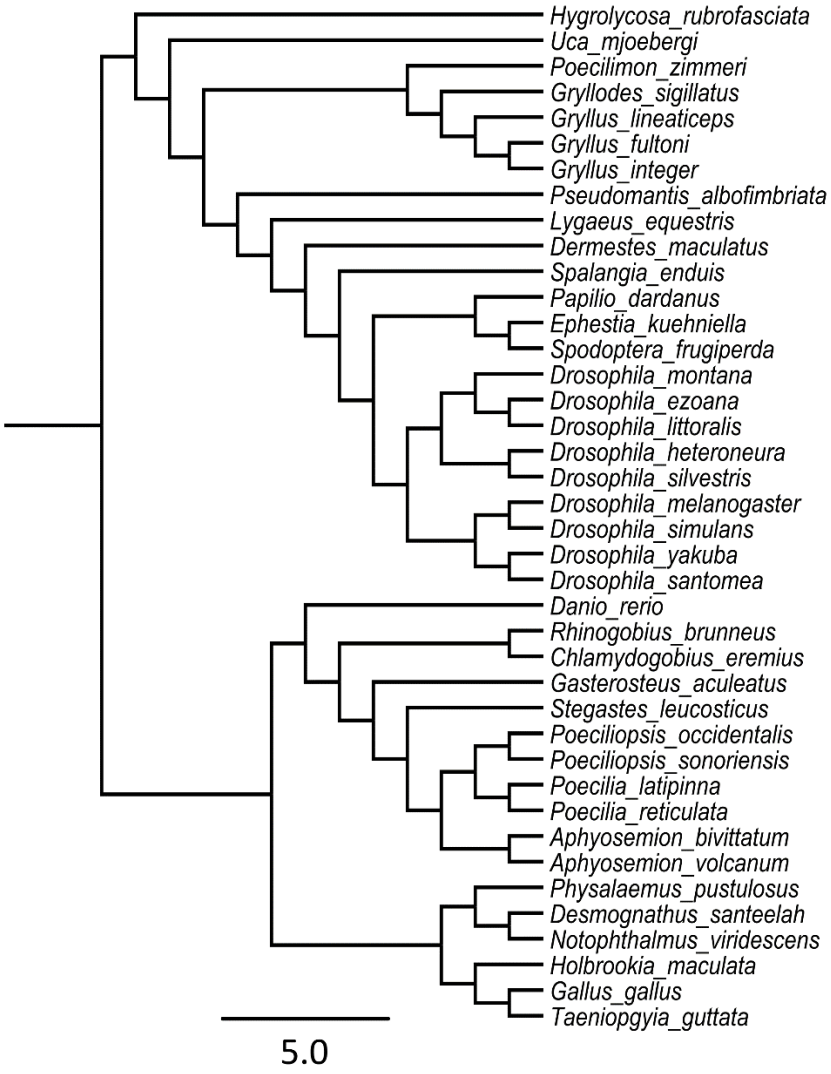

Figure 4.2. Phylogeny used to conduct phylogenetically-controlled meta-analysis.

S30). Most importantly, in most cases the variance component associated with phylogenetic history was zero or very small, indicating that the effect sizes used in the analysis were not phylogenetically restricted, and that the increase in the 95% confidence intervals was almost entirely due to the addition of species and study as random factors. I thus present the simpler meta-analytic models here and present the results of the multivariate models in Appendix 2. Note that the weighted least-squares regression models as described above do include species and study as random factors.

4.2.6 Publication bias

I tested for two types of publication bias: bias due to the underreporting of non-significant results, and bias due to differential publishing patterns over time. To explore the potential for underreporting of non-significant results, I used three approaches. Firstly, I calculated fail-safe numbers using both Rosenberg's method and Orwin's method. Rosenberg's method calculates the number of additional studies (or effect sizes in this case) with a value of zero that would need to be added to the analysis to result in a non-significant mean effect size. These additional effect sizes are also weighted by the average sample size of the dataset (Koricheva *et al*., 2013). Orwin's method calculates the number of additional effect sizes of a given value (set at 0.05) that would be needed to result in a designated 'unimportant' mean effect size (again set at 0.05). I then performed a trim-and-fill analysis to test for funnel plot asymmetry, which calculates a new mean effect size estimate after imputing any potential 'missing' studies (see Duval & Tweedie, 2000). Finally, I tested for the non-parametric correlation between standardized effect size and study variance (Begg & Mazumdar, 1994).

I tested for a potential change in the mean effect size over time in two ways: firstly by testing for the rank correlation between effect size and publication year for each study, and secondly by performing a meta-regression using publication year as a covariate.

4.2.7 Dataset

I was able to obtain data from 38 studies (concerning 40 species), which gave a total of 214 effect sizes, of which 107 were derived from no-choice tests and 107 from choice tests. 95 effect sizes measured female choice and 119 measured male choice. There were no studies on sex-role reversed species, though five of the studies concerned male choice in fish with paternal care only (Rowland, 1982; Jamieson & Colgan, 1989;

Belles-Isles *et al*., 1990; Itzkowitz *et al*., 1998; Wong & Svensson, 2009). 133 effect sizes considered intra-species choice, 18 considered inter-population choice and 63 considered inter-species choice. The analysis includes studies on seven species groups: arachnids, crustaceans, insects, fish, amphibians, reptiles and birds. Insects and fish were the most common taxonomic groups studied (110 and 67 effect sizes respectively); the remaining five groups all contributed less than 12 effect sizes each to the final analysis. 166 effect sizes were derived from proxy measures of preference whereas 48 were derived from choice outcomes. In total, the dataset was based on data from 6322 individual subjects.

Of the 38 papers included in the final analysis, 29 were found using online searches. A further eight studies were found by following references cited in other papers (Wood & Ringo, 1980; Rowland, 1982; Houde, 1987; Hoikkala & Aspi, 1993; Wagner *et al*., 1995; McNamara *et al*., 2004; Coyne *et al*., 2005; King *et al*., 2005). These studies were likely not detected either because the exact experimental design was not mentioned in the abstract and/or the search terms were not used to refer to the tests. I also included data from Chapter 3 which was unpublished at the time of analysis.

4.3 Results

4.3.1 Overall model

Overall, the meta-analysis revealed significant positive mating preferences (mean preference estimate derived from all 214 effect sizes: r= 0.426, 95% CI: 0.375 to 0.474). In fact, mean effect size estimates for all subgroup comparisons were significantly greater than zero, indicating significant mating preferences within all groups (**Table 4.1**).

Table 4.1. Mean effect size estimates resulting from meta-analysis models performed separately using effect sizes derived from no-choice and choice tests from each subgroup. All analyses were performed using Fisher's z transform of the correlation coefficient (*Zr*), and then converted back to *r* for presentation. Mean effect size estimates, 95% confidence intervals and I^2 values were calculated using a random-effects meta-analytic model.

			No-choice tests					Choice tests				
Group	Studies	Species	Effect sizes	Mean *r*	Lower 95% CI	Upper 95% CI	I^2 (%)	Effect sizes	Mean *r*	Lower 95% CI	Upper 95% CI	I^2 (%)
All	38	40	107	0.364	0.297	0.427	85.6	107	0.484	0.409	0.552	89.55
Sex												
Males	20	21	61	0.353	0.259	0.441	86.75	58	0.433	0.318	0.536	90.43
Females	21	25	46	0.376	0.281	0.463	83.75	49	0.535	0.439	0.620	87.72
Trait type												
Intra-species	29	29	68	0.341	0.251	0.425	82.24	65	0.500	0.408	0.582	86.03
Inter-population	4	4	9	0.202	0.096	0.305	51.84	9	0.363	0.152	0.542	75.71
Inter-species	7	11	30	0.446	0.331	0.548	88.94	33	0.480	0.321	0.612	94.19
Taxonomic group												
Arachnid	1	1	1	0.500	-	-	-	1	0.744	-	-	-
Crustacean	2	1	5	0.390	-0.045	0.701	60.19	6	0.430	0.308	0.538	0
Insect	17	21	55	0.322	0.218	0.419	92.54	55	0.449	0.325	0.557	94.76
Fish	12	11	33	0.466	0.387	0.538	29.69	34	0.572	0.475	0.655	56.66
Amphibian	3	3	5	0.332	-0.016	0.608	82.01	4	0.595	0.225	0.815	80.55
Reptile	1	1	4	0.271	0.096	0.430	0	3	0.375	0.030	0.640	68.95
Bird	2	2	4	0.332	0.079	0.544	46.34	4	0.394	-0.086	0.725	83.21

The strength of mate preference was significantly larger when tested using a choice test (r= 0.484, 95% CI: 0.409 to 0.552) compared to a no-choice test (r= 0.364, 95% CI: 0.297 to 0.427; Weighted least-squares regression, main effect of choice design: $F_{1, 168}$= 12.42, P< 0.001; **Figure 4.3a**). The variation in effect sizes was large (Suggested 'high' I^2 values of greater than 75%: Higgins *et al*., 2003) across the whole dataset (I^2= 88.45%), as well as for both no-choice tests (I^2= 85.6%) and choice tests (I^2= 89.55%), as would be expected for data deriving from multiple species and traits. I^2 values for subgroup models can be seen in **Table 4.1**. The difference in effect size between choice designs remained even after excluding studies in which no-choice tests were biased by the inclusion of non-responsive subjects (k= 162, $F_{1, 125}$= 10.01, P= 0.002).

4.3.2 Effect of moderators

There was no difference in the strength of mating preferences between male and female choice ($Q_{M\ 1}$ = 1.83, P= 0.18; **Figure 4.3a**). However, female mating preferences were stronger in choice tests compared to no-choice tests ($F_{1, 68}$ = 18.46, P< 0.001; **Figure 4.4**), while there was no difference in male mating preferences between choice designs ($F_{1, 95}$ = 1.66, P= 0.2; **Figure 4.4**).

Overall, there was no significant difference in the strength of mating preferences between intra-species, inter-population and inter-species choice ($Q_{M\ 2}$ = 2.51, P= 0.29; **Figure 4.3a**). However, intra-species mating preferences were stronger in choice tests compared to no-choice tests ($F_{1, 100}$ = 11.1, P= 0.001; **Figure 4.4**), while there was no difference between choice designs in terms of the strength of inter-population choice ($F_{1, 13}$ = 1.64, P= 0.22; **Figure 4.4**) or inter-species choice ($F_{1, 51}$ = 0.96, P = 0.33; **Figure 4.4**).

There was also no overall difference in the strength of mating preferences across the seven taxonomic groups ($Q_{M\ 6}$ = 6.49, P= 0.37; **Figure 4.3b**). Mating preferences were

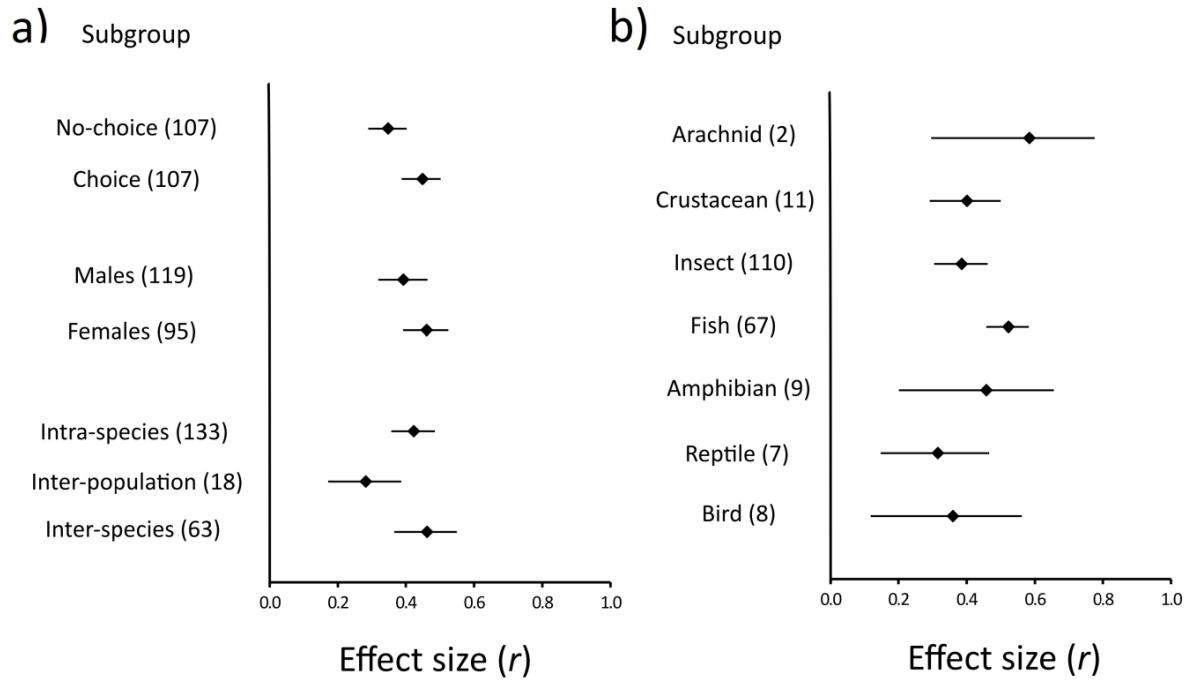

Figure 4.3. Mean strength of mating preferences (correlation coefficient r) for each subgroup analysis, split by **a)** choice design, sex and trait type, and **b)** taxonomic group. Bars show the 95% confidence intervals around the mean effect size estimate. Numbers in parentheses indicate the number of effect sizes in each subgroup.

stronger in choice tests compared to no-choice tests for insects ($F_{1, 87}$ = 6.24, P= 0.014), fish ($F_{1, 52}$ = 4.1, P= 0.048) and amphibians ($F_{1, 5}$ = 11.8, P= 0.02), but not for crustaceans ($F_{1, 8}$ = 0.007, P= 0.94), reptiles ($F_{1, 5}$ = 0.47, P= 0.52) or birds ($F_{1, 5}$ = 0.08, P= 0.78).

There was no significant difference in effect sizes derived from choice outcomes or proxy measures of preference (Mixed-effects meta-analysis, $Q_{M\ 1}$= 0.4, P= 0.53). Mating preferences were stronger in choice tests compared to no-choice tests for both those effect sizes derived from choice outcomes ($F_{1, 29}$ = 13.78, P< 0.001) and those derived from proxy measures of preference ($F_{1, 128}$ = 6.61, P= 0.011).

4.3.3 Publication bias

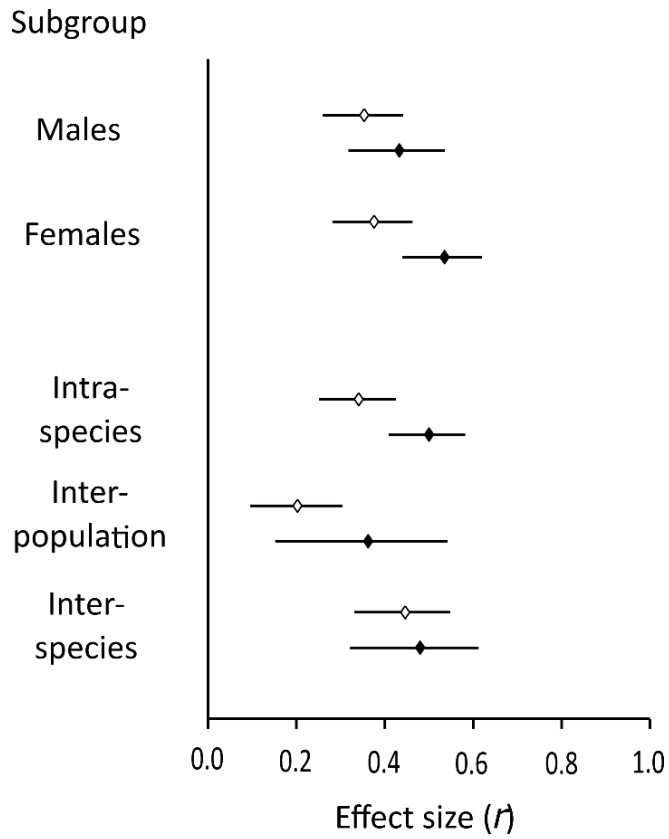

Figure 4.4. Mean strength of mating preferences (correlation coefficient *r*) for sex and trait type subgroups, split by choice design (white diamonds for no-choice tests and black diamonds for choice tests). Bars show the 95% confidence intervals around the mean effect size estimate. See **Table 4.1** for the number of effect sizes for each subgroup.

I found a weak positive correlation between effect size and sample variance (Spearman's rank correlation, r_S= 0.14, *P*= 0.046. However, there was a much stronger correlation between standardized effect size and variance (τ = 0.16, *P*< 0.001). This was true for no-choice tests (τ = 0.18, *P*= 0.006) but not for choice tests (τ = 0.089, *P*= 0.18). The Rosenberg fail-safe number was 108797, suggesting that an unrealistic number of studies with an effect size of zero would need to be added to the sample to give a non-significant result. Orwin's fail-safe number was 1757, so that a large number of studies with effect size 0.05 would need to be added for the mean effect size to be reduced to 0.05. A regression test did not detect significant funnel plot asymmetry (Egger's test, t_{212} = 0.52, *P*= 0.6). However trim and fill analysis detected 33 missing effect sizes on the right hand of the funnel plot (corresponding to large effect sizes, see **Figure 4.5**). This is

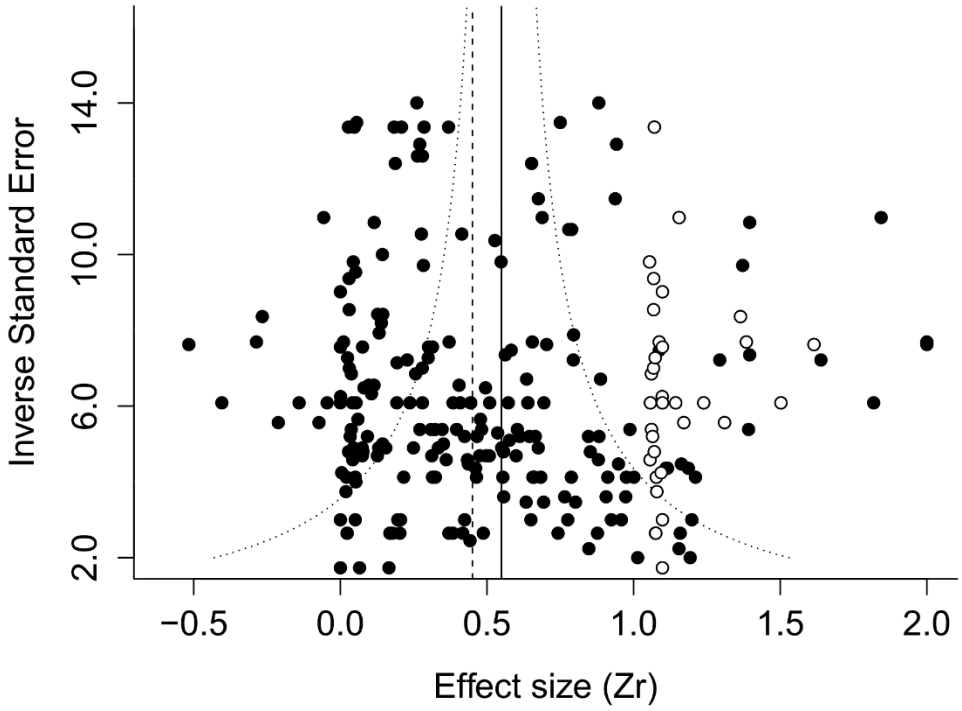

Figure 4.5. Funnel plot showing the relationship between effect size (*Zr*) and inverse standard error. Black dots show the effect sizes included in the meta-analysis (*N*= 214) and white dots show 'missing' effect sizes determined using a trim and fill analysis to test for funnel plot asymmetry (*N*= 33). The solid line indicates the new mean effect size derived from a random-effect meta-analysis after including the 33 'missing' effect sizes.

likely driven by the large number of effect sizes around *Zr*= 0. Running the model after imputing these missing studies nevertheless leads to an increase in the overall mean effect size (*r*= 0.5, 95% CI: 0.45 to 0.54).

There was no significant correlation between effect size and year of publication (r_s= -0.0067, *P*= 0.92). However meta-regression detected a weak negative relationship between effect size and publication year ($Q_{M\,1}$ = 4.82, *P*= 0.028; **Figure 4.6**).

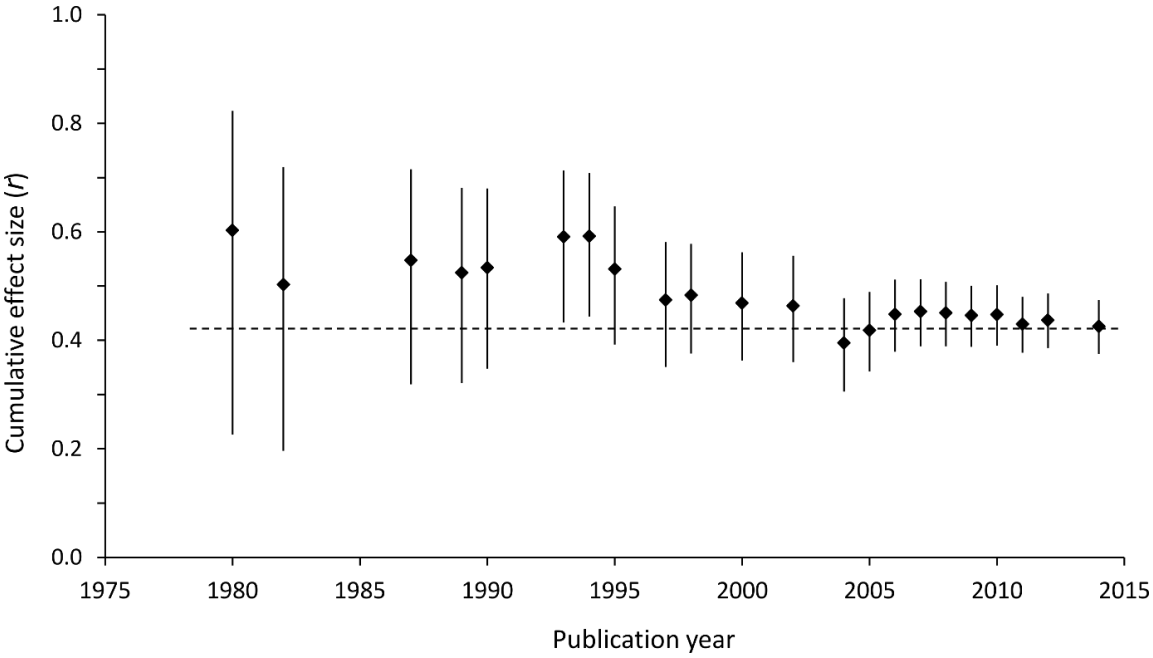

Figure 4.6. Cumulative meta-analysis showing change in mean effect size *r* over time. Diamonds represent mean effect size estimates and error bars represent 95% confidence intervals above and below the mean. The dashed line shows the mean when all effect sizes are included in the model (*N*= 214).

4.4 Discussion

The meta-analysis of 38 studies shows that mating preferences are significantly stronger when tested using a choice test ('medium' effect size of 0.484, see Cohen, 1992) compared to a no-choice test ('medium' effect size of 0.364, see Cohen, 1992), with a difference in mean effect size of 0.12 between the two test designs. Though small, this effect is highly significant and was very consistent across all studies used in the analysis. I have also shown that this result remains after excluding studies in which no-choice tests were biased by the inclusion of non-responsive subjects. This study therefore re-iterates the fact that experimental design is an important factor in the measurement of mating preferences (Wagner 1998), and that social context can strongly influence the strength of sexual selection.

I do not suggest that one experimental design gives a more 'accurate' measure of mating preferences than the other, but rather that these results show that the strength of mating preferences (and thus sexual selection) can vary depending on social context. The use of different choice designs may in part depend on the question an experimenter wishes to ask, and a plurality of approaches may often be useful to tease apart mating preferences. However, I do suggest that the interpretation of future experiments takes this effect into account. Moreover, the two choice designs broadly correspond to the different forms of mate encounter in the wild (sequential versus simultaneous encounter), and thus the strength of choice in natural populations may vary significantly between different social or ecological contexts (Jennions & Petrie, 1997; Coyne *et al*., 2005; MacLaren & Rowland, 2006; Miller & Svensson, 2014). As such, if choice tests are used in the laboratory to test for preferences in species in which mates are mainly encountered sequentially in the wild, then in many cases the strength of mating preference measured may be an overestimate of what occurs in the wild (Barry & Kokko, 2010). Indeed, choice tests appear to be the more common experimental design: Owen *et al*. (2012) estimated that 71% of studies citing Wagner (1998) included choice tests.

Clearly the choice of experimental design should depend on the patterns of mate encounter seen in the wild (Coyne *et al*., 2005; Mendelson & Shaw, 2012). However, in many species we simply do not have the data to be able to assess which choice design is the more ecologically realistic (apart from well-known examples such as lek or harem breeders; e.g. Gibson, 1996). Two studies included in this analysis illustrate the potential to be misled by studies using ecologically unrealistic choice designs. The studies consider male mate choice in the mantid *Pseudomantis albofimbriata* (Barry *et al*., 2010) and in the fiddler crab *Uca mjoebergi* (Booksmythe *et al*., 2010). In both of these species, field data suggest that males are unlikely to encounter more than one female at a time in the wild, and so no-choice tests seem the most ecologically relevant design to use. However, in both cases significant mating preferences were detected in choice tests but not in the corresponding no-choice tests (Barry *et al*.,

2010; Booksmythe *et al*., 2010). Therefore, despite the strong choice observed in experiments, mating preferences are unlikely to lead to significant sexual selection in the wild, except for on the very rare occasions when males encounter females simultaneously.

I consider there to be two important effects that might lead to stronger mating preferences in choice tests. The first is a cognitive effect arising due to differences in the ability to compare options in each design. I suggest that a subject in a choice test may be better able to compare options comparatively when given a choice, either because the method of mate sampling has evolved under such conditions, or because being able to perceive differences between options becomes easier when they can be compared simultaneously (Rowland, 1982; Bateson & Healy, 2005; Beatty & Franks, 2012). This hypothesis assumes that the subject has the ability to actively compare options presented simultaneously, an assumption which may not apply to all species, especially if this requires more "complex" cognitive processes. However, the tactics and decision rules used to make mate choice decisions are unknown for most species, and distinguishing between hypotheses is difficult (Gibson & Langen, 1996). Indeed, it may be that in some species mates are assessed sequentially, perhaps using threshold-based decision rules, even when simultaneous comparison is possible (Gibson, 1996; Kacelnik *et al*., 2011; Chapter 3).

The second factor which may influence the strength of preference is the cost associated with rejecting an option in each test. This is because the perceived mate encounter rate is different under the two choice designs (Valone *et al*., 1996). In a choice test, the cost of rejecting one of the options is zero, as there is always at least one other option available. Conversely, in a no-choice test the potential cost of rejection is higher due the fact that the likelihood of being presented with another option is unknown to the subject (and may depend on how often the subject has encountered mates before the test: in most cases this is never). If subjects in a no-choice test perceive that the risk of remaining unmated is high then they might be less

likely to exhibit any mating preference and be more likely to mate randomly with respect to the stimulus being tested (Werner & Lotem, 2006; Barry & Kokko, 2010; Booksmythe *et al*., 2011). This explanation is more general than the one based on cognition: even if this cost of rejection varies between species it will generally always be higher in a no-choice test (compared to zero for choice tests). This leads to the prediction that we should not see any difference in the strength of preference between designs once this perceived mate encounter rate has been controlled for, for example by giving subjects experience of the same number of mates before choice tests (see Chapter 8). I would also expect that varying the cost of rejection (for example by making the sex ratio more biased, or by varying the age of the subjects) should influence the strength of preference observed in no-choice tests (as is seen for example in sequential choice experiments: Milinski & Bakker, 1992; Shelly & Bailey, 1992; Lehmann, 2007; Beckers & Wagner, 2011) but should have no effect on the strength of preference in choice tests. Finally, I also predict that the difference in the strength of preference between designs should decrease as the costs of mating and/or reproduction increase (for example in species in which females are harmed during mating, or in which females invest heavily in offspring; Halliday, 1983): if this cost is sufficiently high it will outweigh the cost of rejection and so subjects should remain choosy even in the no-choice situation.

I did not find stronger mating preferences overall for female choice compared to male choice as predicted. Kokko & Jennions (2015) recently suggested that this predicted difference will be hard to detect due to research bias. If there is a tendency for mate choice experiments (in either sex) to be performed only in those species for which choice is likely and/or apparent, then this is not a truly representative sample, and so the strength of choice will appear similar in both sexes. This is likely to be a common problem with meta-analysis that may be very difficult to overcome (Koricheva *et al*., 2013). This research bias, along with the relatively small samples sizes in our analysis, means that I would not suggest that this component of sexual selection theory has been refuted by this result. However, I did find that choice design significantly

influenced the strength of female choice, but not the strength of male choice. If the benefits of being choosy are higher for females (due to their larger investment in reproduction) then this may lead to stronger mating preferences in situations where the cost of choosing is small, namely in choice tests. Alternatively, males and females may differ in their mate assessment strategies. For example, if males have a threshold of mate quality above which they will accept all females, so that comparison is not important, then the number of options available will not change the patterns of mate choice observed. However, this explanation only works under the unlikely condition that males are more likely to use threshold-based tactics for choosing mates, whereas females of the same species are more likely to use comparative tactics.

I also found a difference in the effect of choice design depending on the type of choice, so that there was a significant difference between designs for studies considering intra-specific choice but not those considering inter-population or inter-species choice. However, I am cautious to draw strong conclusions from this comparison due to the small sample sizes for the latter two groups. A theory based on the costs of choice would predict the opposite: if mating with the wrong species leads to zero fitness we should expect individuals to be more discriminating when choosing between conspecifics and heterospecifics than when choosing between conspecifics. However, if comparison is not important for species recognition, so that individuals have a threshold above which they accept a partner as a conspecific, the number of options available will not influence the strength of choice. The existence of such a threshold might be more persuasive in terms of con- and hetero-specifics as opposed to some continuous measure of quality for example, as individuals are either conspecifics (so you should consider mating with them) or they are not (so you should ignore them). However, there is still ongoing debate as to whether species recognition and mate choice are different processes or part of a continuum of mate choice (Ryan & Rand, 1993; Mendelson & Shaw, 2004; Phelps *et al*., 2006; Ryan & Taylor, 2015).

I found some evidence for publication bias in the dataset. For example, trim and fill analysis indicated that there are 33 'missing' positive effect sizes. However it is unclear to what extent this represents a signal of publication bias, given that usually the opposite pattern is seen (Koricheva *et al*., 2013), and the non-publication of studies with large effect sizes seems counterintuitive (though see Koricheva, 2003). Rather it seems likely that this result is driven by the large number of effect sizes around zero, as the trim and fill procedure is designed to detect asymmetry and remove it (Duval & Tweedie, 2000). Indeed the main assumption of this analysis (that there is a single symmetric distribution of effect sizes) may be unlikely in this case, as there are several potential moderators and high heterogeneity: Koricheva *et al*., 2013). Meta-regression also detected a negative relationship between effect size and publication year. This is an effect commonly seen in ecological meta-analyses, and may reflect a time-lag in the publication of studies with small effect sizes (Jennions & Møller, 2002; Koricheva *et al*., 2013). However, this decrease is relatively weak, and the overall mean effect size is still highly significantly different from zero following this decrease, suggesting that publication bias is of minor importance to this analysis.

Phylogenetic history was found to have essentially no influence on the mean effect size. This is perhaps unsurprising given the dataset has several features which may make the detection of a phylogenetic signal unlikely. First, mate choice is predicted to be capable of evolving rapidly and thus is highly evolutionarily labile (Blomberg *et al*., 2003). Recent studies suggest it is common for phylogenetic history to have a small or even negligent effect on effect size for analysis of behavioural traits (e.g. delBarco-Trillo, 2011; Santos *et al*., 2011; Garamszegi *et al*., 2012). Second, the analysis includes preference measures for a wide range of traits, and indeed in most cases the preferences tested are different even for closely related taxa. Finally, I obtained data from a range of species with a very wide taxonomic spread (with the exception of nine species of *Drosophila*) so that most species are very distantly related. Indeed the method of constructing a phylogenetic tree used here greatly underestimates the

branch lengths between distantly related species. This makes any potential phylogenetic signal very small (Björklund, 1997).

Because of this wide taxonomic spread, the meta-analysis naturally includes a wide range of studies that vary in many aspects of experimental design, not least due to the specific logistic requirements of working with each study species. As few researchers explicitly set out to test the effect of experimental design on choice, in many cases confounding variables were not fully controlled for. The strength of meta-analysis is in detecting effects in such heterogeneous data (Koricheva *et al*., 2013). However, that is not to say that future experimenters should not attempt to control for such variables. I suggest that where possible experiments be fully randomized, and that the same response traits are used as measures of preference in both kinds of tests. A particularly powerful approach is to test the same subjects in both no-choice and choice tests (with order randomized so as to avoid experience effects; e.g. Reading & Backwell, 2007; Wong & Svensson, 2009). Only three studies in the analysis were able to do this (Rowland, 1982; Verrell, 1995; MacLaren & Rowland, 2006). There are undoubtedly many other aspects of experimental design that may influence the strength of mating preferences seen in the laboratory, including for example how animals are kept prior to testing, how preferences are scored (e.g. whether subjects who do not respond to stimuli are included in the analysis; Kokko & Jennions, 2015), and even the personality (exploratory tendency) of subjects in tests that use association time as a preference measure (e.g. David & Cezily, 2011). The influence of these factors on the strength of mating preferences is outside the scope of this study, but I suggest that quantification of these effects will be possible.

4.5 Conclusion

In conclusion, I find that female, intra-specific mating preferences are significantly stronger when tested using choice tests compared to no-choice tests. I suggest that this is due to the increased cost of rejection in no-choice tests. If confirmed, this effect

may not be limited to mate choice, but may indeed also be applicable to other areas of behavioural research in which these kinds of choice designs are used, such as studies of foraging (Kacelnik *et al*., 2011) or predation (Beatty & Franks, 2012). I also show that the effect of experimental design on preferences depends on both the type of preference and the sex of the subject used in a test. This suggests that these groups may fundamentally differ either in how they choose mates or in the relative costs and benefits of choosing. Importantly, choice tests in the laboratory may systematically inflate estimates of the strength of mating preferences in species in which this situation is demographically unrealistic in the wild. For this reason I recommend that studies of mate choice do not start with two-choice tests by default. Instead, no-choice designs may be the most sensible starting point unless knowledge of the natural behaviour of the study species suggests otherwise. Further, only by measuring mate choice in more natural social contexts will we fully understand its role in sexual selection and speciation.

Chapter 5

Sexual selection on male processus length in *Lygaeus equestris* and *Lygaeus simulans*

Abstract

In this chapter I investigate the strength of sexual selection acting on male processus length in *Lygaeus equestris* and *Lygaeus simulans*. I first test for pre- and post-copulatory selection on processus length in *L. equestris* using the large sample of males used in Chapter 3. I find negative linear pre-copulatory selection on processus length, but only in mating trials in which two males were present. This selection likely arises indirectly due to selection on a correlated trait, as processus does not interact with the female prior to copulation. I also detect significant stabilising post-copulatory selection on processus length in terms of the likelihood of successful insemination. This selection was unaffected by choice design. By combining both episodes of selection I show that overall selection on processus length is stabilising. Second, I investigate pre- and post-copulatory selection on processus length in *L. simulans* in an attempt to replicate the results of Tadler (1999), using a no-choice design only. I could not detect significant pre- or post-copulatory selection in this population of *L. simulans*. I discuss why this might be the case. I then use a formal meta-analysis, combining the results of three of my experiments with two previously published studies, to show that overall there is significant stabilising selection on processus length in *L. simulans*.

Acknowledgments

I thank Michael Morrissey for advice on calculating selection gradients.

5.1 Introduction

In many insects the male intromittent organ ends in an extremely thin, elongate tube through which sperm are transferred (e.g. Tadler, 1999; Aspöck & Aspöck, 2008; Matsumura & Akimoto, 2009; van Lieshout & Elgar, 2011; see Chapter 1). Terminology for such structures varies between species, but it is commonly referred to as a flagellum or processus (Snodgrass, 1935). An extremely elongate intromittent organ is also seen in *Lygaeus equestris* and *L. simulans*. The male intromittent organ consists of two distinct parts: a soft proximal region which I refer to as the vesica, and a much longer distal processus (known as the *processus gonopori*, but hereafter referred to as the processus) which is around two-thirds of a male's body length (Tadler, 1999). The processus is a simple, sclerotized, hollow tube through which the ejaculate is transferred via fluid pressure at the base (Ludwig, 1926). This structure is threaded along the female spermathecal duct, and for insemination to be successful it appears that sperm has to be released at the entrance to the spermatheca at the end of this duct (Tadler, 1999).

Despite their widespread occurrence, few studies have formally investigated selection acting on exaggerated genitalia. *L. equestris* and *L. simulans* are notable exceptions to this pattern, with selection acting on male processus length being investigated in several studies (Tadler, 1999; Tadler *et al*., 1999; Higgins, 2009). The may be because of the relative simplicity of the processus, which makes it amenable for study without the need to quantify subtle aspects of genital size and shape which may be difficult to interpret. Tadler (1999) performed a formal analysis of the strength and pattern of sexual selection acting on processus length in *L. simulans*, and was able to detect significant post-copulatory selection using insemination success as the measure of male fitness. Higgins (2009) also found evidence for negative post-copulatory selection on aedeagus length (the total length of both the processus and vesica) in *L. equestris*, detecting a negative relationship between aedeagus length and the average number of fertilised eggs produced after mating with a median of three females. However this

relationship is driven by a single male with a very short aedeagus and so does not seem to be very robust.

Another relatively well-studied species in this regard is the tortoise beetle *Chelymorpha alternans*, in which males possess a long, thin flagellum that is inserted into the female spermathecal duct during mating (Rodriguez, 1995; Rodriguez *et al*., 2004). Rodriguez (1995) used experimental manipulation to show that flagellum length is important for sperm transfer, as females are less likely to reject sperm from males with a longer flagellum. Additionally, in competitive mating tests males with a longer flagellum fathered more offspring (Rodriguez *et al*., 2004), suggesting that male flagellum length is under positive directional selection.

Though it seems obvious that sexual selection may play a role in the evolution of such exaggerated genitalia (Andersson, 1994; Eberhard, 1996), this needs to be explicitly tested before alternative explanations can be ruled out. For example, if processus breakages are common, perhaps during mating, then the extreme length of the intromittent organ may be a form of insurance, allowing a male to successfully inseminate a female even after a breakage (see Chapter 6).

In this chapter I present two experiments that investigate selection acting on processus length in both *L. equestris* and *L. simulans*. First I measured the length of the processus for the sample of *L. equestris* males used in Chapter 3, for which I obtained measures of both mating and insemination success. Data from these males came from four different experimental choice designs, and so I was additionally able to assess the effect of social environment on the strength of sexual selection on processus length. Second, I performed a similar experiment to Tadler (1999) in an attempt to replicate the finding that there is significant post-copulatory selection on processus length in *L. simulans*. In this experiment I also record male mating success as before, but in this case only in a no-choice context. By recording both male mating success and insemination success I am able to estimate both pre- and post-copulatory episodes of

selection on processus length. I also combine both these episodes of selection, by examining the insemination success of all males (including those that did not mate) to get an estimate of the overall selection acting on processus length in the population. Finally, I use formal meta-analysis to combine selection gradients reported for *L. simulans* in previous studies with those found here.

5.2 Sexual selection on processus length in Lygaeus equestris

5.2.1 Methods

The individuals used in this experiment were a subset of males used in Chapter 3. Therefore experimental methods for the mating trials are identical to those already described. After mating trials, all males were euthanized and the male processus was removed and measured as described in Chapter 2. Accidental breakages during dissection meant I obtained processus measurements from 174 males.

My analysis considered the relationship between male processus length and three estimates of male reproductive success. I first considered pre-copulatory selection on processus length by using male mating as the measure of fitness. Second I considered post-copulatory selection by comparing male insemination success for those males that mated. Finally I attempted to estimate overall, population-level selection using insemination success as the response variable, but this time for all males, including those that failed to mate.

I tested for sexual selection on morphological traits using a classic regression-based approach (Lande & Arnold, 1983) using generalised linear models, with mating success or insemination success as a binary response variable. Processus length and experimental choice treatment were included as explanatory factors in all models. I also included male and female body length and copulation duration as factors where

appropriate. As many copulations were ended manually (26 out of 64), I fitted copulation duration as a categorical factor with two levels: either long (copulations were ended manually) or short (copulations ended naturally). I first fitted full models, including quadratic and interaction terms where appropriate. To avoid over-parameterisation, I then removed any non-significant quadratic and interaction terms and re-ran models.

To visualise the shape of selection on processus length I produced fitness surfaces using cubic-splines, as in Chapter 3. Curves were calculated using general additive models, with a single predictor variable (processus length, male body length or female body length). All statistical analyses were performed in R version 3.1.0 (R Development Core Team, 2014).

5.2.2 Results

I obtained processus measurements for 174 males in total (*N*= 39, 54, 26 and 55 individuals from treatments 1, 2, 3 and 4 respectively), of which *N*= 64 mated (*N*= 15, 17, 9 and 23 individuals from treatments 1, 2, 3 and 4 respectively). 50 processi were also re-measured to assess repeatability, using the same images but blind to the original measurements. Analysis of variance (Lessells & Boag, 1987) indicated that repeatability was very high (r = 0.98). Average processus length was 7.22 mm (s.d. = 0.17 mm), which is over two-thirds the total body length of males (mean= 10.22 mm, s.d.= 0.32 mm). Average female body length was 11.25 mm (s.d.= 0.38 mm, *N*= 64). Processus length was significantly correlated with male body length (r_{172} = 0.4, P< 0.001).

I first considered pre-copulatory selection on male processus length arising from differential mating success. Across all choice treatments there is no significant difference in processus length between mated and unmated males (Binary logistic GLM; χ^2_1 = 1.39, P= 0.24; **Figure 5.1a**). However, there is a significant interaction

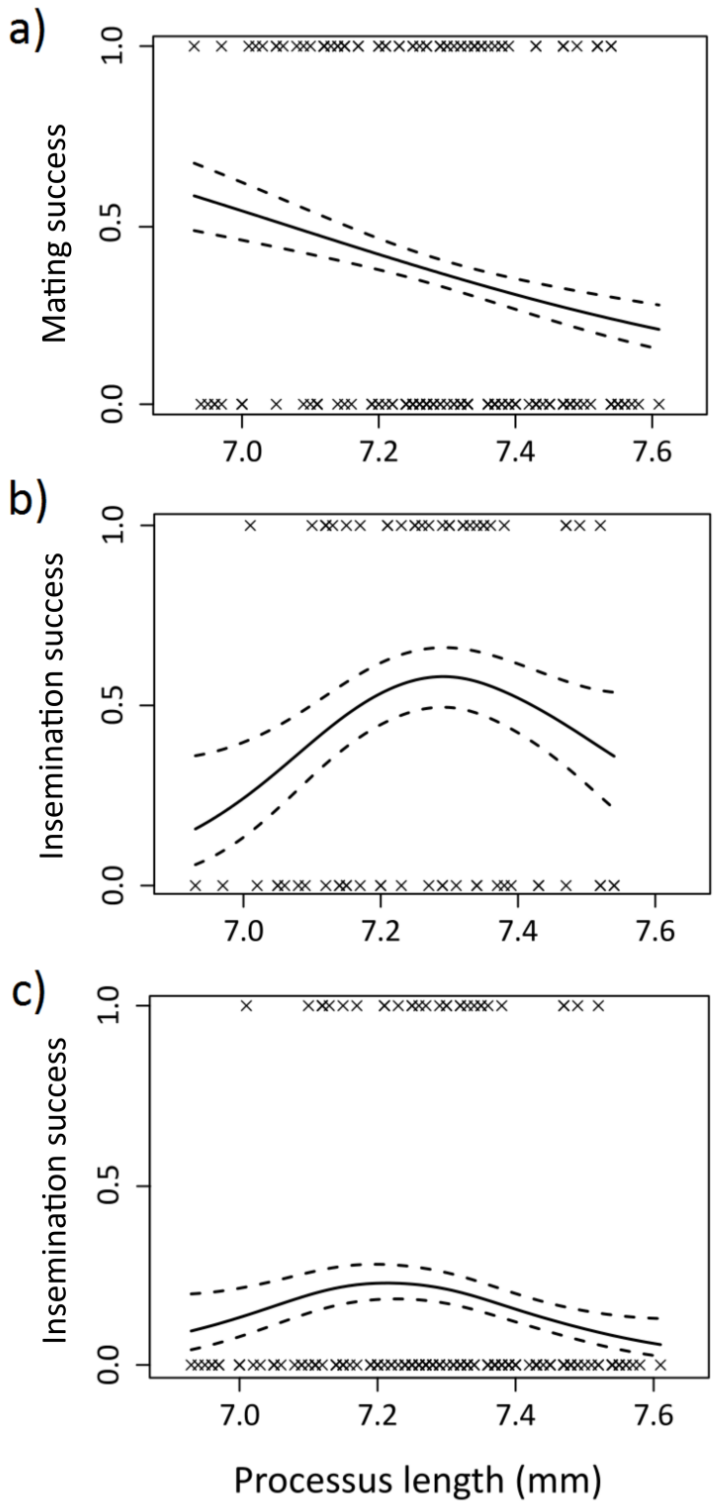

Figure 5.1. Selection on male processus length in *L. equestris* for three measures of male reproductive success: **a)** mating (mated or non-mated, *N* = 174); **b)** insemination success (production of offspring) for mated males (*N* = 64); and **c)** insemination success (production of offspring) for all males, including those that did not mate (*N* = 174). Dashed lines indicate 1 standard error above and below the predicted line.

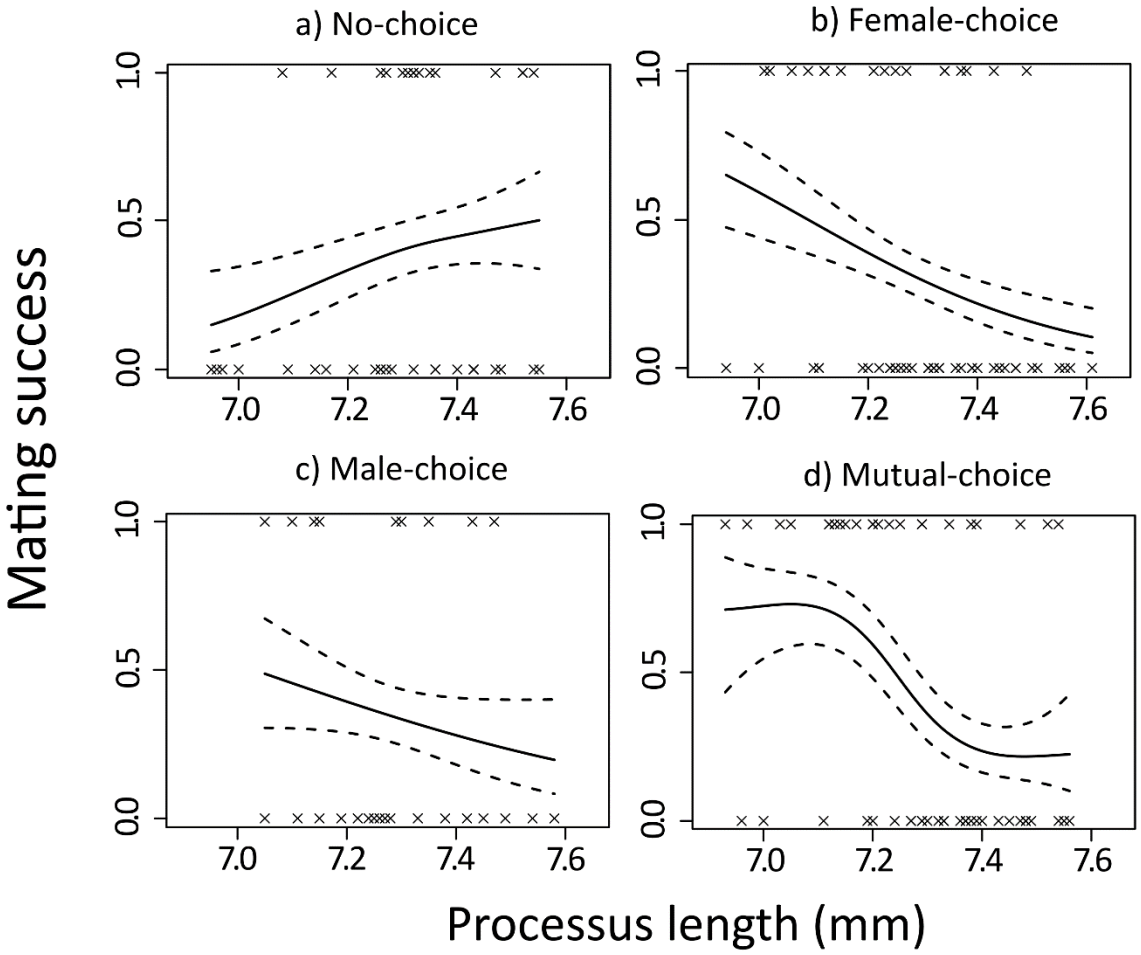

Figure 5.2. Pre-copulatory selection on male processus length in *L. equestris* for the four experimental choice treatments: a) No-choice (1 male and 1 female per dish, *N*= 39); b) Female choice (2 males and 1 female per dish, *N*= 54); c) Male choice (1 male and 2 females per dish, *N*= 26); and d) Mutual choice (2 males and 2 females per dish, *N*= 55). Dashed lines indicate 1 standard error above and below the predicted line.

between processus length and choice treatment (χ^2_1 = 6.1, *P*= 0.014), so that a significant negative relationship was only seen in the two choice treatments in which two females were present (**Figure 5.2**). Male mating frequency was also significantly influenced by choice treatment (χ^2_1 = 6.15, *P*= 0.013; Chapter 3). As in Chapter 3, there was no significant effect of male body length on pre-copulatory success across all choice treatments (χ^2_1 = 0.055, *P*= 0.82).

Second, I considered post-copulatory selection on processus length arising from differential insemination success. 30 out of 64 mated females laid fertile eggs. For those males that mated, processi of intermediate length were significantly more likely to lead to the production of offspring (χ^2_1 = 7.84, *P*= 0.005; **Figure 5.1b**). Importantly, post-copulatory selection on processus length did not vary according to experimental treatment (χ^2_1 = 1.48, *P*= 0.22). Insemination success was also significantly lower for males of intermediate body length (χ^2_1 = 10.92, *P*= 0.001; **Figure 5.3**). In contrast to Chapter 3, insemination success was not significantly influenced by female body length (χ^2_1 = 3.26, *P*= 0.07). Insemination success was also not significantly influenced by choice treatment (χ^2_1 = 1.48, *P*= 0.22). Finally, longer copulations were also significantly more likely to result in successful insemination (χ^2_1 = 5.68, *P*= 0.02), with only a single copulation shorter than 200 minutes resulting in offspring. Males did not have to copulate for as long in order to successfully inseminate small females compared to large females (interaction between female body length and copulation duration, χ^2_1 = 6.08, *P*= 0.014; see Chapter 3). There was no significant correlation between processus length and copulation duration for mated males (r_s = -0.2, *N* = 64, *P*= 0.11).

Third I considered 'overall' selection by repeating the analysis of male insemination success but including all males, including those that did not mate. Again males with an intermediate processus length were significantly more likely to gain a successful insemination (χ^2_1 = 7.35, *P*= 0.007; **Figure 5.1c**). Overall insemination success was not influenced by male body length (χ^2_1 = 0.19, *P*= 0.67). Finally, there was also a significant effect of choice treatment on overall selection on processus length (χ^2_1 = 4.3, *P*= 0.038), and insemination success was significantly higher in the no-choice treatment compared to the remaining treatments (χ^2_1 = 4.21, *P*= 0.04). However both of these results may be driven by stochastic effects due to the small number of inseminations in each treatment.

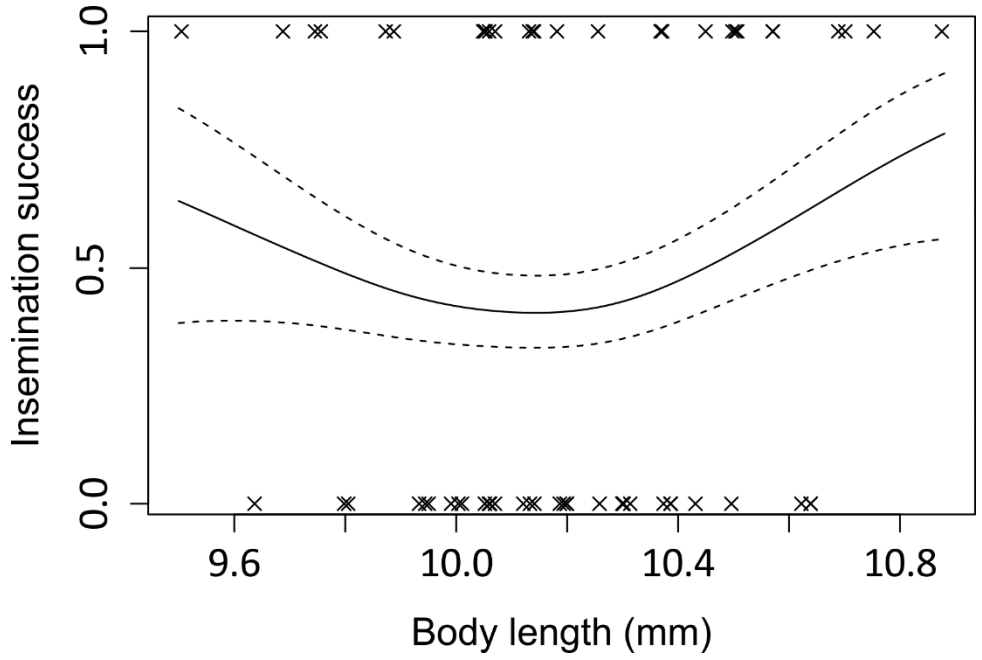

Figure 5.3. Post-copulatory selection on male body length in *L. equestris* for mated males (*N*= 64). Dashed lines indicate 1 standard error above and below the predicted line.

5.3 Sexual selection on processus length in Lygaeus simulans

5.3.1 Methods

I performed no-choice mating trials (one male and one female per dish) using virgin, sexually mature individuals (between 8 and 11 days old). Trials were performed on the bench (21-25°C) in plastic dishes (see general methods for details). Pairs were watched continuously for two hours, and mating attempts and copulations were recorded. Pairs were then checked every ten minutes for up to eight further hours (10 hours total), or until a copulation ended. Pairs were separated manually if they were still in copula at the end of the trial. Copulations were considered sufficient if they lasted 20 minutes or longer (this is likely to be conservative: see Chapter 2), and pairs were only allowed one such copulation to ensure an accurate measure of insemination success. I performed 140 such trials.

At the end of the mating trial, mated females were isolated in tubs and given two weeks to oviposit. If nymphs were present after one week, females were transferred to a new tub with fresh water. Tubs and nymphs were frozen after two weeks and the number of nymphs produced by each female was counted. This gives an additional measure of post-copulatory success (offspring production) that was not measured in the previous experiment. Body length was also recorded for all males and most mated females (though some died early and so body lengths could not be measured accurately) as in Chapter 3. All males were euthanized at the end of the trial, and the processus was then removed and measured as described in Chapter 2.

I determined the strength of sexual selection on morphological traits using generalised linear models as above, with mating success and insemination success as binary response variables. Processus length was included as a factor in all models. I first constructed models that included quadratic effects, and then followed a model simplification rationale in which non-significant interactions were removed. As above, copulation duration was included in all models as a categorical factor with two levels, as 25 of 102 matings were ended manually. I did not estimate a combined measure of pre- and post-copulatory selection as in the previous experiment (because results were not significant), however I did use this method to produce a fitness function (see below).

By counting the number of offspring (and unhatched, fertile eggs) produced after two weeks I have an additional measure of post-copulatory reproductive success which I refer to as ‘fertilisation success’. I tested for determinants of fertilisation success using a general linear model with total fertility (total number of nymphs and unhatched fertile eggs produced after two weeks) as the response variable (this variable is normally distributed). I included only those matings that produced offspring or fertile eggs.

I visualised the shape of selection on male and female traits using cubic splines as described above. Again general additive models included only a single predictor variable (see above). All statistical analyses were performed in R version 3.0.1 (R core development team, 2014).

5.3.2 Results

I performed mating trials using 140 pairs, of which 102 mated. Males had an average processus length of 6.76mm (s.d.= 0.19mm). One male had a very short processus (5.61mm with tip still intact), though removal of this outlier did not change the mean greatly (N= 139, mean= 6.77mm, s.d.= 0.16mm). This male was included in the analysis but was removed when plotting cubic splines, as outliers can have a strong effect on curve fitting. As the male was unmated it was only included in the analysis considering male mating success; nevertheless removing this outlier had no effect on the significance of the model results. Average male body length was 10.19 mm (s.d.= 0.36 mm), and average female body length was 11.44 mm (s.d.= 0.39 mm, *N*= 86). There was a significant correlation between male body length and processus length (t_{138}= 6.07, *r*= 0.46, *P*< 0.001).

I first considered pre-copulatory selection on morphology arising from differential mating success There was no significant pre-copulatory effect of male processus length on mating success (Binary logistic GLM; χ^2_1 = 0.31, *P*= 0.58; **Figure 5.4a**). However, larger males were more likely to achieve a mating (χ^2_1 = 5.9, *P*= 0.015; **Figure 5.5**).

I next considered post-copulatory selection using mated pairs. I obtained fertility data for 101 mated females, and body length measurements for 86 of these. Copulation duration was the strongest determinant of insemination success (χ^2_1 = 36.02, *P*< 0.001). Contrary to previous studies, there was no significant quadratic effect of processus length on insemination success (χ^2_1 = 0.08, *P*= 0.77; **Figure 5.4b**), nor was there a significant linear effect (χ^2_1 = 1.48, *P*= 0.22). Insemination success was not

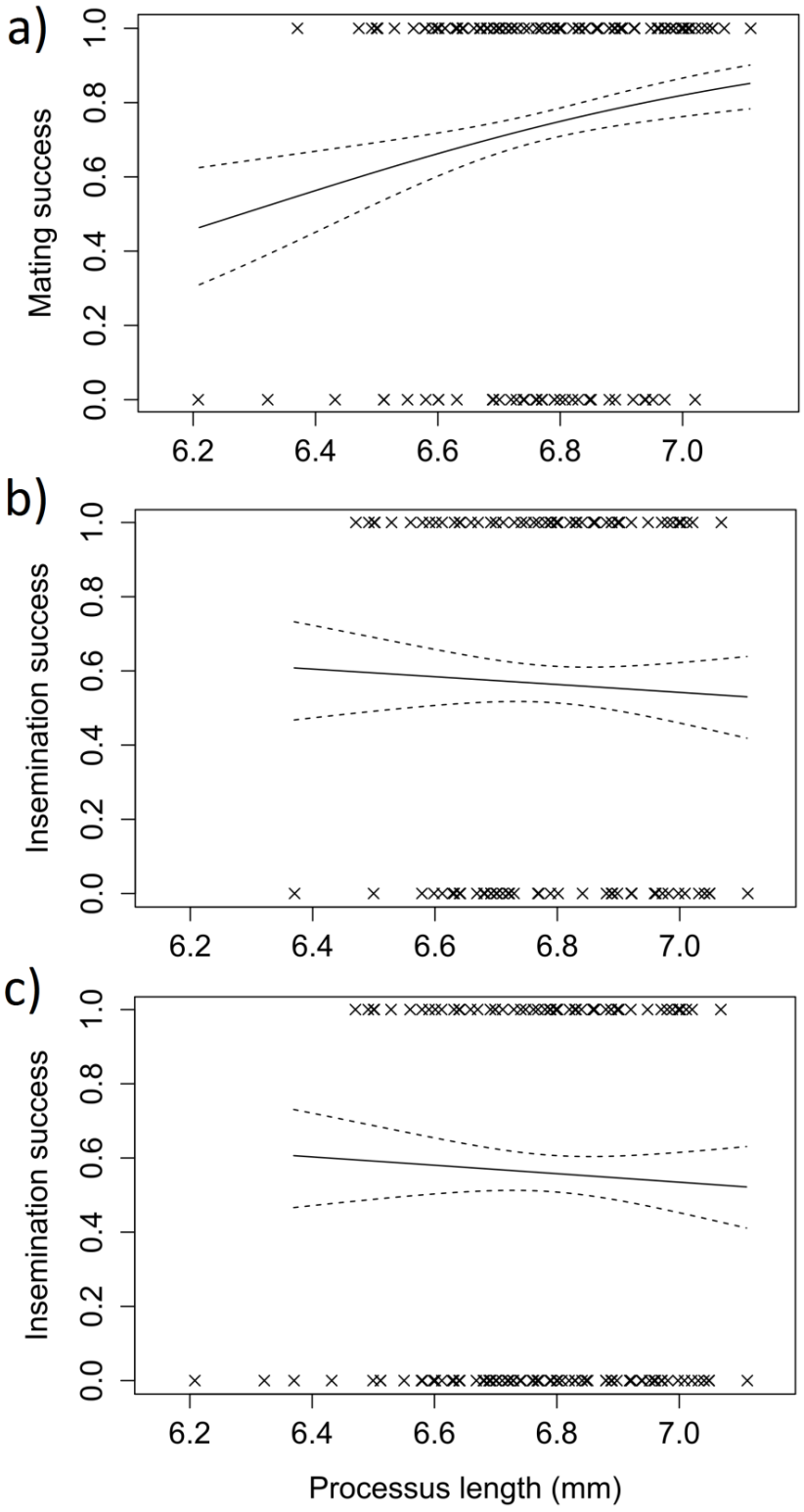

Figure 5.4. Selection on processus length in *L. simulans* for three measures of reproductive success: **a)** male mating (*N*= 140), **b)** insemination success for mated males only (*N*= 101), and **c)** insemination success for all males (N= 140). Dashed lines indicate 1 standard error above and below the predicted line.

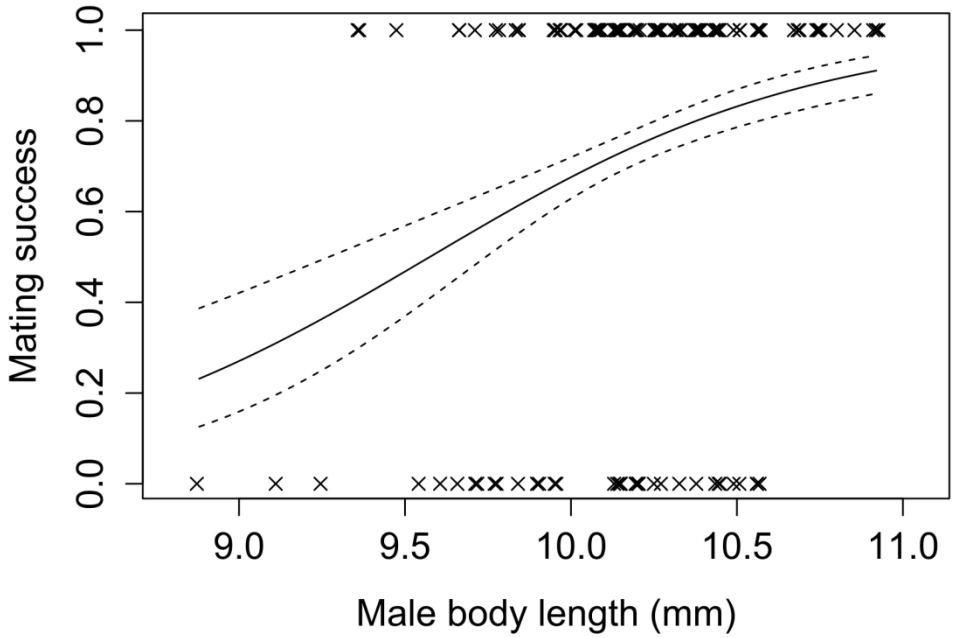

Figure 5.5. Pre-copulatory selection on male body length in *L. simulans* for all males (*N*= 140). Dashed lines indicate 1 standard error above and below the predicted line.

influenced by male body length (χ^2_1 = 0.14, *P*= 0.71). However, larger females were significantly more likely to be successfully inseminated (χ^2_1 = 10.64, *P*= 0.001; **Figure 5.6a**). This is likely driven by the positive correlation between female body length and copulation duration (Spearman's rank correlation, *N*= 86, r_s= 0.23, *P*= 0.032). Considering insemination success for all males (including those that did not mate) did not alter the shape of selection on processus length (**Figure 5.4c**).

Finally I also considered post-copulatory sexual selection arising through differential offspring production, in those pairs that successfully produced offspring. Larger females produced significantly more offspring ($F_{1, 43}$= 8.61, *P*= 0.005; **Figure 5.6b**). Offspring number was not significantly influenced by copulation duration ($F_{1, 43}$= 0.48, *P*= 0.49), male body length ($F_{1, 43}$= 3.07, *P*= 0.09) or male processus length ($F_{1, 43}$= 0.01, *P*= 0.91; **Figure 5.7**).

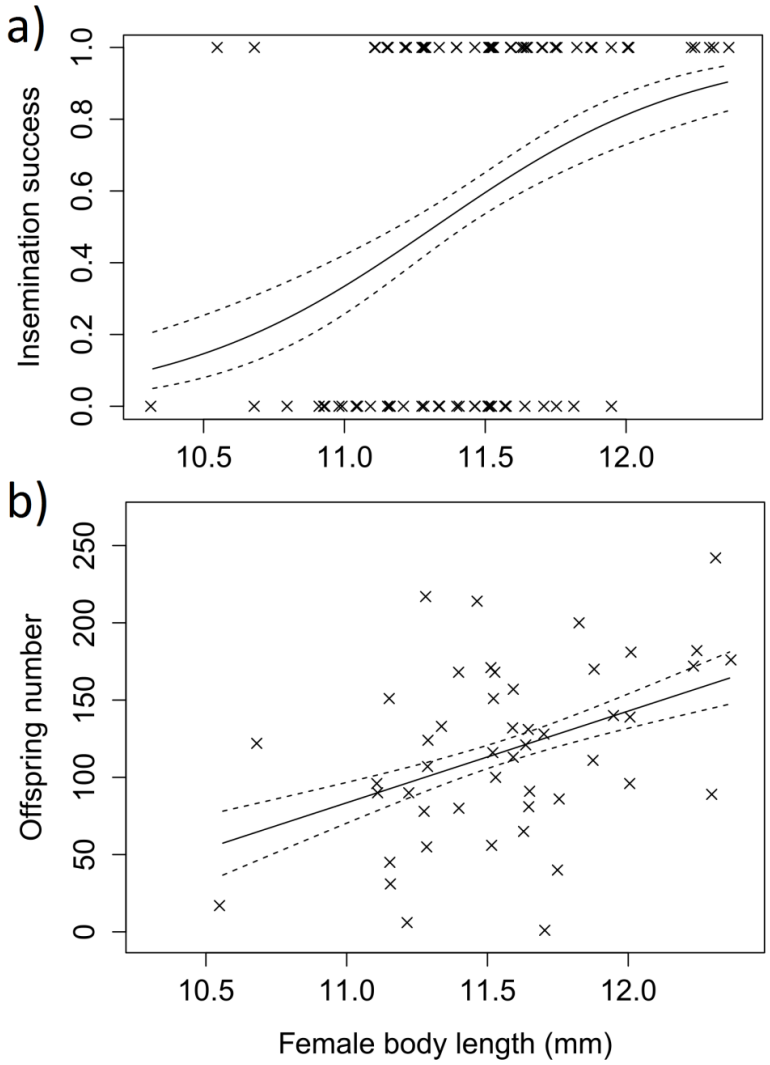

Figure 5.6. Post-copulatory selection on female body length in *L. simulans*, in terms of **a)** insemination success for mated females (*N*= 86), and **b)** offspring production for fertile matings only (*N*= 48). Dashed lines indicate 1 standard error above and below the predicted line.

5.4 Post-copulatory selection in Lygaeus simulans: a meta-analysis

5.4.1 Methods

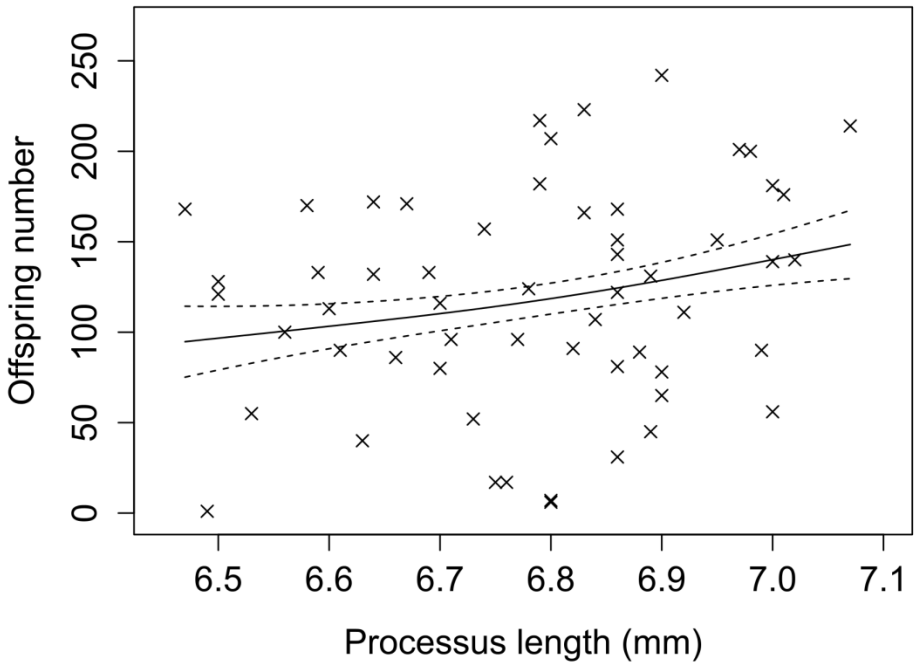

Figure 5.7. Post-copulatory selection on male processus length in *L. simulans* in terms of the number of offspring produced following a fertile mating (*N*= 57). Dashed lines indicate 1 standard error above and below the predicted line.

The strength of selection acting on a trait is commonly calculated using variance-standardised selection gradients (Lande & Arnold, 1983; Arnold & Wade, 1984). By standardising in this way, the strength of selection can be compared across multiple studies, and standardised selection gradients can be seen as a measure of effect size (Kingsolver *et al*., 2012). If studies also present the standard error of the selection estimate, then selection can be analysed using formal meta-analyses which take sampling error into account (Morrissey & Hadfield, 2011). Meta-analysis of such standardised selection gradients has been used to assess how the strength of selection in wild populations varies temporally (Morrissey & Hadfield, 2011), spatially (Siepelski *et al*., 2013) and when acting on different traits (Kingsolver *et al*., 2012).

Standardised selection gradients for the strength of quadratic selection on processus length in *L. simulans* have been reported previously in two studies: Tadler (1999) detected significant stabilising selection on processus length, whereas Tadler et al.

(1999) failed to detect significant selection using a slightly different experimental design (Tadler *et al*., 1999). Though there were small methodological differences between these studies, there is nevertheless surprising variation in selection on processus length in the lab. One reason for the failure to detect selection is that some studies may have insufficient power to detect the small effect sizes typically associated with the strength of selection.

I therefore used formal meta-analyses to assess the mean strength of quadratic selection on processus length in *L. simulans* detected across these multiple studies. I used the standardised quadratic selection gradient as the effect size. I used only selection gradients for which the associated standard error was reported. I used selection gradients taken from univariate regression models, i.e. models do not include covariates (but they do include both linear and quadratic terms), as I am interested in total selection acting on processus length, including indirect selection arising through selection on correlated traits (these may be more correctly referred to as selection differentials: Kingsolver *et al*., 2012).

I first calculated the standardised linear and quadratic selection gradients acting on male processus length in both *L. equestris* and *L. simulans* (Morrissey & Sakrejda, 2013). I also obtained similar estimates using data from two experiments presented in Chapter 6. I used only those males that did not have processus length manipulated ('sham males'). I present the full methods and results of these analyses in Appendix 2.

I therefore obtained five effect sizes: two derived from previous published studies (Tadler, 1999; Tadler *et al*., 1999) and three from my own experiments (from Chapters 5 & 6). All effect sizes considered the strength of post-copulatory selection in mated males; however fitness was recorded in different ways. For my experiments, selection was calculated using the successful production of offspring (as a binary trait) as the measure of fitness. Tadler (1999) used the actual presence of sperm in the spermatheca as the measure of insemination success. Finally, Tadler *et al*. (1999)

presented an estimate of selection derived from the regression of processus length against the number of offspring produced after a single mating (selection gradients arising through insemination success are presented but without standard errors).

I used a random-effects meta-analysis to determine the mean effect size across the five studies, and the associated 95% confidence intervals. I first calculated the variance associated with each effect size as SE^2. I considered the mean effect size estimate to be significantly different from zero if the 95% confidence intervals around the mean did not include zero. I used the I^2 statistic to determine the amount of heterogeneity in effect sizes across studies; this gives the percentage of variation in effect sizes due to heterogeneity rather than by chance (Higgins *et al*., 2003). I used a mixed-effects meta-analysis (random-effects model with a categorical fixed factor: Nakagawa & Santos, 2012) to test if mean effect size differed due to author (Dougherty or Tadler), and a meta-regression to test if effect size was significantly affected by study year. All statistical analyses were performed in R 3.0.1 (R Development Core Team, 2014) using the Metafor package v1.9-2 (Viechtbauer, 2010).

5.4.2 Results

Overall the quadratic selection gradient was significantly negative, indicating significant stabilising selection on processus length across all studies (Mean= -0.19, 95% CI lower= -0.31, 95% CI upper= -0.06; **Figure 5.8**). The percentage heterogeneity (I^2) was 44%, which is suggested as moderate (Higgins *et al*., 2003). There was no significant effect of author ($Q_{M\,1}$ = 0.004, P= 0.95) or study year ($Q_{M\,1}$ = 0.003, P= 0.96) on the mean effect size estimate.

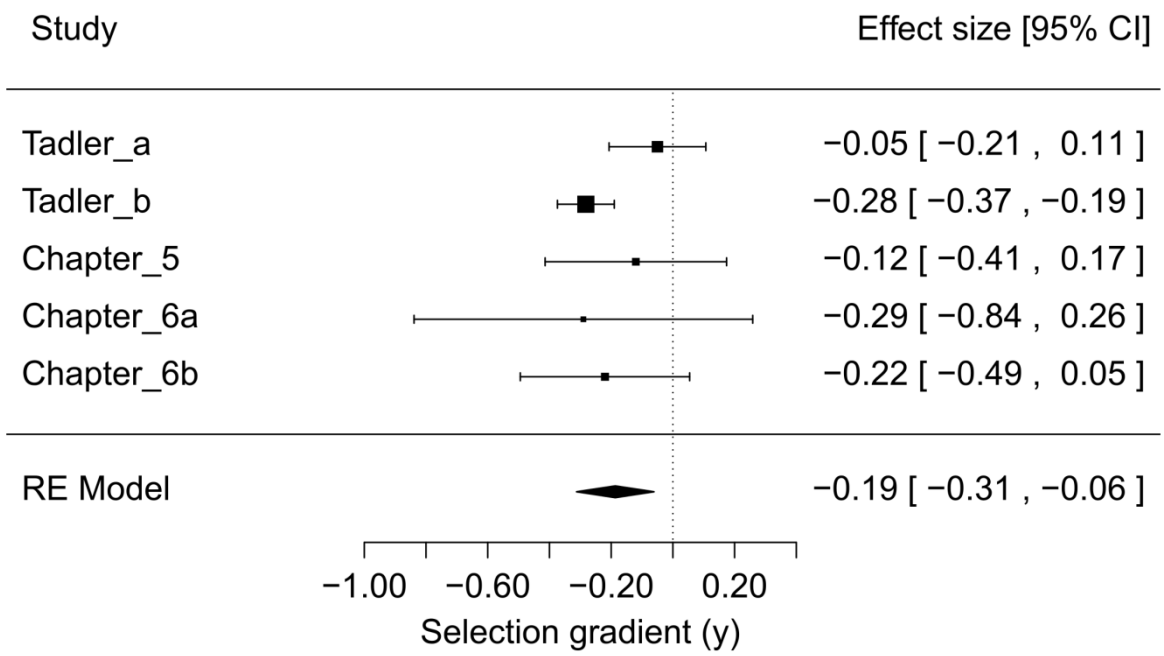

Figure 5.8. Forest plot showing the quadratic selection gradient (γ) and associated 95% CI of the effect sizes included in the meta-analysis. The sizes of the squares represent the relative weightings of each effect size in the model. The mean effect size estimate produced using a random-effects model is represented by the centre of the diamond, with the width of the diamond representing the 95% CI of the estimate.

5.5 Discussion

I have shown that male processus length in *L. equestris* and *L. simulans* is subject to contrasting selection which may act at different stages of mating. Prior to mating there is significant negative, linear selection on processus length in *L. equestris*, but only in choice tests in which two males were present. During mating there is significant stabilising selection on processus length in both species. However such selection was only apparent in *L. simulans* when combining multiple estimates of selection. The net outcome of both pre- and post-copulatory episodes of selection is significant stabilising selection on processus length. Below I will consider selection acting on both species at each stage of mating in turn.

5.5.1 Pre-copulatory selection

I detected significant negative pre-copulatory selection on processus length in *L. equestris*, but only for males that were used in mating trials for which rivals were present. This effect was not seen in *L. simulans*, however this is unsurprising as all mating trials in that experiment were performed using a no-choice design (but see Chapter 7). Significant pre-copulatory selection is present in *L. equestris* despite the fact that the processus is stored inside the male genital capsule before mating. I can think of no way in which the processus is able to interact with, or be assessed by, the female until intromission has been achieved. One explanation could be that longer processi are more difficult to uncoil prior to intromission, however the majority of mating attempts appear to fail long before the processus is uncoiled. Instead the most likely explanation for this is that selection is arising indirectly, due to selection on a correlated trait. Determining whether a trait is actually the target of selection is a major problem in selection analysis (Lande & Arnold, 1983; Endler, 1986; Mitchell-Olds & Shaw, 1987). I can rule out male body length as the cause of this indirect selection, as the analysis controls for this.

One possibility is that processus length is strongly correlated with another male genital trait. For example, the male genital claspers are used to open the female ovipositor prior to mating, and could potentially be under strong selection as males need to overcome female resistance in this species (Sillén-Tullberg, 1981; Shuker *et al*., 2006). This could also explain why selection on processus length was only seen in the choice treatments in which two males were present. Male-male competition is predicted to be stronger in these contexts, so that male traits that are important during such competition will be under stronger selection. For example, claspers may be important for deciding the winner of mating scrambles. I investigate pre-copulatory selection acting on clasper morphology further in Chapter 7.

An alternative explanation is that pre-copulatory selection arises via female choice, as mating preferences tend to be stronger when two options are available compared to one (Chapter 4). Again though, the target trait in this case is unclear, though certainly male body size and leg lengths do not seem to be important (Chapter 3).

Nevertheless, this purported indirect pre-copulatory selection will still able to influence genital evolution if it is consistent, especially if genital traits are strongly correlated. Although there are many studies that have investigated pre-copulatory selection on external genital traits (e.g. Kahn *et al*., 2009; Grieshop & Polak, 2012), few have detected pre-copulatory selection on internal genital traits (Simmons *et al*., 2009; Xu & Wang, 2010). Pre-copulatory selection on genital morphology might be expected for traits involved in maintaining genital contact during mating, and these traits may remain outside the female or be associated with the intromittent organ (e.g. Simmons *et al*., 2009). However for traits such as the processus, which is clearly not involved in maintaining genital contact, such selection is unexpected, and was only tested because I had a sample of males for which mating history had already been recorded. I suggest that where possible pre-copulatory selection should be tested as a mechanism of genital evolution, if only to rule out its effect. This should not require too much of a change in experimental design: researchers are already essentially recording male mating success in any study of post-copulatory selection in which some males fail to mate, but these males are typically discarded before genitalia are measured.

Importantly, the strength of pre-copulatory selection on processus length varies according to social context. This is in contrast to results from Chapter 3, in which choice design had no effect on selection on a measure of overall size. Until this pre-copulatory selection on processus length is explained it is hard to interpret why I have detected an effect of social context on processus length but not on other morphological traits. However, one important implication from this result is that the strength of pre-copulatory selection on processus length in the wild will thus depend on how often *L. equestris* males encounter mates in isolation or in the presence of

rivals. If many rivals are present during the breeding season (see Chapter 2) then pre-copulatory selection on processus length may be negative for a significant proportion of the population (though see below for discussion of overall selection acting in the population).

Though I found no significant pre-copulatory selection on processus length in *L. simulans*, I did find significant positive, linear selection on male body length in a no-choice context. This is in contrast to the stabilising selection on overall male size (combining body length, leg length and antenna length) seen in *L. equestris* (Chapter 3). This could arise if larger males are better able to overcome females during mating struggles. However, it is not clear why there is apparently such strong selection on male body length in *L. simulans* but not in *L. equestris*, though perhaps the use of multiple choice designs in the *L. equestris* experiment masked selection for some reason.

5.5.2 Post-copulatory selection

I was unable to detect significant post-copulatory selection on male processus length in *L. simulans* in the second experiment. This is unexpected in light of previous results in this species (Tadler, 1999). However I did detect significant stabilising selection on processus length in *L. simulans* after combining multiple estimates from this thesis and other published studies. The forest plot (**Figure 5.8**) shows that though there is some variation in the effect size detected in the five experiments, the largest difference appears to be the variance associated with each estimate. The variance in this case is derived from the reported standard error of each selection estimate, and so reflects both the sample size of the experiment and also the variability in success across male phenotypes. For the three effect sizes derived from my experiments, variance is high, suggesting that statistical power is lower than in the Tadler studies. One of the strengths of meta-analysis is its ability to combine several effect size estimates of low power (Koricheva *et al*., 2013). One reason why the second Tadler study especially has

a very low variance might be that related females were deliberately used to reduce the variation in female preferences (Tadler, 1999).

Overall then, I also detected significant stabilising post-copulatory selection on processus length in both *L. equestris* and *L. simulans*. Furthermore, in both cases selection is relatively strong (for a review of the strength of selection in natural populations see: Kingsolver *et al*., 2001). Why do males with processi either significantly longer or shorter than the population average have reduced insemination success? Such a relationship would make intuitive sense if the length of the female reproductive tract was similar to that of the male processus, but that is not the case. Instead, the female spermathecal duct is significantly shorter than the processus (around 1.9mm long in *L. simulans*: Gschwentner & Tadler, 2000), so that a large proportion of the processus remains in the bursa during mating (see Chapter 6). For some reason then, males with short processi cannot simply thread more of the processus into the spermathecal duct, despite appearing to have plenty to spare. Some males also appear to have processi that are too long, even though sperm appear to be released fine even when the tip of the processus reaches the corkscrew region of the spermathecal duct (Gschwentner & Tadler, 2000).

I suggest two other mechanisms in which processus length could be important for insemination success. The first is that the length of the processus may be important in a structural sense, for example if it makes the structure more flexible or more rigid, or if the number of coils made within the bursa is important for positioning the tip at the entrance to the spermathecal duct (See Chapter 6). Alternatively, this result, coupled with the presence of a valve at the entrance to the spermatheca, could be explained as the result cryptic female choice, with females actively preventing unwanted males from achieving insemination (Eberhard, 1996; Gschwentner & Tadler, 2000).

I assume in these experiments that failure to produce offspring following mating reflects a failure to inseminate a female. However this may not be true; instead, sperm

may be successfully deposited in the spermatheca but not utilised by the female. For example Tadler *et al*. (1999) found that in 12 of 67 matings (18%) sperm was present in the spermatheca but no fertile eggs were produced after 45 days. It is unclear why these sperm were not used to fertilise the female's eggs. The proxy measure of insemination success used here therefore probably underestimates the likelihood of a male's sperm reaching the spermatheca.

I also detected significant post-copulatory selection on female body length in *L. simulans*, with larger females being both more likely to be inseminated and also producing more offspring following a single mating. This was also seen in Chapter 3. This could be due to the fact that body size correlates with female fecundity in insects (Honěk, 1993). Alternatively, males may allocate more sperm to large (high quality) females (Bonduriansky, 2001; Kelly & Jennions, 2011). I am unable to distinguish between these explanations without some estimate of male sperm allocation and subsequent sperm use by the female. I did not find a significant relationship between female body length and insemination success in *L. equestris*, though note that there was a non-significant positive trend, suggesting that a relationship is present but weak.

Finally, I also detected weak, disruptive post-copulatory selection on male body length in *L. equestris*, so that males of an intermediate size seem to be the least likely to inseminate a female. This was not seen in *L. simulans*. This result seems counterintuitive. If insemination success arises partly through female choice, then this would mean that females prefer both large and small males over intermediate-sized males for some reason. An alternative way for this pattern to arise would be if male ejaculate allocation was different for males of different sizes, perhaps representing alternative mating tactics. For example, small males could transfer more sperm to make up for reduced pre-copulatory success.

5.5.3 Combining selection

Sexual selection may act both before, during or after copulation (Kvarnemo & Simmons, 2013). However, historically these different episodes of selection have been considered in isolation. Yet if we want to understand how selection acts on populations in the wild then we need to estimate total selection arising from multiple episodes, for example by combining intrasexual and intersexual selection (Hunt *et al*., 2009), or pre- and post-copulatory selection (Bangham *et al*., 2002; Pélissié *et al*., 2014). Importantly, individual behavioural or morphological traits may be subject to contrasting episodes of selection (Bonduriansky & Rowe, 2003; Bailey, 2008). For example, in the water strider *Gerris lacustris*, pre-copulatory selection favours large males, whereas post-copulatory selection favours small males (Danielsson, 2001). Despite this, studies looking at multiple episodes of selection acting on genital traits are rare. A recent study in *Drosophila simulans* used experimental evolution to show that natural and sexual selection both influence the shape of the posterior and ventral lobes of the genital arch in complex ways (House *et al*., 2013). However, I have been unable to find any studies that consider the strength of both pre- and post-copulatory selection acting on either an internal or external genital trait. This is surprising given that genital traits may have complex functions that can influence both pre- and post-copulatory reproductive success. For example, external grasping structures could potentially function both to initiate copulation and to extend duration so that sperm transfer can take place (Eberhard, 1985; Arnqvist & Rowe, 2005; Simmons, 2014).

I combined pre- and post-copulatory measures of selection by quantifying the strength of selection arising through male insemination success for all males, including those who failed to mate. In *L. equestris* this combined measure indicated strongly stabilising selection on male processus length. This suggests that the linear pre-copulatory selection on processus length seen in *L. equestris* is insufficient to overcome (or in part contributes to) the strong stabilising post-copulatory selection in the population. Note also that the quadratic selection gradients detected for the two post-copulatory measures of success (and presented in Appendix 2) are similar, despite the fitness surface appearing shallower in the former case (**Figure 5.1**). This is due to the

population mean fitness being lower when all males are included (Michael Morrissey, *pers. comm.*).

This method of combining both measures of selection requires that morphological data is also measured for individuals that do not mate during mating trials, which will of course require more data collection. However, I suggest that this will be a useful way to assess 'total sexual selection' on a trait arising from selection before and after mating in single mating experiments. I note that this approach will not be suitable for double-mating experiments, as mating success is controlled during the experiment. However it could be extended to more natural competitive situations if methods for identifying offspring are available (Simmons, 2001). This is analogous to recent studies that attempt to estimate the overall contributions of pre- and post-copulatory reproductive success to total fitness, without correlating this to specific phenotypic traits (Rose *et al*., 2013; Pélissié *et al*., 2014).

In the case of *L. equestris* this approach reveals that overall pre-copulatory selection makes on a very small contribution to the overall relationship between processus length and fitness, despite highly significant selection being detected in some contexts. More generally, the results of this chapter highlight the fact that sexual selection may vary across multiple stages of mating, and across different social contexts. Selection on morphological (or indeed any other) traits can be complex, and therefore considering episodes in isolation is clearly insufficient in order to understand how traits evolve in natural populations.

Chapter 6

Functional morphology of processus length in *Lygaeus simulans*

Abstract

In this chapter I investigate the functional morphology of processus length in *Lygaeus simulans* in three ways. First, in collaboration with Dr Imran Rahman (University of Bristol) I used micro-CT scanning of a flash-frozen mating pair to reconstruct in high resolution both the internal reproductive anatomy of the female and the path of the male intromittent organ inside the female. Next I performed an experiment in which males were allowed to mate with females for three weeks, and show that the processus may commonly break at the tip under these conditions, though it is not clear whether breakages happen during or after mating. I then confirm that male processus length influences sperm transfer by experimental shortening, and show that males with shortened processi had significantly reduced reproductive success. Importantly though, the sperm transfer ability of the processus is not impaired by cutting *per se*. I thus present rare, direct experimental evidence that an internal genital trait functions to increase reproductive success, and suggest that the extreme length of the processus does not function as an insurance policy against breakage during mating.

Acknowledgments

This work would not have been possible without the help of Imran Rahman, who arranged, paid for and supervised the micro-CT scans, and produced reconstructions and all figures and videos. I thank Dan Sykes and Rebecca Summerfield at the London Natural History Museum for assistance with micro-CT and advice on staining, and Irvine Davidson for taking SEM images. I thank Emily Burdfield-Steel and Ginny Greenway for help with mating trials and freezing of bugs prior to CT scanning. Ginny Greenway also helped with the breakage experiment.

6.1 Introduction

Studies commonly investigate sexual selection acting on genitalia by correlating natural phenotypic variation with reproductive success (Simmons, 2014; see Chapter 5). However, such correlations are rarely confirmed experimentally. Additionally, distinguishing between mechanisms of selection is difficult without knowledge of the functional morphology of genital traits (Werner & Simmons, 2008b; Simmons, 2014). Detailed knowledge of exactly how male and female genitalia interact during copulation is lacking for most species. Determining the relevant functions of male traits during copulation is difficult partly because most interactions occur inside the female during copulation, and these interactions can be incredibly complex. However such investigations have been performed for some arthropod species (e.g. Miller, 1991; Tadler, 1996; Hosken *et al*., 1999; Huber, 2002; Fairbairn *et al*., 2003; Jagadeeshan & Singh, 2006; Werner & Simmons, 2008b). Such studies may benefit from multiple experimental methods, including behavioural observations, dissection and imaging techniques, artificial selection, and manipulation experiments. In this chapter I use a variety of experimental techniques in order to investigate the functional morphology of the male processus in *L. simulans*; a trait which is under significant post-copulatory selection (Chapter 5).

6.1.1 Micro-CT

In order to understand the function of male genital traits it would be useful to be able to visualise the interactions between male and female genitalia whilst in copula. The study of functional anatomy has traditionally combined many experimental techniques, including dissection, histology and microscopy (Friedrich et al, 2014). In order to record the interactions between male and female genitalia for most species we need to be able to see inside the female, and this usually requires destructive sampling of some form. However, genital interactions can be very delicate, especially

in insects, so that even the most careful dissections of copulating pairs may alter the normal positions of male and female genitalia before morphology can be accurately described. A modern alternative is to use non-destructive imaging techniques such as confocal-laser scanning microscopy (CLSM), magnetic resonance imaging (MRI), or micro-computed tomography (micro-CT; Ziegler *et al*., 2010). Micro-CT uses x-rays fired through an object to reconstruct a three-dimensional virtual model of the object at a very fine spatial scale, and has been widely used to describe the morphology of fossil organisms (e.g. Cunningham *et al*., 2014; Sutton *et al*., 2014). The high energy x-rays are able to pass through biological material, and the detector is able to reconstruct the density of the material each beam passes through (Holdsworth & Thornton, 2002). In recent years micro-CT has also become increasingly prominent in anatomical studies of extant species (Friedrich *et al*., 2008; Ziegler, 2010; Friedrich, 2014), particularly when used in combination with contrast-enhancing agents (Metscher, 2009). Recent studies have even begun to use micro-CT scans of live insects (e.g. butterfly pupae: Lowe *et al*., 2013), and have developed methods to reconstruct sample movements in real time (Walker *et al*., 2014).

Micro-CT thus allows taxonomists to carry out non-destructive “virtual dissections” of taxonomically important characters, such as genitalia (Perreau & Tafforeau, 2011; Simonsen & Kitching, 2014). In the last few years researchers have begun to investigate the evolution of genital traits across species (McPeek *et al*., 2008; 2009; Arbuthnott *et al*., 2010; Herdina *et al*., 2015) and the functional morphology of genital traits within species (Wojcieszek *et al*., 2012; Wulff *et al*., 2015; Mattei *et al*., 2015). Most importantly, though micro-CT scanning is commonly used to produce high-resolution reconstructions of external morphology, it can also be used to visualise internal anatomical structures (Metscher, 2009; Lowe *et al*., 2013). This technique is now beginning to be used to reconstruct the interactions between male and female genitalia inside the female during mating (Wulff *et al*., 2015; Mattei *et al*., 2015).

6.1.2 Natural breakage of the processus

In the previous chapter I illustrated how male genital traits may be subject to multiple episodes of selection acting both before and during/after mating. Additionally, genitalia will always be subject to natural selection to some extent, as normal function is required for successful reproduction. Damage or breakage of the intromittent organ may impair normal function, and so we should expect adaptations to protect genitalia against such damage. However, the long, thin intromittent organs possessed by some insects appear to be very fragile. In the earwig *Euborellia plebeja*, males possess a pair of extremely elongate intromittent organs (or virgae), which are able to remove rival sperm from the female reproductive tract (Kamimura, 2005). Males with broken virgae have been observed in the wild, though at a very low frequency, and breakages can be induced experimentally by interrupting matings (Kamimura, 2003). Experimental manipulations have shown that a female can be successfully inseminated even with a broken virga in the reproductive tract, suggesting that such breakages do not function as mating plugs (Kamimura, 2003). Breakages are likely a by-product on selection for extreme virga length, and the fact that males possess paired organs reduces the cost of breakage significantly (Kamimura, 2003). In other species such breakages may even be adaptive, as broken genitalia that remain in the female tract after mating can act as a mating plug, preventing rival males from being able to inseminate the female (Page, 1986; Knoflach & van Harten, 2001; Uhl *et al*., 2010).

In *L. simulans* the processus is very long and highly sclerotized. If the processus is likely to break during mating, and sperm transfer is still possible with a broken processus, then the more 'excess' processus a male has the more breakages he will be able to sustain and still be able to mate. This will lead to natural selection for increased processus length so that normal processus function can be maintained. I hereafter refer to this idea as the 'breakage insurance' hypothesis. Visual inspection of processi removed from males in a previous experiment appears to show a very low frequency of missing tips (only 1 of the 140 *L. simulans* males used in Chapter 5 had the tip of the

processus missing). However, these experiments all involved males that were relatively young and only mated once. In the wild, it is likely that both males and females may mate multiple times (Chapter 1). A potential way to estimate the upper limit of breakage frequency would be to allow males to mate many times across their lifespan. I therefore performed such an experiment to assess the frequency of natural breakages in a sample of males allowed to mate constantly during their lifetime.

6.1.3 Ablation studies

Evidence for the role of sexual selection in genital evolution comes primarily from studies correlating intraspecific variation in morphology with reproductive success (for examples in insects see Table 1 in Simmons, 2014). An alternative approach is to experimentally manipulate male genitalia and record how reproductive success is influenced by such manipulation (Simmons, 2014). This has the advantage of establishing that the targeted trait actually functions to influence reproductive success (although of course other functions cannot be ruled out).

Studies in which genital structures are removed or reduced in some way are known as genital ablation studies, and such studies have become much more sophisticated in recent years. For example, Hotzy *et al.* (2012) used micro-laser surgery to ablate male genital spines in the seed beetle *Callosobruchus maculatus*. This manipulation, along with artificial selection lines, showed that males with longer spines gain more fertilisations in a competitive context, and that this may be due to more seminal fluid passing into the haemolymph of the female after mating (Hotzy *et al*. 2012). The traits targeted by such ablation studies tend to be tough sclerotized structures such as spines (e.g. Hotzy *et al*., 2012; Grieshop & Polak, 2012), teeth (Briceño & Eberhard, 2009) and claspers (e.g. Moreno-García & Cordero, 2008) that are amenable to manipulation. Manipulation of structures directly associated with sperm transfer is likely not possible in most species, as such structures tend to be highly complex (so

that manipulation impairs function; Arnqvist & Rowe, 2005) and vascularised (so that manipulation leads to injury and the loss of blood/haemolymph).

Moreover, this approach has recently come under criticism, with Simmons (2014) noting that:

> "[Experimental manipulation] tells us little about how selection acts on genital morphology. By analogy, although removing the legs of a long distance runner would show us that legs are required to run, it would tell us little about how stride length contributes to running speed. Nonetheless, such approaches can sometimes be useful in identifying the functional mechanisms by which male genitalia might impact fitness"

However, if a genital trait can be manipulated in a more subtle way, whilst keeping normal reproductive functions intact, the major drawbacks of this potentially powerful approach are resolved. In continuing the analogy above, whilst removing a leg would not inform us of how selection acts on leg length in a long distance runner, experimental shortening of a runner's legs (without disrupting the complex interactions between neurons, muscles and bones which allow running to occur) certainly would. Though this is clearly impossible for very complex traits such as legs, this may not be the case for more simple structures. Indeed this kind of manipulation has been successfully performed on a genital trait, in the tortoise beetle *Chelymorpha alternans* (Rodriguez, 1995; Rodriguez *et al*., 2004). Males of this species possess an extremely long, thread-like flagellum that enters the female spermathecal duct, and experimental reduction of the flagellum leads to an increased incidence of sperm droplet formation after mating, a behaviour which may be under female control (Rodriguez, 1995; Rodriguez *et al*., 2004). I suggest that this is a potentially powerful approach to studying the functional morphology of genitalia that may be possible for some simple traits.

6.1.4 Chapter outline

In this chapter I investigate the functional morphology of the male processus in *L. simulans* in three ways. First, I obtained micro-CT scans of flash-frozen copulating pairs, and show that this technique can be used to non-destructively visualise the interactions between male and female genitalia during mating. Second, I attempt to estimate the potential for natural processus breakages by allowing a male to mate multiple times over several weeks and then checking the processus for breakage. Finally, I investigate the effect of experimental reduction of processus length on male reproductive success over three experiments. If experimental reduction has no effect on male reproductive success this would give support to the hypothesis that processus length is longer than is required for successful insemination, possibly as an insurance against natural breakages. Alternatively, a reduction in male reproductive success following manipulation is strong evidence that processus length influences sperm transfer. This would support the significant post-copulatory selection on processus length in *L. simulans* (Chapter 5). I consider four measures of reproductive success: male mating (whether the male mated or not, or the proportion of observations seen mating), copulation duration, 'insemination success' (for those males that mated, whether the mating resulted in offspring) and 'fertilisation success' (for those males that mated and produced offspring, the number of offspring produced).

6.2 Micro-CT

6.2.1 Methods

To obtain samples for micro-CT scanning I performed mating trials using virgin, sexually mature *L. simulans* individuals. First, a male was allowed to initiate mating with a female, and then copulation was interrupted after approximately five minutes using a fine paintbrush. This causes the male to disengage from the female with his

intromittent organ everted from the genital capsule. This mated male was then immediately flash-frozen in liquid nitrogen. This male was stored in 70% ethanol and unstained (as the genitalia are much easier to visualise when outside of the body).

A second pair was allowed to copulate for two hours, and then carefully moved into an Eppendorf tube and flash frozen in liquid nitrogen while still in copula. This sample was fixed by placing in Alcoholic Bouin's solution for four hours. The fixative was then washed out using 70% ethanol, and then the pair was stained with 1% iodine in 100% ethanol (I2E) for four days prior to scanning. This serves to enhance the X-ray attenuation contrast of non-mineralised tissues, which are otherwise difficult to distinguish using micro-CT (Metscher, 2009). Prior to transportation to the scanning facility, the sample was washed several times in 70% ethanol to remove excess I2E, and then all ethanol was pipetted out (ethanol residue on the sides of the tubes was sufficient to prevent the samples from drying out).

Three scans were performed in total: one of a single male, and two of different mating pairs. Micro-CT was performed on a Nikon XT H 225 cabinet scanner at the Natural History Museum, London by Dr Imran Rahman (University of Bristol). Samples were scanned dry, in an Eppendorf tube mounted on florist's foam. The scan of the single male was performed using a current/voltage of 95 kV/190 µA and 3142 projections. This gave a dataset with a voxel size of 5.7 µm. The scan of the mating pair was performed using a current/voltage of 105 kV/190 µA and 3142 projections. This generated datasets of slice images with voxel sizes ranging from about 5 to 7 µm.

Digital visualization was undertaken using the freely available SPIERS software suite (Sutton *et al*., 2012). For each scan, a global linear threshold was applied to the dataset, creating binary images in which all pixels brighter than a user-defined grey level were turned "on" (white). The "on" pixels identified as belonging to the bugs were then manually assigned to distinct regions-of-interest, which corresponded to important anatomical characters (e.g. processus, aedaegus, claspers, spermatheca and

bursa). Finally, these regions-of-interest were rendered as separate isosurfaces, producing interactive three-dimensional virtual reconstructions in which the different anatomical structures could be independently manipulated. High-quality images were produced in the open-source program Blender (www.blender.org). All visualisations were created by Imran Rahman.

6.2.2 Results

Two views of the male genitalia obtained via micro-CT scanning can be seen in **Figure 6.1**, showing the external claspers and male intromittent organ post-mating. The processus and base of the aedeagus can be seen uncoiled following separation, and the claspers are in the open position. This scan proved useful in identifying structures in the mating pair sample.

Figure 6.2 shows several views of the interaction between male and female genitalia during copulation, obtained via micro-CT scanning of a mating pair. Iodine staining served to greatly enhance the contrast of non-mineralised tissues, allowing the entire male intromittent organ to be visualised, including the processus and fleshy base of the aedeagus, within the female tract. The sclerotized nature of the processus meant that it was clearly differentiated from the surrounding tissues in micro-CT images (**Figure 6.2**), and hence its path could be traced both inside the female, and also posteriorly within the vesica (**Figure 6.2a**). Female internal reproductive morphology could also be reconstructed in detail; specifically, the bursa (which appears as a large cavity) and the spermatheca, which is sclerotized (**Figure 6.2b-c**). The positions of the male aedeagus and processus within the female bursa have not previously been reported, and physical dissection invariably causes distortion of the natural shape of the bursa which is very fragile; consequently, this virtual approach was an ideal way of imaging these structures *in situ*. It appears that the processus is coiled inside the bursa for slightly more than half of its length (**Figure 6.2b-c**). The high-resolution of the scans

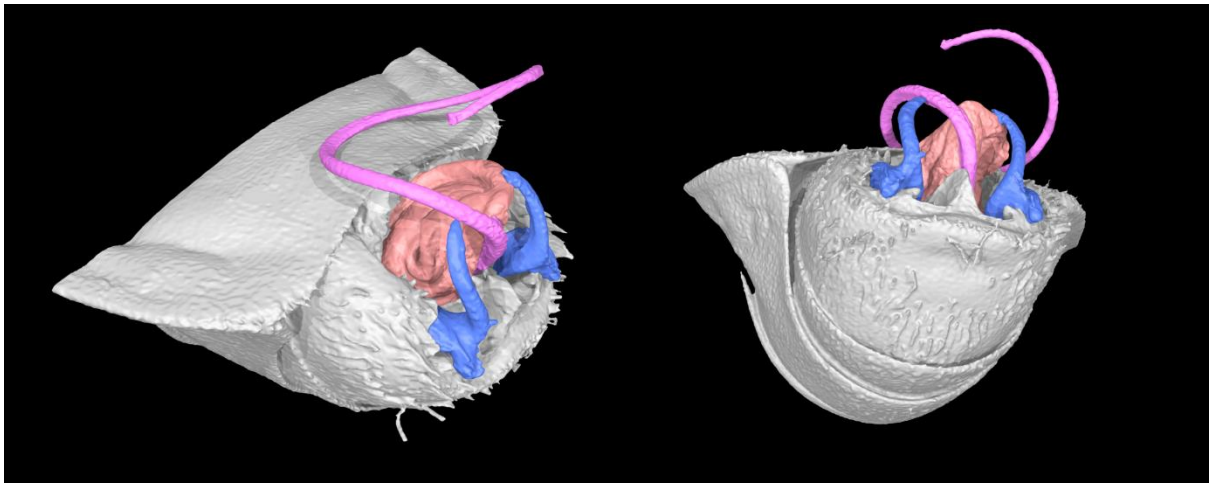

Figure 6.1. Reconstructions of external reproductive anatomy of a male *L. simulans* obtained from micro-CT scanning, showing the paired claspers (blue), fleshy aedeagus (red) and the long processus (pink). The aedeagus and processus are everted following mating.

(down to about 5–7 µm) means that very fine-scale anatomical features can be seen, such as the tight corkscrew region at the entrance to the spermatheca (Point D in **Figure 6.2b**; Gschwentner & Tadler, 2000). However, the resolution of the scans was insufficient to reveal the fine-scale structure of the processus tip, which is better resolved using SEM imaging (**Figure 6.3**).

Scans also confirm that the male processus is able to reach the spermatheca after copulation for two hours, and can thus be inferred to extend all the way along the spermathecal duct (as previous studies have reported: Micholitsch *et al*., 2000). However, the spermathecal duct could not be distinguished from the male processus; this may be because the spermathecal duct is a very fine structure, and hence is difficult to resolve with micro-CT, even after the use of contrast-enhancing agents to increase differential attenuation (Metscher, 2009).

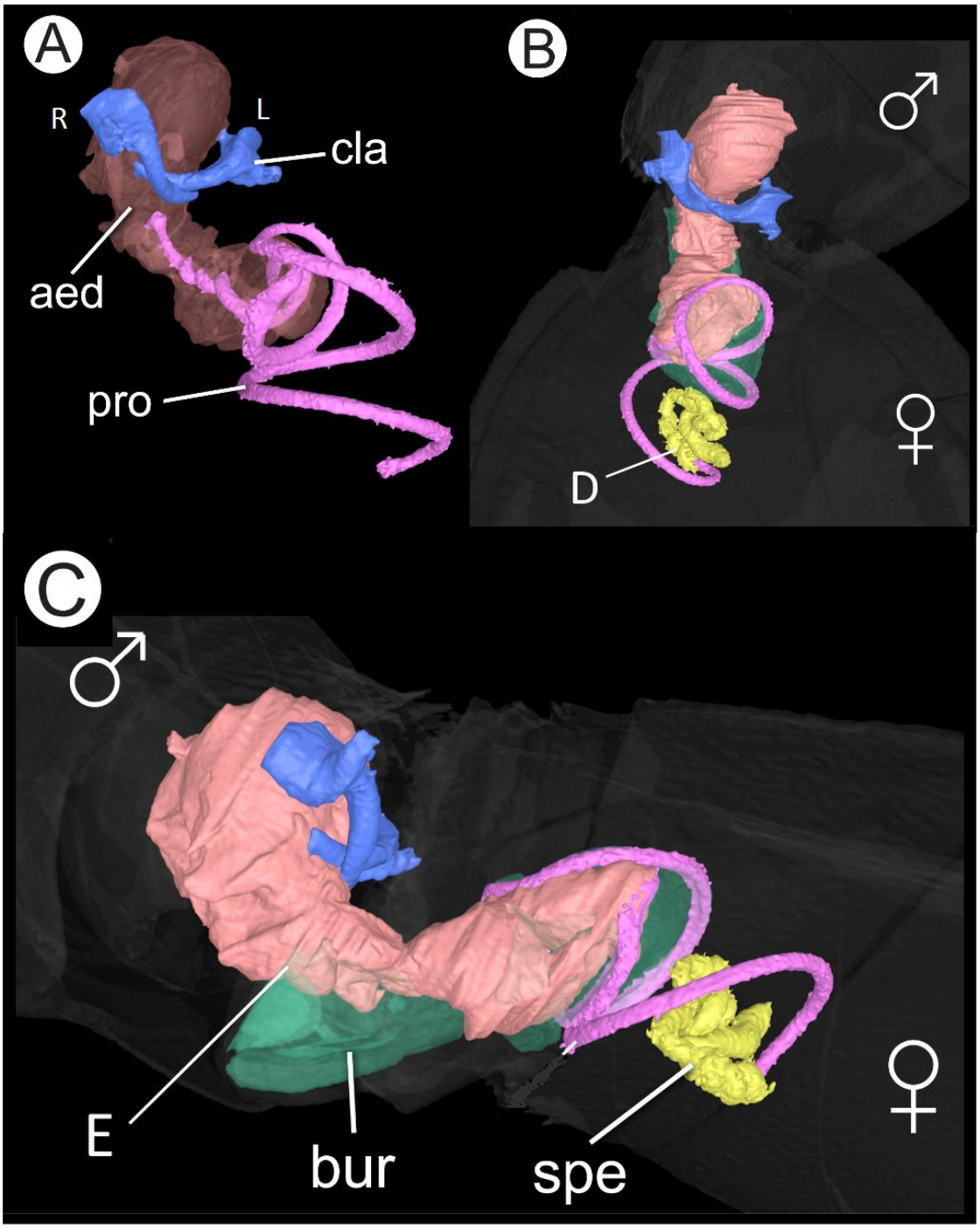

Figure 6.2. Reconstructions of reproductive anatomy of *L. simulans* obtained from micro-CT scans, showing male and female genitalia during mating. Inset A shows the male genitalia in isolation, and insets B and C show the interaction between the male and female genitalia (with the body transparent) in dorsal and lateral view respectively. The fleshy base of the aedeagus can be seen in red (aed), and the coiled processus in pink (pro). The paired male claspers are shown in blue (cla). The female bursa is shown in green (bur), and the spermatheca in yellow (spe). The corkscrew region at the entrance to the spermatheca is shown at point D. The aedeagus enters the female at point E. The left and right claspers are also highlighted (see Chapter 7).

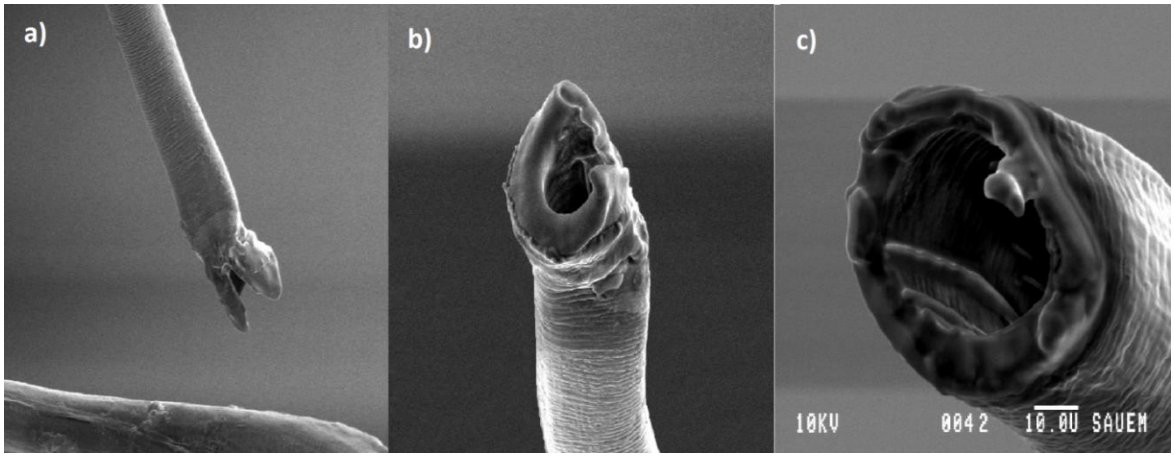

Figure 6.3. Scanning electron micrographs showing: **a-b)** normal processus tip morphology in *L. simulans*, and **c)** the intact lumen after experimental manipulation.

6.3 Breakage experiment

6.3.1 Methods

In order to assess whether processus breakages occur naturally, I placed virgin pairs in isolated tubs with food and water ad libitum for three weeks, or until the male died. Males and females were able to interact freely for the duration of the experiment. Pairs were checked three times a day for copulation. Any dead females were replaced with a mature, virgin female. Adults were moved into new tubs each week if nymphs were present to prevent mould build-up. After three weeks all individuals were frozen, and the male processus was dissected out and measured as in Chapter 2. All processi were checked for the presence of tips. For males possessing broken processi, I removed the reproductive tract of any females that had come into contact with the males and checked for processi in the bursa and spermathecal duct. I recorded male and female lifespan and the proportion of observations pairs were seen mating. All statistical analysis was performed in R version 3.0.1 (R Development Core Team, 2014).

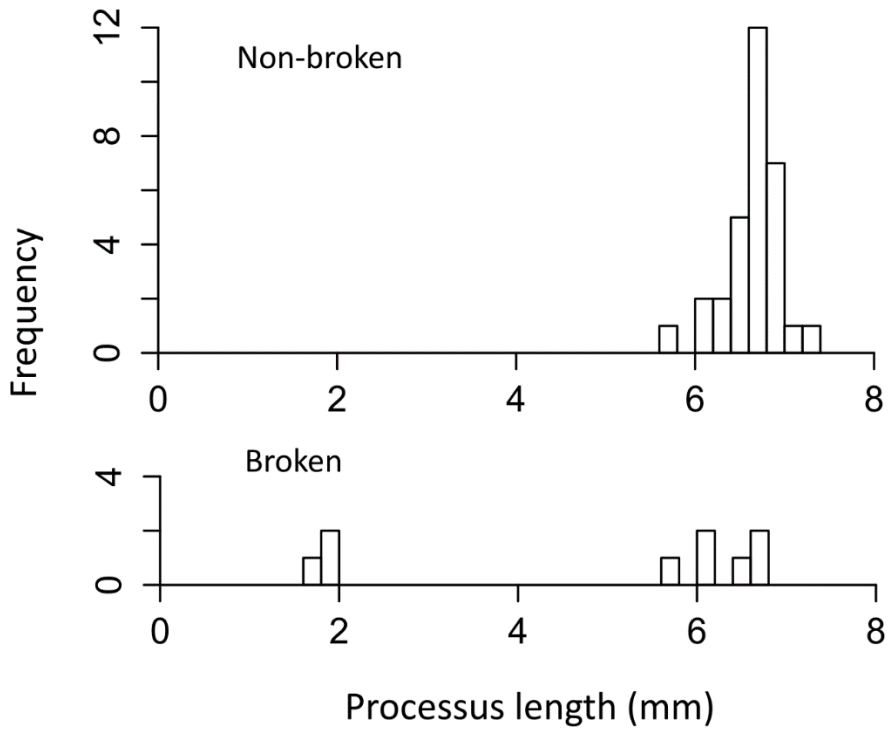

Figure 6.4. Histogram showing the frequency distribution of processus lengths (mm), for non- broken processi (top panel) and broken processi (bottom panel) in the breakage experiment.

6.3.2 Results

Most males (34 out of 40) died before the end of the experiment: the average male lifespan was 11.58 days (s.d.= 6.16), with only six males surviving the full 21 days. Pairs were seen mating during 19% of observations on average. Eight males were never seen mating, however four of these lived less than ten days. Three of the remaining males had broken processi at the end of the trial.

Nine males (22.5%) appeared to have broken processi (of which three were never seen mating). The average length of non-broken processi was 6.66 mm (s.d.= 0.3 mm). Of the nine broken processi, three were missing over 50% of the normal processus length (**Figure 6.4**). The average length of the remaining six broken processi was 6.25 mm

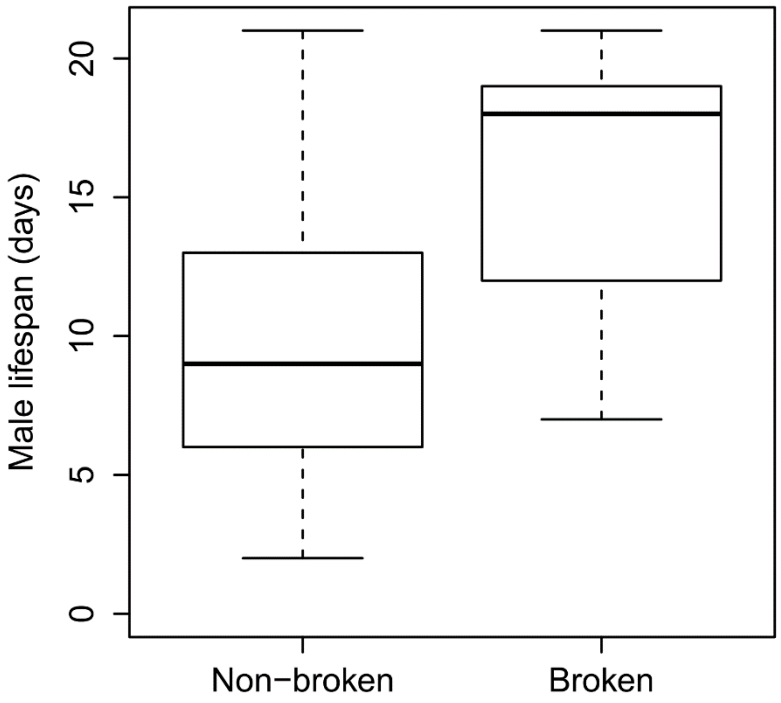

Figure 6.5. Male lifespan (days) for males with broken (*N*= 9) and non-broken (*N*= 31) processi in the breakage experiment. Boxes show the mean (thick line) and interquartile range, and whiskers extend up to 1.5 times the interquartile range above and below the box.

(s.d.= 0.42 mm). There was no difference between broken and non-broken males in the proportion of observations they were seen mating (Mann-Whitney test; U= 137.5, *N*= 40, *P*= 0.961). Males with broken processi lived significantly longer than those without broken processi (U= 72, *N*= 40, *P*= 0.029; **Figure 6.5**). Importantly, I was unable to find any processus fragments in the reproductive tract of any female kept with the nine males with broken processi.

6.4 Experimental manipulation of processus length

6.4.1 Methods

In order to confirm the correlations between processus length and insemination success seen in Chapter 5, I performed three experiments in which I experimentally shortened the processus of *L. simulans* males by varying amounts, and then compared the reproductive success of manipulated and non-manipulated males.

6.4.1.1 Processus cutting

In order to manipulate male processus length, I first placed virgin males and females together in a mating arena and allowed pairs to couple and achieve intromission. Each pair was allowed to mate for approximately five minutes, after which copulation was interrupted using a fine paintbrush. This causes the male to disengage from the female with his intromittent organ everted from the genital capsule. The male was then sedated by placing in a freezer at –18 °C for four minutes, and then the processus was cut using a pair of micro-scissors (**Figure 6.6**). The removed portion of the processus was kept for measurement. I also performed a sham treatment in which males were placed in the freezer and the processus manipulated but not cut. As highlighted by the results above, processus breakage is likely to occur occasionally in the wild, and so this manipulation is not as unnatural as it may first appear. I note that the processus possesses no vascularisation, so that cutting does not lead to fluid loss, and males do not appear harmed in any way following cutting. Additionally, across all three experiments, processus cutting did not obviously alter male mating behaviour (see below).

Following both procedures males were given at least one day to recover before being introduced to new, naive females: the females used for this pre-trial stage were not re-used. Prior to the experiment the lumen of the processus was confirmed as remaining open after cutting by taking sample images using both a dissecting microscope and a scanning electron microscope (**Figure 6.3c**). During the experiment each processus was checked by eye following cutting to ensure the cut was performed cleanly.

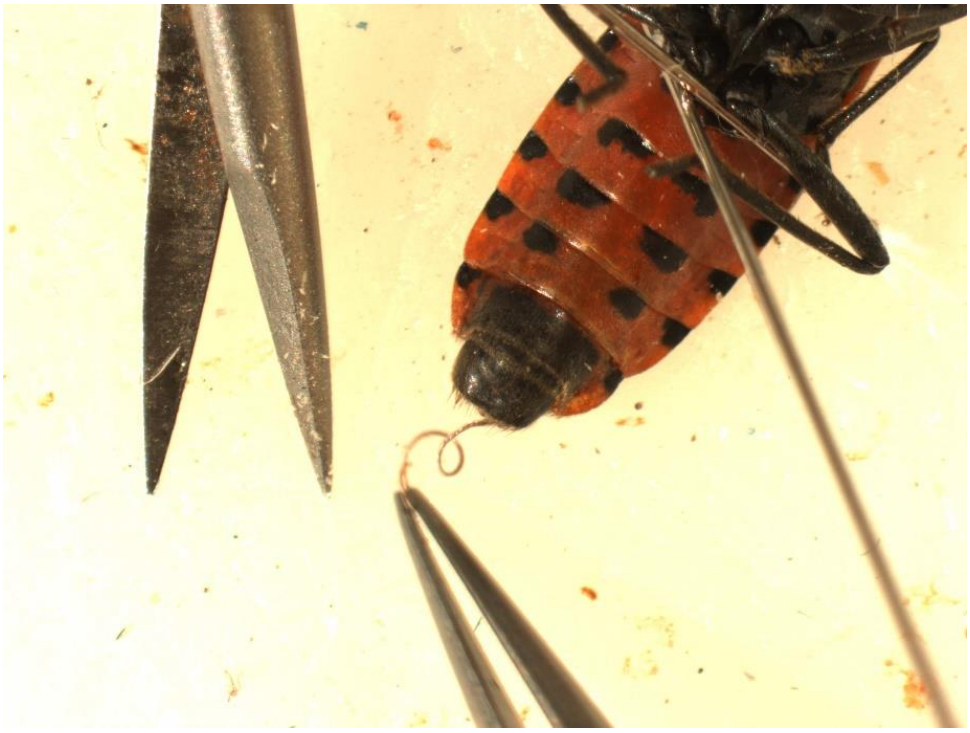

Figure 6.6. The experimental setup for processus cutting. The processus was held with fine forceps and cut using micro-scissors.

6.4.1.2 Experimental design

I performed three manipulation experiments. In the first experiment ("large reduction experiment"), I shortened the processus of 39 males by an average of 2 mm, which is 29% of the total processus length. This is far outside the natural phenotypic range of the processus (see Tadler, 1999; Chapter 5). I subjected a further 39 males to the same procedure but without cutting (sham males). Males were then given the opportunity for a single mating with a virgin female.

In order to confirm that sperm transfer was possible after experimental manipulation of the processus, I performed a second experiment ("medium reduction experiment") in which males were housed with a single virgin female for two weeks after the treatment, thus allowing the opportunity for multiple matings. I shortened the

processus of 13 males, this time by an average of 1 mm (14% of total length), while 12 males were left untreated.

Finally, I performed a third experiment ("small reduction experiment") in which the treated males had their processi reduced by only 0.4 mm, which is within the natural phenotypic range of the processus. I also added a treatment in which only the very tip of the processus was removed, for two reasons. First, this controls for the cutting procedure without reducing the processus length substantially. Second, the processus ends in a cup-like structure with a v-shaped cleft (**Figure 6.3**) which may be important for normal sperm transfer. Males were thus given one of three treatments: a) reduction by 0.4 mm (5.7% of total length, N = 56), b) reduction by 0.1 mm (N = 54), or c) no reduction (sham males, N = 55). Males were then given the opportunity for a single mating with a virgin female as before.

Table 6.1 shows the average processus lengths following manipulation for all three manipulation experiments.

6.4.1.3 Measures of reproductive success

For the large and small reduction experiments, I performed no-choice mating trials in which virgin males were introduced to a virgin female in small plastic Petri dishes (55 mm diameter). All mating trials were performed when males and females were sexually mature (7-14 days post adult eclosion). Dishes were observed continuously for two hours, and then checked every ten minutes for a further eight hours. If copulation ended during the trial, the pair was separated so as to restrict the female to a single mating. This was done for any copulation that lasted 15 minutes or more. Copulations that did not end during the trial were separated manually using a fine paintbrush.

Table 6.1. Table showing mean processus lengths for all three manipulation experiments, split by experimental treatment.

Experiment	Treatment	*N*	Amount removed (mm)	s.d.	Length after cutting (mm)	s.d.	Proportion of total removed
Large reduction	Sham	39	0.00	-	6.90	0.22	-
	Reduced	39	2.00	0.24	4.84	0.30	0.29
Medium reduction	Sham	12	0.00	-	6.92	0.26	-
	Reduced	13	1.02	0.39	5.80	0.48	0.16
Small reduction	Sham	55	0.00	-	6.80	0.16	-
	Tip removed	54	0.10	0.03	6.74	0.20	0.01
	Reduced	56	0.39	0.13	6.48	0.20	0.05

In the large and small reduction experiments the proportion of males that mated was recorded, as well as the copulation duration of all mated pairs. For the medium reduction experiment, each male were housed with a single virgin female in a tub with food and water *ad libitum* for two weeks. Therefore in this treatment multiple mating was very likely. I checked whether pairs were mating in all tubs 2-3 times a day. For this treatment I used the proportion of observations a pair was seen in copula as a proxy for male mating frequency.

All males were euthanized once mating trials were finished. I then dissected and measured all male processi as described in Chapter 2. Male body length was measured for males in the large and small reduction eperiments as described in Chapter 3. Mated females were kept in isolated tubs with food and water for two weeks to oviposit. After two weeks all mated females and offspring were frozen, and the number of offspring produced was recorded. As in Chapter 5, I refer to whether a female produced offspring or not as 'insemination success', and the number of offspring produced by a female as 'fertilisation success'.

6.4.1.4 Statistical analysis

Analyses were performed separately for each measure of male reproductive success. All models (with the exception of those concerning copulation duration for the small reduction experiment; see below) were first run including treatment, male body length and their interaction as response variables. In all cases the interaction was not significant and so was removed from the model. Male body lengths were not measured for the medium reduction experiment, so those models include only experimental treatment as a response variable.

Determinants of male mating were tested in two ways. For the large and small reduction experiments logistic regression was used, with male mating as a binary response variable (whether a male mated or not). For the medium reduction experiment general linear models were used, with the proportion of times a male was seen mating (square-root transformed) as the response variable. Determinants of copulation duration were tested in two ways. For the large reduction experiment a general linear model was used, including both experimental treatment and male body length as response variables. However, the residuals for the small reduction experiment were not normally distributed, and so the effects of treatment and male body length were tested separately, using non-parametric tests. The effect of experimental treatment was tested using a Kruskall-Wallis test, and the effect of male body length using Spearman's rank correlation. Determinants of insemination success were tested using logistic regression with insemination as a binary response variable (whether a mating resulted in offspring or not). Finally, determinants of fertilisation success were tested using general linear models, with offspring number as the response variable. For the small reduction experiment additional pairwise comparisons were performed between the three experimental treatments using Tukey tests, using the multcomp package in R (Hothorn *et al.*, 2008).

Additionally, for the small reduction experiment logistic regression was used to estimate the relationship between male processus length and insemination success (as a binomial response) separately for each of the three experimental treatments.

Processus length was included as both a linear and quadratic term. This relationship was visualised using cubic splines as described in Chapter 3. The curve was estimated using a general additive model, with insemination success as a binomial response (whether the mating resulted in offspring or not) and processus length as the predictor variable.

All statistical analyses were performed in R version 3.1.0 (R Development Core Team, 2014).

6.4.2 Results

6.4.2.1 Reduction by 2 mm

The proportion of males that mated did not differ between the two experimental treatments (Logistic regression; $\chi^2_1 = 0.6$, $P = 0.44$). However, larger males were more likely to mate ($\chi^2_1 = 6.58$, $P = 0.01$).

Copulation duration was significantly shorter for males with a shortened processus compared to sham males (Binary logistic GLM; $F_{1, 56} = 7.04$, $P = 0.01$; **Figure 6.7a**). Larger males also copulated for longer ($F_{1, 56} = 4.23$, $P = 0.044$). Males with a shortened processus also had significantly reduced insemination success ($\chi^2_1 = 12.44$, $P < 0.001$; **Figure 6.7b**): only 2 out of 28 matings by manipulated males led to offspring, compared to 15 out of 31 matings for sham males. Insemination success was not influenced by male body length ($\chi^2_1 = 1.96$, $P = 0.16$).

For those matings that produced offspring, there was no significant difference in the number of offspring between reduced and sham males ($F_{1, 14} = 3.22$, $P = 0.09$; **Figure 6.7c**), which is likely due to the small number of successful inseminations by

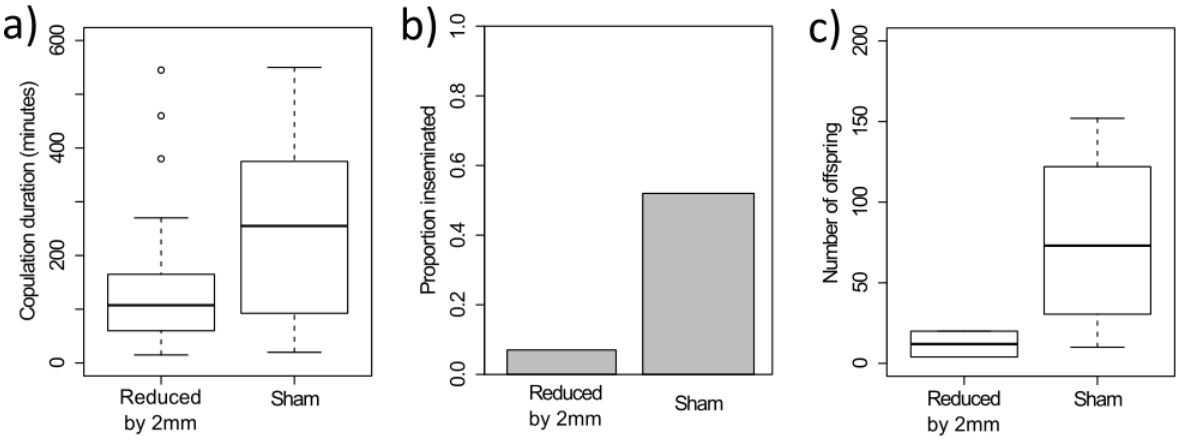

Figure 6.7. Male reproductive success following manipulation of processus length in the large reduction experiment, showing: **a)** copulation duration for mated males (*N*= 28 for manipulated males and 31 for sham males), **b)** insemination success for mated males and **c)** the number of offspring produced for fertile matings (*N*= 12 for manipulated males and 15 for sham males). For boxplots, boxes show the mean (thick line) and interquartile range, and whiskers extend up to 1.5 times the interquartile range above and below the box.

manipulated males. Additionally, larger males produced more offspring following fertile matings ($F_{1, 14}$= 6.03, *P*= 0.027).

6.4.2.2 Reduction by 1 mm

There was no significant difference in male mating frequency (proportion of observations seen in copula) between the two treatments ($F_{1, 23}$= 0.95, *P*= 0.34). Reduction of processus length by 1 mm led to no difference in male insemination success (including all males, even those that were not seen mating) compared to sham males (χ^2_1 = 2.59, *P*= 0.11; **Figure 6.8a**). However the sample size for this experiment is small, and there is a non-significant trend towards a reduction in the insemination success of manipulated males. Nevertheless, this confirms that males can successfully transfer sperm after experimental manipulation, at least when the processus has been shortened by around 1 mm and males have the opportunity to mate multiple times.

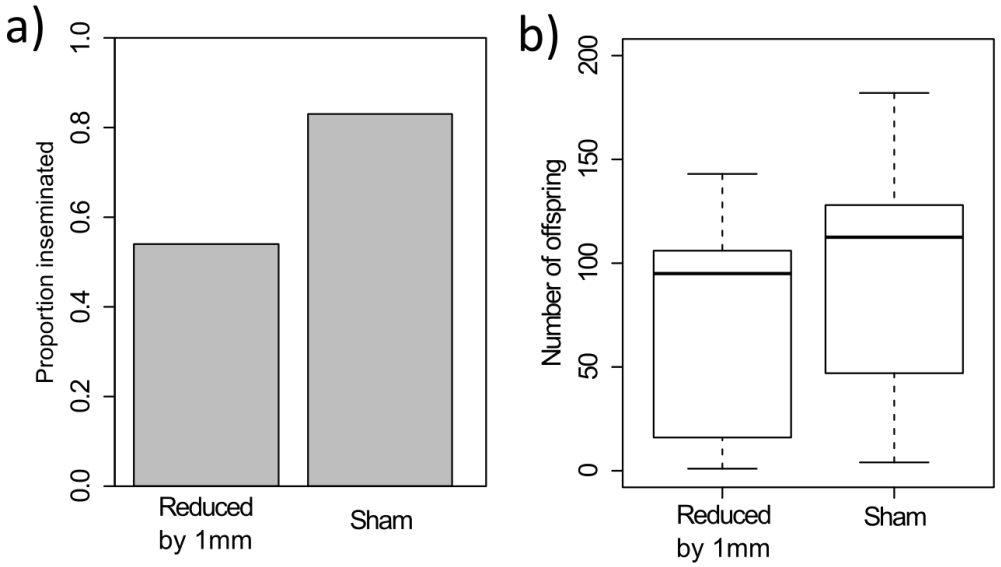

Figure 6.8. Male reproductive success following manipulation of processus length in the medium reduction experiment, showing: **a)** insemination success and **b)** the number of offspring produced, for all males (*N*= 13 manipulated males and 12 sham males). For boxplots, boxes show the mean (thick line) and interquartile range, and whiskers extend up to 1.5 times the interquartile range above and below the box.

There was also no significant difference in the fertilisation success of manipulated males compared to sham males ($F_{1, 15}$= 1.14, *P*= 0.3; **Figure 6.8b**).

6.4.2.3 Reduction by 0.4 mm

The proportion of males that mated was not significantly influenced by experimental treatment (χ^2_1 = 0.13, *P*= 0.94) or male body length (χ^2_1 = 0.84, *P*= 0.36). Copulation duration was also not significantly influenced by experimental shortening (Kruskal-Wallis test, H_2 = 0.54, *P*= 0.76; **Figure 6.9a**). However, larger males copulated for longer (Spearman's rank correlation, $r_{s\,1}$= 0.18, *P*= 0.026).

Insemination success was not significantly influenced by experimental shortening (χ^2_1 = 0.028, *P*= 0.99; **Figure 6.9b**), though matings with larger males were more likely to

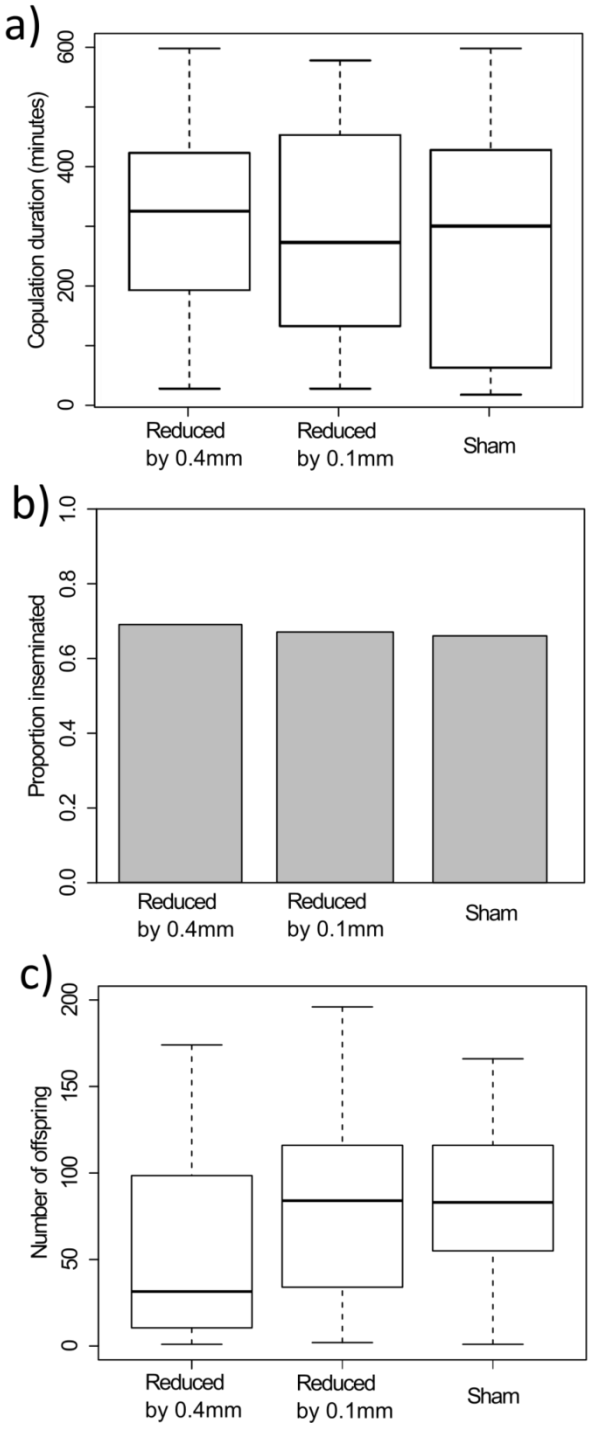

Figure 6.9. Male reproductive success following manipulation of processus length in the small reduction experiment, showing: **a)** copulation duration for mated pairs (*N*= 52 reduced by 0.4mm, 49 reduced by 0.1mm and 50 sham), **b)** insemination success for mated males (*N*= 52 reduced by 0.4mm, 49 reduced by 0.1mm and 50 sham) and **c)** offspring production following fertile matings (*N*= 36 reduced by 0.4 mm, 33 reduced by 0.1mm and 33 sham). For boxplots, boxes show the mean (thick line) and interquartile range, and whiskers extend up to 1.5 times the interquartile range above and below the box.

result in insemination (χ^2_1 = 5.8, *P*= 0.016). Amongst the males that produced offspring, there is a positive relationship between processus length and insemination success for males that had 0.4 mm of processus removed (χ^2_{52}= 5.16, *P*= 0.023; **Figure 6.10c**), but no relationship for sham males (χ^2_{50}= 0.1, *P*= 0.75; **Figure 6.10a**) or those that had just the tip removed (χ^2_{49}= 2.003, *P*= 0.16; **Figure 6.10b**).

The number of offspring produced following a fertile mating was not influenced by male body length ($F_{1, 98}$= 1.89, *P*= 0.17), but was significantly influenced by the experimental treatment ($F_{2, 98}$= 4.59, *P*= 0.012; **Figure 6.9c**). Post-hoc tests show that removal of the tip did not influence the number of offspring produced compared to sham males (t_{65}= 0.35, *P*= 0.94), however females mated to males with a processus shortened by 0.4 mm had significantly fewer offspring compared to both sham males (t_{68}= 2.4, *P*= 0.046) and those with just the tip removed (t_{68}= 2.76, *P*= 0.019).

6.5 Discussion

6.5.1 Micro-CT

I used micro-CT to produce high-resolution virtual dissections of male and female reproductive anatomy *in copula*. This is the first study to my knowledge which attempts to visualise the interaction between male and female genitalia in this way. I was successfully able to trace the path of the processus inside the female, inside both the female bursa and the spermathecal duct. The most striking feature of these images is how much of the processus remains coiled inside the bursa once the tip of the processus has reached the entrance to the spermatheca. This emphasises the difference in length between the processus and duct. Though Gschwenter & Tadler (2000) claim that the spermathecal duct can expand to be up to 6 mm in length, this does not fit with the results seen here. I was also able to infer the normal size and

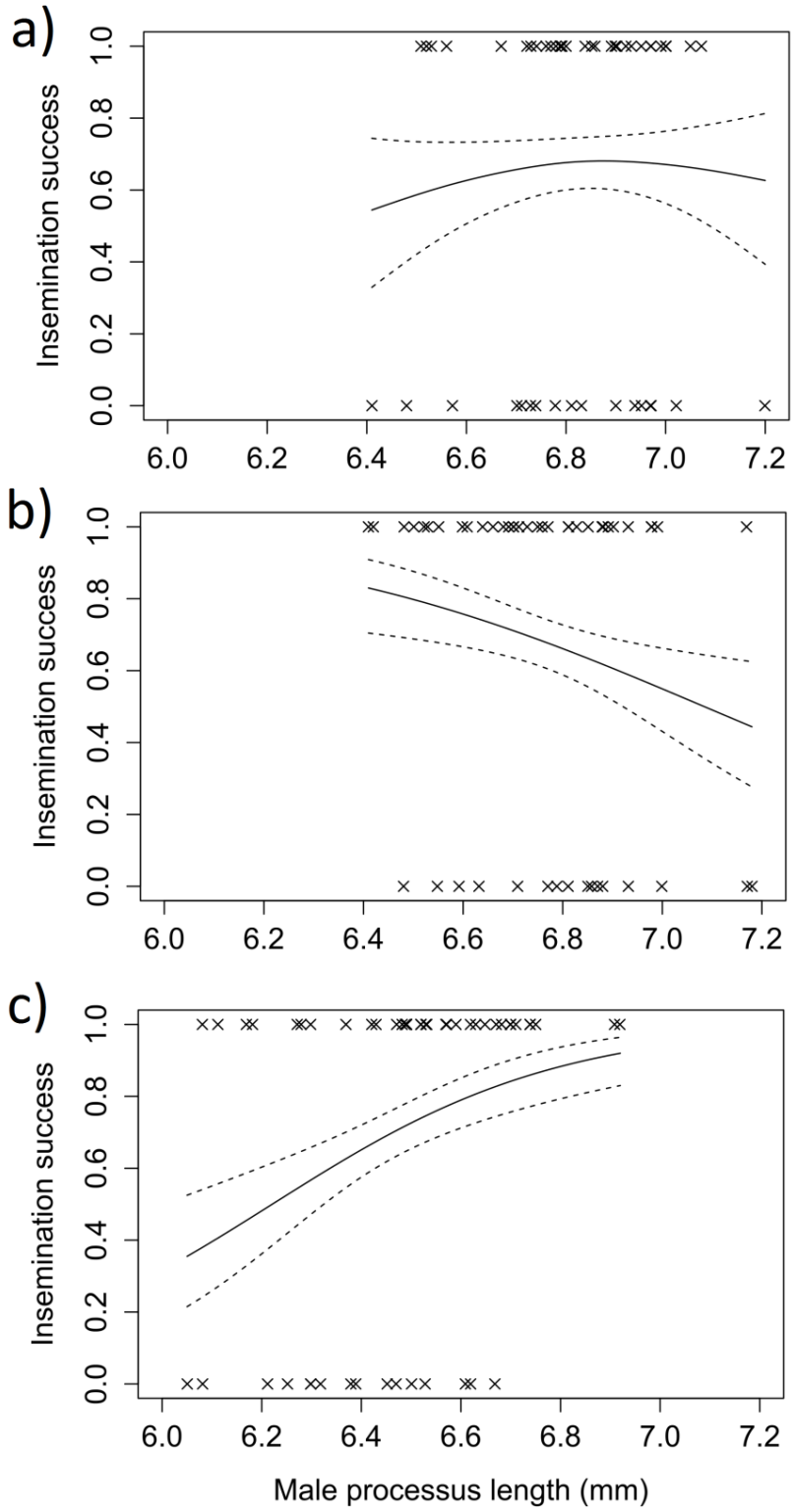

Figure 6.10. Post-copulatory selection on male processus for **a)** sham males (*N*= 50), **b)** control males (0.1 mm of processus removed, *N*= 49), and **c)** manipulated males (0.4 mm of processus removed, *N*= 52) in the small reduction experiment. Dashed lines indicate 1 standard error above and below the predicted line.

shape of the bursa cavity during copulation. This structure is very fragile and is easily broken during dissection (*pers. obs*.), so the ability to images such structures is a key advantage of non-destructive imaging techniques such as this. The accuracy with which micro-CT is able to reconstruct such cavities (for which the contrast between tissue and the empty cavity is high) may be very useful in the future for visualising complex shape changes of female internal anatomy during mating which may be important for successful sperm transfer and uptake.

My results show that it is possible, with relatively simple staining procedures, to distinguish accurately between soft (non-sclerotized) structures in medium-sized invertebrates such as *Lygaeus*; with the scans able resolve structures less than 10 µm long. Importantly, I was able to distinguish between male and female tissues successfully (with the exception of the spermathecal duct), despite the close-fitting nature of the male intromittent organ and female reproductive tract. This method may be especially useful when coupled with flash-freezing to investigate the positioning of genitalia at different stages of copulation (Micholitsch *et al.*, 2000), and also to determine the normal shape of internal structures (such as the female bursa). This has traditionally been investigated using serial sections; however micro-CT has the advantage of not requiring the destruction of samples, and allows the production of distortion-free reconstructions (Holdsworth & Thornton, 2002; Ziegler, 2010; Friedrich *et al.*, 2014).

One caveat is that this study was performed using a species for which genital morphology is reasonably well studied (e.g. Ludwig, 1926; Tadler, 1999; Gschwentner & Tadler, 2000, Micholitsch *et al.*, 2000). Previous reference material was very useful during the reconstruction process, and allowed me to confirm the accuracy of the final virtual models. Attempting such reconstructions on species for which reproductive morphology has not been thoroughly described may be more difficult. Though of course, any hypotheses concerning functional anatomy will be stronger if informed by

multiple lines of evidence, and so micro-CT will be an especially powerful method when used in tandem with other complementary techniques (such as dissections).

In conclusion, my results confirm that micro-CT is an excellent tool for the visualisation of insect internal reproductive morphology, and importantly can be used to visualise the interaction between male and female genitalia in copula. I suggest that these methods should be further adopted in the field of evolutionary ecology in order to understand the proximate mechanisms underlying genital evolution.

6.5.2 Natural breakages

I attempted to assess the frequency of natural processus breakages in a sample of males allowed to mate multiply for several weeks. Following this experiment I observed a much higher frequency of broken processi than in previous experiments (see above), with evidence for at least some breakage in almost a quarter of males. This can be assessed easily as the morphology of the processus tip is distinctive (**Figure 6.3**). However I was unable to confirm that breakages occurred during mating, as I could not find any processus fragments in the corresponding female tracts. Furthermore, I have never found broken processi in the female bursa or spermathecal duct when dissecting mated females. Therefore I suggest that processus breakages are not an adaptation to prevent rival inseminations.

Older males were more likely to exhibit broken processi in my experiment, but I am unable to confirm whether this is due to male age itself or the number of matings a male performs. My method of recording the frequency of copulation naturally underestimates the actual number of intromissions a male performs across his life, which would be the most important determining factor if processi are broken during the act of mating. Though the number of matings a male achieves during his lifetime will be expected to correlate with age (unless there is a strong decline in mating success with age), alternative explanations cannot be ruled out. For example, processi

may become more brittle and liable to break in older males, irrespective of the number of intromissions. Indeed processi become very brittle if kept outside of the body for an extended period of time (per. obs.). The most likely time for breakages to occur is probably as the processus is being re-coiled into the genital capsule following mating (see Chapter 2).

One limitation of this study is that we did not check the processus morphology prior to performing the experiment, so we cannot be certain that breakages did not occur prior to males being introduced to females. However, as noted above, less than 1% of 140 processi checked following a single mating were damaged in any way. This strongly suggests that the breakages seen here did occur during the experiment. Additionally, it must be noted that the average male lifespan in this experiment is likely much shorter than in the wild (Solbreck & Sillén-Tullberg, 1990a). This is probably due to the experimental conditions: mating and harassment costs appear to be high for both males and females and lead to significant reductions in longevity (Sillén-Tullberg, 1981; Shuker *et al.*, 2006). Therefore the average male age in the wild may be significantly older, although the lifetime number of matings may be comparable.

6.5.3 Experimental reduction in processus length

The three manipulation experiments presented in this chapter resulted in direct, experimental evidence that processus length influences male insemination and fertilisation success in *L. simulans*, in a non-competitive mating context. Importantly, I have shown that this effect is not due to manipulation *per se*, as removal of the tip of the processus had no detectable effect on either of the four measures of male reproductive success. Reduction in processus length to a level both within and significantly beyond the natural phenotypic range in *L. simulans* shows that reproductive success decreases in relation to the proportion of processus removed. Furthermore, the reduction in reproductive success observed depends on which proxy measure was used: if 0.4 mm of the processus is removed there is no significant

reduction in insemination success, but there is a significant reduction in the number of eggs fertilised (fertilisation success). In contrast, reduction of the processus by 2 mm leads to a significant reduction in all three proxy measures (copulation duration, insemination success and fertilisation success).

Across all three manipulation experiments, the manipulation of processus length had no effect on the proportion of males seen mating, or male mating frequency. By removing only the tip of the processus in the small reduction experiment I also show that the experimental ablation itself does not influence post-copulatory reproductive success. This result, and the fact that processus morphology is the same over the region manipulated here, suggests that the reduction in reproductive success seen following shortening by 1 mm and 2 mm is not due to injury caused by cutting, but rather a direct result of the reduction in processus length. Additionally, in the medium reduction experiment I show that insemination success when the processus is reduced by around 15% (which is still outside the natural phenotypic range) is comparable to that from a non-manipulated processus, when males were allowed to mate multiple times. However it is not clear if males mated significantly more often following this manipulation. Interestingly, the relationship between processus length and insemination success is positive and linear following reduction by 0.4mm (**Figure 6.8c**), in contrast to the stabilising selection found over the normal range of the processus (Tadler, 1999). This could represent the left-hand side of a stabilising fitness function, and demonstrates how directional selection may act strongly following perturbation to return a trait to its optimum.

Such an experimental approach to the study of genital function has rarely been taken, and this is likely due to the perceived difficulties of manipulating traits while maintaining function (Arnqvist & Rowe, 2005; Simmons, 2014). This is likely an insurmountable problem in most species, but here I have demonstrated that this approach may be fruitful in certain specific cases, for example in species for which sperm transfer is achieved using a simple, sclerotized, tube-like structure as in *Lygaeus*.

The results of these experiments also add support to one of my conclusions in Chapter 5, namely that the significant pre-copulatory selection on processus length detected in *L. equestris* does not arise through selection on processus length directly. Across all three experiments, reduction in processus length had no significant effect on male mating success, suggesting both that males are not adversely harmed by the experimental procedure, and that females do not assess processus length prior to intromission. Being able to show causation is a key advantage of using such an experimental approach, and in that respect *L. simulans* is a useful model organism for studying post-copulatory processes. These results also support the conclusion of the meta-analysis reported in Chapter 5 that there is significant post-copulatory selection on processus length in *L. simulans*. I was not able to experimentally lengthen processus length, and so cannot show that longer processus lengths result in reduced reproductive success.

Finally, the results of these three experiments cast doubt on the breakage insurance hypothesis for the extreme length of the processus. Importantly, they suggest that processus breakage will reduced male reproductive success due to a decrease in length. Therefore the excess processus that does not enter the spermathecal duct is required for normal sperm transfer, rather than functioning as 'excess' length available to guard against breakages. I discuss the potential mechanical benefits of a long processus in Chapter 5. Three of the males in this experiment had lost an even greater proportion of the processus than in the large reduction experiment (up to 75% lost), and so I suggest these males would be very unlikely to achieve a successful insemination if they were to mate again. However, the average length of the six remaining broken processi is only 6% shorter than the average length of normal processi, and removal of approximately 5% of processus length in the manipulation experiment reduced the number of offspring following fertile matings, but did not influence the likelihood of insemination. Therefore, though there is strong post-copulatory selection on processus length, a small reduction in length following a

natural breakage may not impair male insemination success fully. Males may even be able to compensate for such loss by attempting to perform more intromissions, though this could impact male lifespan (see below). Of course this relies on the breakage not blocking the release of sperm from the tip of the processus in any way.

6.6 Conclusion

In this chapter I have investigated the functional morphology of the male processus in *L. simulans*. I used micro-CT scanning to confirm that the majority of the processus does not enter the female spermathecal duct during mating. Natural processus breakages in this population appear to be reasonably common when males are able to mate multiple times. However, processus fragments do not appear to break off whilst in the female reproductive tract, so that I can reject one adaptive explanation for such breakages. Most importantly, experimental manipulation shows conclusively that processus length directly influences sperm transfer. Therefore I have experimentally confirmed that there is strong selection to maintain the extreme length of the processus length, despite the disparity in length between it and the female reproductive tract.

Chapter 7

Sexual selection on male genital clasper size and shape in *Lygaeus simulans*

Abstract

In this chapter I investigate whether there is significant sexual selection acting on male genital clasper size and shape in *Lygaeus simulans*. I first use experimental ablation to show that mating cannot occur when both claspers are removed. I then perform mating trials in both no-choice and female-choice contexts, and measure the size and shape of male claspers for all males. I describe clasper shape in two dimensions using a geometric morphometric approach. I first show that there is no clear relationship between male processus length and clasper size or shape. I then show that there is significant sexual selection on left clasper shape, but only in no-choice trials: mated males have a tooth section that is both straighter and thinner than in unmated males. Therefore selection on clasper shape is influenced by the social environment, but cannot explain the significant pre-copulatory selection on processus length detected when rival males are present during mating trials.

Acknowledgments

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7.1 Introduction

In Chapter 5 I found evidence that male processus length was under significant, negative pre-copulatory selection in *L. equestris*. This result seems counterintuitive given that the processus is coiled inside the male genital capsule prior to mating, and so should not directly influence male mating success. I suggested that this effect may arise due to selection on another trait correlated with processus length. Such correlations may be likely to occur between internal and external male genital structures. In this chapter I investigate whether selection on the external genital claspers of males may be leading to indirect selection on processus length in *L. simulans*.

In many species, males possess specialised grasping structures that appear to function to hold females during mating (Thornhill, 1980; Thornhill & Sauer, 1991; Westlake *et al.*, 2000; Eberhard, 2001; Arnqvist & Rowe, 2005). In insects such structures may derive from modified external genitalia (Darwin, 1871; Eberhard, 1985; 1996; Arnqvist & Rowe, 2005). These structures have been suggested to have a range of different functions, from holding females and preventing male takeovers during mating, to sense organs used by the male to orient prior to mating (Moreno-García & Cordero, 2008). They are given different names in different species, including claspers (Moreno-García & Cordero, 2008), parameres (Deckert, 1990), gonostyli (Bonhag & Wick, 1953) and cerci (Vahed & Carron, 2008; Vahed *et al.*, 2011), and may have different developmental origins across insect orders (Snodgrass, 1935). I hereafter refer to any male genital structures which serve to grasp the female near her genital opening as 'genital claspers'.

There is some evidence that suggests that claspers influence male reproductive success in several animal groups. For example, forced mating in bushcrickets (Tettigoniidae) is rare, but is observed in four species in which the cerci are used to grab the female abdomen, rather than the specialised grooves in the sub genital plate

typical of those species without forced matings (Vahed & Carron, 2008). Eberhard (1996) suggests that genital claspers may function in copulatory courtship in many species, and indeed the male claspers in the fly *Dryomyza anilis* seem to influence fertilisation success after mating by tapping the female (Otronen, 1998). However, the functional morphology of genital claspers has been explicitly investigated in only a few insect taxa. Moreno-García & Cordero (2008) showed using experimental manipulation that at least one intact clasper is needed for successful intromission in the bug *Stenomacra marginella*. Males of the sagebrush cricket *Cyphoderris strepitans* possess two abdominal spines which are able to grasp the female during mating (Sakaluk *et al.*, 1995). Experimental manipulation showed that these spines are not required for copulation, but can be used by males to perform coercive matings (Sakaluk *et al.*, 1995).

Fewer studies have investigated whether clasper size or morphology influences male mating success. In the water strider *Gerris odontogaster* male clasper length is positively associated with the ability of males to withstand female rejection behaviour, and mating males have significantly longer claspers than non-mating males in the wild (Arnqvist, 1989). Mated males also had significantly longer genital claspers (gonostyli) in the lygaeid *Nysius huttoni* (Yang & Wang, 2004). Additionally, clasper symmetry is also correlated with male mating success in the zygaenid moth *Elcysma westwoodii* (Koshio *et al.*, 2007), and with fertilisation success in the fly *Dryomyza anilis* (Otronen, 1998).

In *Lygaeus* the claspers are external, paired and fit into grooves in the genital capsule when at rest (Ludwig, 1926). They can be separated into a proximal 'body' which is primarily internal, and a distal 'tooth' that is visible externally and actually contacts the female (**Figure 7.1**). The male uses his claspers to open the female ovipositor prior to intromission and then lock into place (**Figure 6.2**), and observations suggest that these structures are necessary for genital coupling. As such, they are also used to maintain genital contact during copulation, and locking of the claspers in place may give males

significant control over copulation duration. However, it is unclear whether these functions are influenced by clasper size or shape. Interestingly, the most obvious difference in genital morphology between *L. equestris* and *L. simulans* (and the easiest way to distinguish the two species) is in the shape of the notches on the clasper body (see **Figure 2.1**). The main tooth region that physically interacts with the female during mating also seems to differ in size and shape between the two species. It is unclear how or if selection has led to the divergence in clasper morphology. The fact that the claspers appear important for genital coupling is suggestive that sexual selection may be driving the evolution of clasper shape. Specifically, if there is variation in clasper size among males, then clasper size or shape could possibly influence male mating success or the length of time that genital contact can be maintained.

In this chapter I consider the functional morphology of the male claspers, and selection acting on their size and shape, in *Lygaeus simulans*. I experimentally ablated both claspers in order to compare male mating success for males with intact or ablated claspers. The male claspers are solid, sclerotized structures (though the base appears to be hollow: *pers. obs*.) which lack vascularisation, therefore no haemolymph is lost following ablation. I am thus confident that the male is not harmed significantly from these manipulations (see below). I then assess how natural variation in clasper size and shape relates to the likelihood of male mating, using both no-choice and female-choice mating trials. I use both choice designs because the presence of rival males influenced the strength of pre-copulatory selection on male processus length in Chapter 5. The main aim of this chapter is to determine whether the significant pre-copulatory selection on processus length seen in Chapter 5 (in *L. equestris*) can be explained as arising from selection on male clasper morphology. I therefore also measure male processus length of males in order to investigate whether clasper size or shape is related to processus length. I assess selection on clasper shape in two dimensions using a geometric morphometric approach. In the no-choice experiment I also record copulation duration for males that mate, and examine whether duration is related to clasper size or shape. As copulation duration correlates strongly with insemination

success, such a relationship could be considered a form of post-copulatory sexual selection (Eberhard, 1996).

In analysing the results of these experiments I ask four main questions. Firstly, are claspers necessary for mating? Secondly, does clasper size or shape correlate with processus length? Thirdly, does clasper size or shape influence male mating success or copulation duration? And lastly, is the strength of sexual selection on clasper size or shape influenced by social context?

7.2 Methods

7.2.1 Clasper ablation experiment

I ablated both male claspers from 35 males using the following procedure. Virgin, 7-10 day old males were sedated by placing in a plastic dish in a freezer at -13 °C for three minutes and 30 seconds. Males were then taken out and placed head-first into a hollow in a block of florist's foam. Both claspers were cut using micro-scissors as close as possible to the base of the tooth (**Figure 7.1**). Any males that did not recover from sedation after 4 minutes were discarded. As a control 35 males were subjected to the same procedure but claspers were not cut; these are referred to as sham males. After this procedure males were allowed one day to recover.

I then performed no-choice mating trials in which these males were presented to 7-10 day old virgin females (see Chapter 2 for details). Pairs were observed continuously for five hours. I recorded male mating success and copulation duration during the trial; pairs that were still copulating after five hours were separated manually using a paintbrush. I also recorded all male mating attempts, but using the stricter criteria (compared to those recorded in Chapter 3) that the male claspers needed to physically contact the female genital region in order to be classed as an attempt. Following

Figure 7.1. Comparison of clasper morphology before and after ablation, showing: **a)** claspers following dissection (with the main clasper anatomical regions highlighted), and **b)** claspers still attached to the genital capsule.

mating trials, all individuals were frozen, and females were discarded. I then removed the claspers from manipulated and unmanipulated males. Visual inspection confirmed that the length of the tooth was reduced at least by half following cutting in all cases.

7.2.2 Clasper morphology and male reproductive success in a no-choice context

I next tested for an effect of clasper size and shape on male mating success. I performed no-choice mating trials using 7-11 day old virgin individuals. In order to record copulation duration, dishes were watched continuously for ten hours. I allowed

for multiple matings during the trial. I classed a mating as sufficient if it lasted longer than 20 minutes. After ten hours any pairs still copulating were gently separated using a paintbrush. Both males and females were frozen post-trial. I then recorded total male body length, and then removed and measured processus length (see Chapter 2).

7.2.3 Clasper morphology and male reproductive success in a choice context

To test whether selection on clasper morphology is different in the presence of rival males, I repeated the previous experiment but using a female-choice design, with two males and one female per dish. In order to follow individual males during trials, each male was marked on either the left or right side of the pronotum with a small spot of enamel paint (see Chapter 2). All dishes were watched continuously for two hours (compared to ten hours in the no-choice experiment). I recorded the copulation duration of mating pairs, and measured male body length and processus length as above.

7.2.4 Clasper imaging

Following mating trials, both claspers were carefully removed from the genital capsule using fine forceps, and stored in 80% ethanol prior to imaging. Claspers were then carefully placed onto a microscope slide, and paper tissue was used to remove excess liquid from the samples, as the surface tension of the ethanol was seen to affect the position. The claspers are curved and so cannot be laid flat (**Figure 7.2**). Instead a stable position was found by resting the clasper on the dorsal surface, so that the tooth portion curved toward the camera slightly. Care was taken to place all claspers in this same position relative to the camera, and in the centre of the frame. Once the correct position was obtained, an image was taken using an Olympus SZX10 stereo microscope and attached camera, against a white background to maximise contrast.

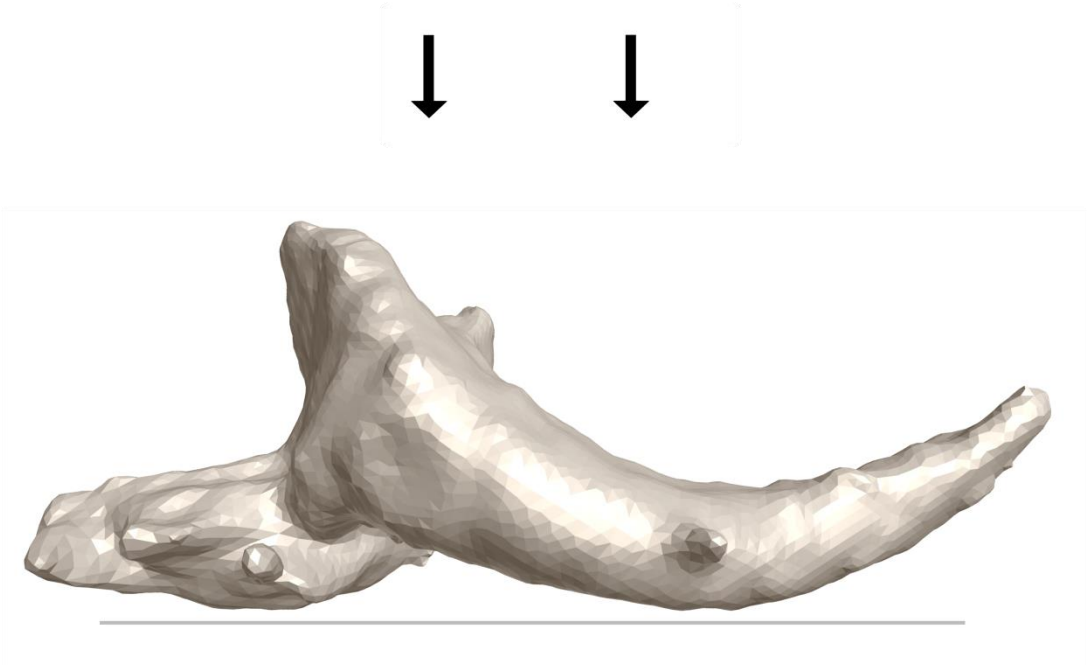

Figure 7.2. 3D reconstruction of clasper shape derived from micro-CT scanning (Chapter 6), showing the curvature in the clasper tooth when laid flat. The arrows represent the direction the microscope was pointing when images were taken.

Both left and right claspers were imaged at the same time in most cases; in some cases one clasper was damaged and so only one side was used. In order to assess the repeatability of clasper placement, all claspers were re-placed onto a slide and imaged twice.

7.2.5 Clasper size and shape measurement

I first obtained a measure of clasper size by measuring the widest distance along the tooth (**Figure 7.3a**). Measurements were made using the image analysis program Cell^D (Soft Imaging System, Olympus Corp; see Chapter 2). I measured a subset of 18 males (32 claspers) a total of 5 times and assessed the repeatability of these measures (see below).

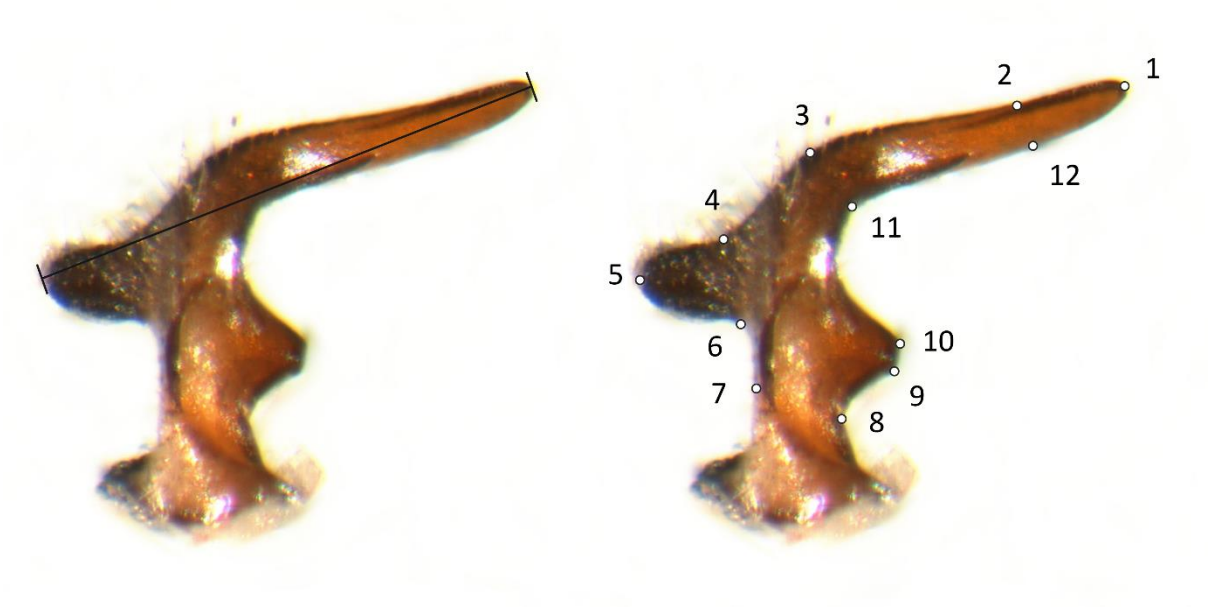

Figure 7.3. Clasper size and shape measurements. Left: total clasper length at the widest point was used as a measure of clasper size. Right: the 12 landmarks used for geometric morphometric analysis.

The complex shape of the claspers means that a lot of information is lost if only certain length and width measurements are taken. An alternative approach is to use a geometric morphometric approach based on landmarks; that is, specific structures that can reliably be located in all samples (Klingenberg, 2010; Lawing & Polly, 2010). A shape-based geometric morphometric approach is commonly used to study selection on genital traits (e.g. Simmons *et al.*, 2009; Holwell *et al.*, 2010; Wojcieszek & Simmons, 2011; Simmons & García-Gonzaléz, 2011; House *et al.*, 2013). Modern geometric morphometric procedures extract shape information using a procedure called Procrustes superimposition, in which variation between samples in size, position and orientation is removed. Any remaining variation following Procrustes superimposition is thus exclusively shape variation (Klingenberg, 2010). This initial shape data is known as the 'Procrustes fit'. Differences in shape between samples are then assessed using multivariate statistics. It is important to emphasise that these analyses of shape are performed independent of size differences between samples, which is why size and shape need to be analysed separately.

All clasper images were first combined into a single file using tpsutil software (James Rohlf, 2013). I then used tpsdig2 (James Rohlf, 2013) to digitise landmarks. I located a total of 12 landmarks (**Figure 7.3b**; see Appendix 2 for descriptions of all 12 landmarks).

7.2.6 Statistical analysis

I performed separate analyses for clasper size and clasper shape. For clasper size I first assessed the repeatability of clasper measurement using analysis of variance (Lessells & Boag, 1987), using the rptR package in R (Nakagawa & Schielzeth, 2010). I then calculated the average length of the two measurements taken for each clasper. All subsequent analyses considering clasper length use these average scores.

I investigated variation in clasper length by combining data from all claspers measured in both the no-choice and choice experiments (*N*= 152 left and 151 right claspers). To assess the difference between the left and right clasper length I used a linear mixed-model with clasper length as a response variable and clasper side (left or right) as a categorical response variable. As the lengths of left and right claspers for each male are highly correlated, male identity was added as a random factor to these models. I found that there was a significant difference between left and right claspers in length (see below). However across all males left and right clasper lengths are strongly correlated with each other (r_{196}= 0.88, P< 0.001). For males for which both claspers were measured, I obtained a measure of average clasper length by taking the average of left and right clasper length for each male. For all remaining analyses of clasper length I therefore used this average measure. Additionally, I determined the degree of clasper asymmetry by subtracting right clasper length from left clasper length for each male.

I tested for a significant relationship between clasper length and male mating success in two ways, with analyses performed separately using males from the no-choice and choice experiments. For the no-choice experiment, I used generalised linear models with male mating as a binary response variable (mated or unmated). I used mating success after two hours so that the results could be compared to those from the choice experiment. For the choice experiment, I controlled for potential non-independence of male mating between the two males in each same dish using generalised mixed models, with female added as a random effect. Male mating (after two hours) was fitted as a binary response variable. In the choice experiment, one (unmated) male possessed a very short processus (5.01mm long) which is far outside the normal phenotypic range. This male was removed from the analysis so as not to influence any significance tests. For both experiments I first fitted full models including male body length, processus length and clasper length, and the associated quadratic terms, as predictor variables. I then performed model simplification by removing non-significant quadratic terms. To visualise the shape of pre-copulatory selection on male processus length I produced fitness functions using cubic-splines as described in Chapter 2. Splines were generated using general additive models with male mating as a binary response variable, and a single predictor variable (processus length).

For the no-choice experiment I also considered two additional sources of selection on clasper length. First I tested for a relationship between clasper length and the number of matings a male achieved over ten hours, using a generalised linear model with mating number as a categorical variable with a Poisson distribution. For mating number I considered all times a given male was seen to couple with a female, including matings that lasted for less than twenty minutes. Second, I tested for a relationship between clasper length and the total time a male spent in copula over ten hours, using a generalised linear model with copulation duration as a binary logistic response variable. Because copulation duration is bimodal, I converted total duration into a binary categorical variable, with a total copulation of less than 300 minutes being classed as ‘short’ and a total copulation of greater than 300 minutes classed as ‘long’.

Note that this is different from the duration classifications used in previous chapters. This is because multiple matings were allowed in this experiment (unlike in previous chapters), which means that manually-separated matings are not necessarily longer then naturally separating matings. I included males that were seen mating for at least five minutes in this analysis. Again, I first fitted full models including male body length, processus length and clasper length, and the associated quadratic terms, as predictor variables, and then performed model simplification by removing non-significant quadratic terms until I obtained the model with the minimum AIC score (while still including all three predictor variables).

To investigate clasper shape I used a geometric morphometric approach. Firstly, all landmark data was imported into the morphometrics package MorphoJ (Klingenberg, 2011). I first analysed all claspers imaged in both the no-choice and choice experiments (N= 152 left and 151 right claspers). I assessed the repeatability of my landmark measurements across the two images of each clasper in MorphoJ using Procrustes ANOVA. This method can be used to quantify the amount of variance in shape accounted for by different factors, and is useful for quantifying the variance associated with fluctuating asymmetry and different levels of measurement error (Goodall, 1991; Klingenberg and McIntyre, 1998; Klingenberg *et al.*, 2002). I ran a Procrustes ANOVA with male ID, clasper and measurement as nested factors. As this analysis suggested that measurement error was small relative to between-individual variation, I used MorphoJ to obtain the average landmark positions for each clasper by combining the two sets of landmark coordinates. The remaining analyses all use this average shape data. I tested for shape differences between left and right clasper shape using discriminant function analysis. This is used to find shape features that best distinguish samples from two groups, when the membership to each group is already known (Timm, 2002). The associated *P* value (derived from the t^2 statistic, which is a multivariate form of the *t* statistic) tells us whether the multivariate transformation that maximises between-group variance relative to within-group variance (the discriminant function) can allocate samples into their correct groups. Discriminant

function analysis of side indicated significant differences in average shape between left and right claspers. Therefore, as with clasper length, the remaining analyses were performed separately for left and right claspers.

In order to investigate whether clasper shape is related to other aspects of male morphology, I performed principal component analyses in MorphoJ separately for left and right claspers. This transforms the original shape variables into new variables which are uncorrelated with each other and successively account for the maximum amount of variation in multi-dimensional space. Information on the 20 principal components extracted for each clasper is presented in Appendix 2. I tested for significant correlations between male body length, processus length, and the first five principal components extracted for each clasper (which explained approximately 67% of the variance in both left and right claspers) using Pearson's product-moment correlation. To correct for multiple testing I used the sequential Holm-Bonferroni method (Holm, 1979), with significance determined using the associated adjusted *P* values.

Finally, I used discriminant function analysis in MorphoJ to assess variation in clasper shape associated with male mating and copulation duration (as a binary factor as above) for the no-choice experiment, and male mating for the choice experiment. For the choice experiment I used only males from dishes in which one male mated during the mating trial (N= 39 trials). Tests were performed separately for left and right claspers.

Unless otherwise stated, all statistical analyses were performed in R version 3.0.1 (R Development Core Team, 2014).

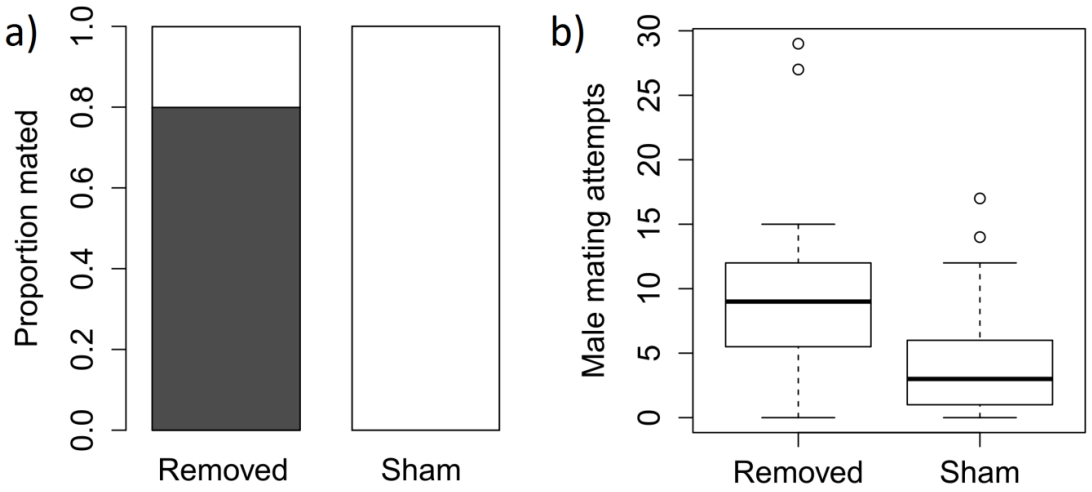

Figure 7.4. The effect of clasper ablation on male mating behaviour, showing: **a)** the proportion of males that mated, and **b)** the average number of mating attempts performed, for normal males (sham treatment, *N*= 35) and those with claspers ablated (removed treatment, *N*= 35). For boxplots, boxes show the mean (thick line) and interquartile range, and whiskers extend up to 1.5 times the interquartile range above and below the box.

7.3 Results

7.3.1 Clasper ablation

Males were significantly more likely to mate when they possessed intact claspers (Chi-square test, χ^2_1= 46.67, *P*< 0.001): none of the 35 males with cut claspers mated, whereas 28 of 35 unmanipulated males mated (**Figure 7.4a**). This difference was not due to males with cut claspers being less motivated to mate; in fact, manipulated males attempted to mate significantly more than sham males (Mann-Whitney test, U= 294, *P*< 0.001; **Figure 7.4b**).

7.3.2 Clasper morphology

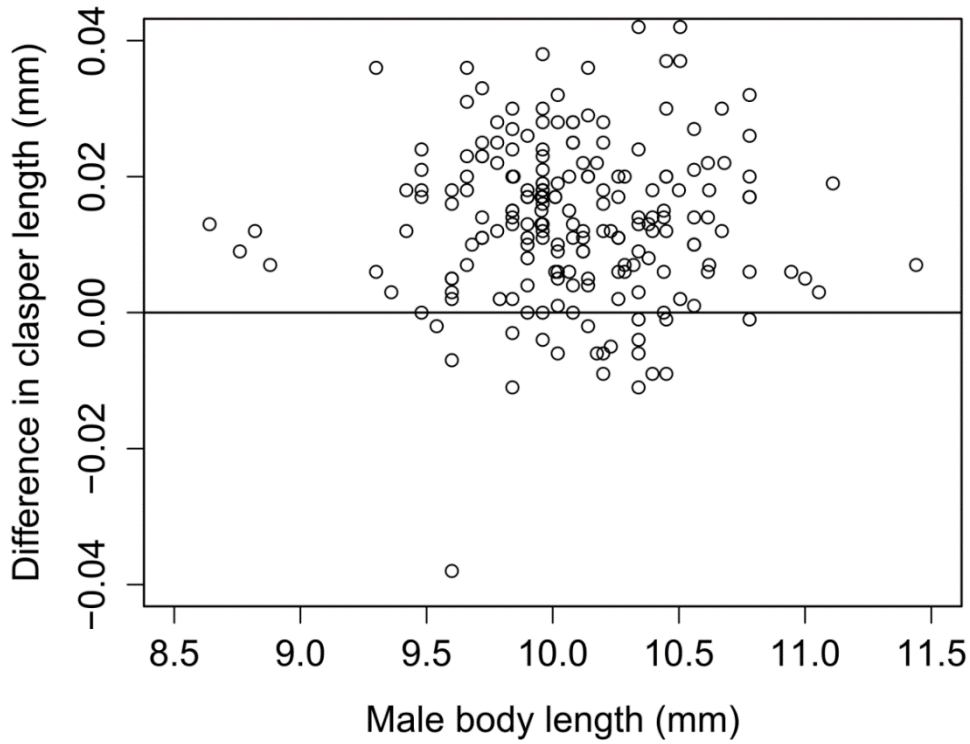

Figure 7.5. The relationship between male body length (mm) and the degree of clasper asymmetry in *L. simulans*, calculated as the difference in right and left clasper length (right minus left; in mm), for males for which both claspers were measured (males from both the no-choice and choice experiment, *N*= 198)

The measurement of total clasper length was highly repeatable (*N*= 32 claspers, 5 observations of each; *R*= 0.835, 95% CI= 0.726- 0.943). Right claspers are significantly larger than left claspers (Linear mixed model; χ^2_1= 235.58, *P*< 0.001; Left clasper: mean= 0.83 mm, s.d.= 0.023 mm, *N*= 152; Right clasper: mean= 0.84 mm, s.d.= 0.023 mm, *N*= 151). Furthermore, clasper asymmetry is directional, with almost all males having a right clasper that is larger than the left (**Figure 7.5**). There is a significant positive correlation between clasper length and body length for both left (r_{150}= 0.29, *P*< 0.001) and right claspers (r_{149}= 0.24, *P*= 0.003). However processus length did not correlate with left (r_{149}= 0.06, *P*= 0.45) or right clasper length (r_{148}= 0.1, *P*= 0.21).

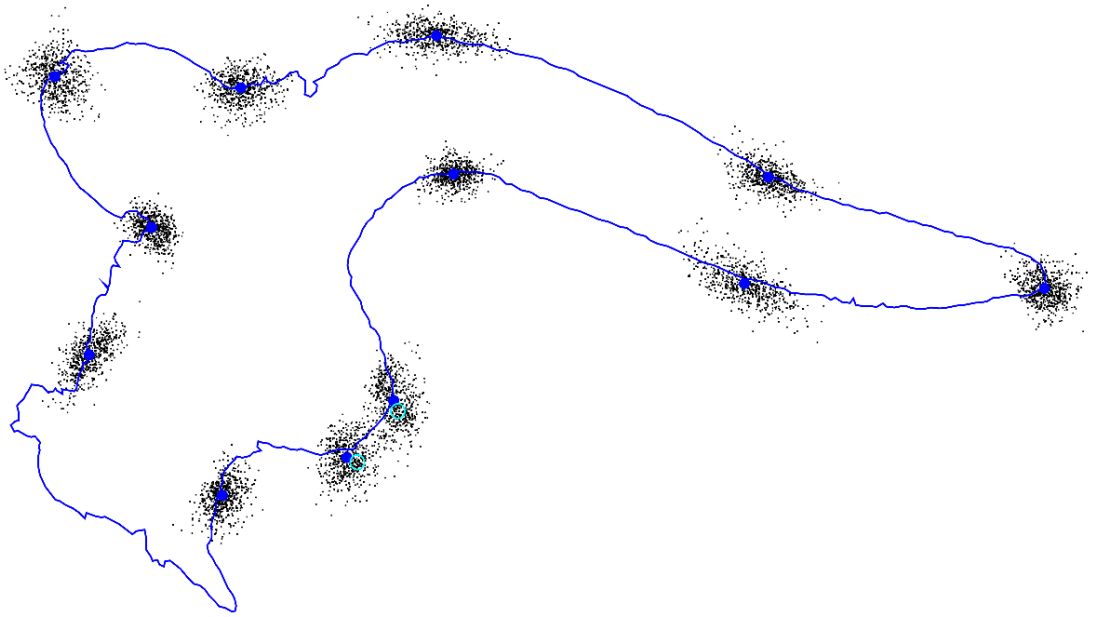

Figure 7.6. The amount of scatter associated with each of the 12 landmarks used in the geometric morphometric analysis. Data are for both claspers (with some exceptions) for males in both the no-choice and choice experiment (*N*= 303).

There was significant variation in the position of all landmarks across the 160 males from both experiments (**Figure 7.6**). Procrustes ANOVA indicated that the variation associated with measurement error was much smaller than that between individuals or between the left and right claspers (**Table 7.1**). Left and right claspers differed significantly in shape (Discriminant function analysis; *N*= 152 left and 151 right, Procrustes distance= 0.054, Mahalanobis distance= 3.25, t^2= 797.93, *P*< 0.001), with right claspers being significantly wider than left claspers (**Figure 7.7**).

For left claspers, the third principal component of shape was significantly positively correlated with body length (Pearson's correlation, r_{150}= 0.33, Adjusted *P*= 0.001) and processus length (r_{149}= 0.29, Adjusted *P*= 0.005). This component explains 12.85% of variance in left clasper shape. There were no other significant correlations for the other four principal components tested (**Table S43**). For right claspers, the fourth principal component of shape was significantly positively correlated with body length

Table 7.1. Procrustes ANOVA results, showing the amount of variance in centroid size across individuals, clasper side (left or right) and clasper image. Data are for both claspers (with some exceptions) for males in both the no-choice and choice experiment (*N*= 606).

	SS	MS	*d.f.*	*F*	*P*
Individual	246556.9	1712.2	144	3.47	<0.001
Side	17146.26	17146.26	1	34.78	<0.001
Individual * Side	70988.92	492.98	144	5.57	<0.001
Image	25677.76	88.54	290		

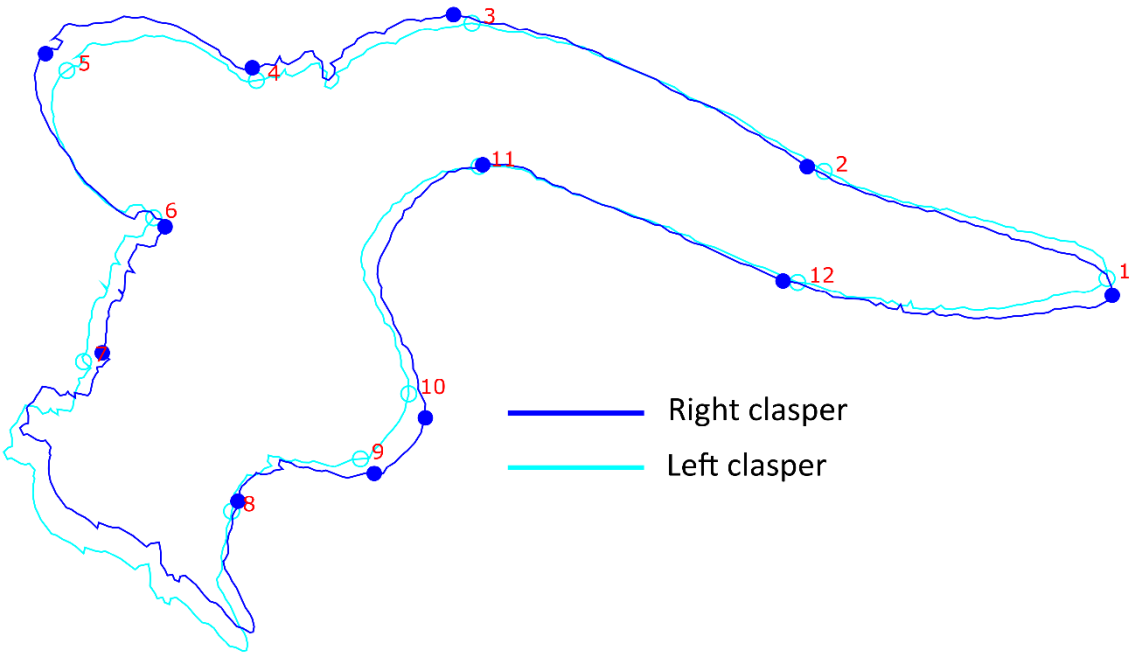

Figure 7.7. Average shape (Procrustes fit) of left and right claspers, for in both the no-choice and choice experiment (*N*= 152 left and 151 right).

(r_{149}= 0.34, Adjusted *P*< 0.001).This component explains 8.94% of variance in right clasper shape. There was no significant correlation for the other four principal components tested (**Table S43**).

7.3.3 Clasper morphology and mating success

Of the 80 males tested in a no-choice test, 42 (52.5%) mated after two hours. Of the 132 males tested in choice tests, 43 (32.6%) mated after two hours. Male mating success in no-choice tests was not significantly influenced by male body length (Binary logistic GLM, quadratic effect of body length; χ^2_1= 3.22, *P*= 0.073), processus length (χ^2_1= 0.91, *P*= 0.34; **Figure 7.8a**) or mean clasper length (χ^2_1= 0.91, *P*= 0.34). Male mating success in female-choice trials was not significantly influenced by mean clasper length (*z*= -0.68, *P*= 0.5) or male body length (*z*= 0.17, *P*= 0.87), but was significantly influenced by male processus length (Generalised linear mixed-model; *z*= 2.81, *P*= 0.005). There is a positive linear relationship between processus length and the likelihood of a male mating in female-choice trials (**Figure 7.8b**).

Discriminant function analysis detected a significant difference in left clasper shape between mated and unmated males in no-choice trials (**Table 7.2**). Comparison of the average shape between mated and unmated males suggests that the shape difference is primarily in the tooth region: mated males have a tooth section that is both straighter and thinner than in unmated males (**Figure 7.9**). However there was no significant pre-copulatory selection on left clasper shape in female-choice trials, or on right clasper shape in either choice design (**Table 7.2**).

Males mated a median of 2 times (IQR= 1-3) over ten hours in no-choice trials. For those males that were seen mating at least once, mating frequency was significantly associated with the quadratic term for body length (Generalised linear model; χ^2_1= 4.23, *P*= 0.039), but not with processus length (χ^2_1= 0.25, *P*= 0.61) or linear or quadratic mean clasper length (Linear: χ^2_1= 3.33, *P*= 0.68, Quadratic: χ^2_1= 3.35, *P*= 0.067).

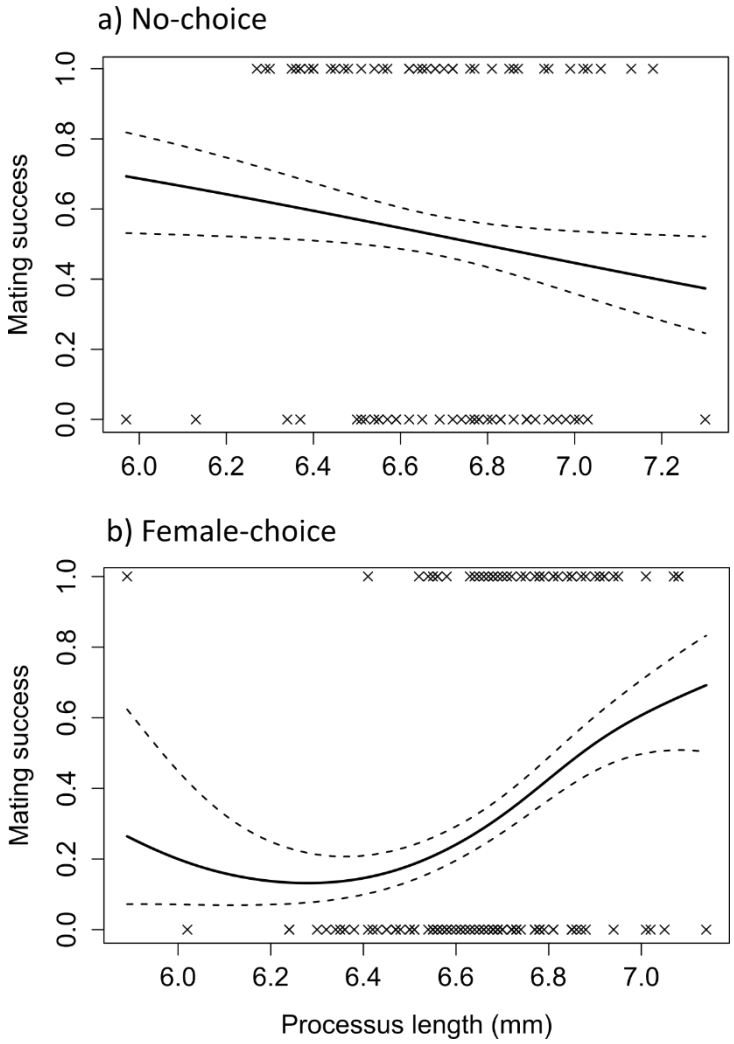

Figure 7.8. The relationship between male mating success and processus length in *L. simulans* using: **a)** a no-choice design (*N*= 80), and **b)** a choice design (*N*= 131).

Table 7.2. Results of discriminant function analyses comparing the difference in clasper shape between mated and unmated males for both choice designs.

Choice design	Clasper	*N* Mated	*N* Unmated	Procrustes distance	Mahalanobis distance	t^2	*P*
No-choice	Left	40	37	0.019	1.67	53.84	0.02
	Right	40	36	0.015	1.38	36.26	0.19
Choice	Left	36	37	0.009	0.94	16.15	0.9
	Right	38	39	0.008	0.67	8.58	0.99

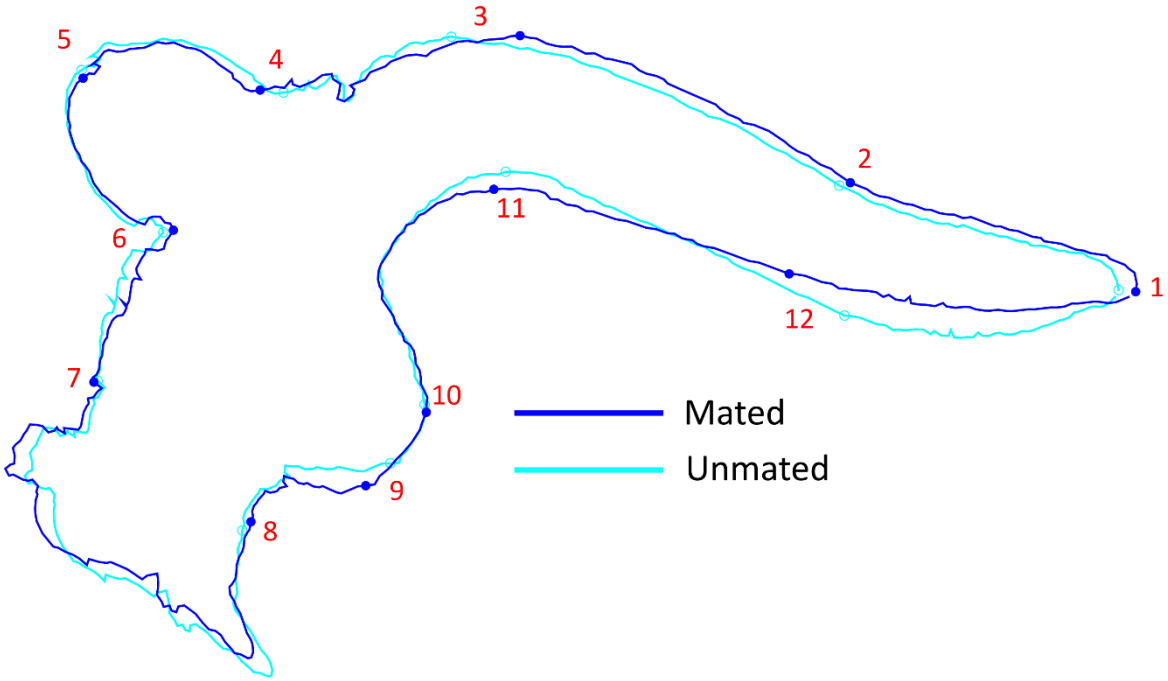

Figure 7.9. Average shape of left claspers for mated and unmated males in the no-choice experiment (*N*= 77).

7.3.4 Clasper morphology and copulation duration

Median copulation duration for males that mated after two hours was 495 (IQR= 406-547 minutes). Total time spent in copula over ten hours was not significantly influenced by male body length (χ^2_1= 1.1, *P*= 0.3), processus length (χ^2_1= 2.57, *P*= 0.11) or mean clasper length (χ^2_1= 1.13, *P*= 0.29). Additionally, discriminant function analysis did not detect any significant differences in (left or right) clasper shape between males that copulated for less than 300 minutes and males that copulated for more than 300 minutes (**Table 7.3**).

Table 7.3. Results of discriminant function analysis comparing the difference in clasper shape between short and long copulations for mated males in the no-choice experiment.

Choice design	Clasper	*N* Long	*N* Short	Procrustes distance	Mahalanobis distance	t^2	*P*
No-choice	Left	36	26	0.014	1.42	30.14	0.46
	Right	36	25	0.01	1.25	23.16	0.71

7.4 Discussion

In this chapter I investigated selection acting on clasper size and shape in *L. simulans*. I first confirmed experimentally that male genital claspers are necessary for mating to be achieved. I then show that left (but not right) clasper shape is subject to significant pre-copulatory sexual selection, albeit only in no-choice trials. In female-choice mating trials I detected significant pre-copulatory selection on male processus length, but in the opposite direction to that found for *L. equestris* (Chapter 5). Male processus length is significantly correlated with one aspect of clasper shape, but not with clasper size. However I was unable to detect any pre-copulatory selection on clasper morphology in a female-choice context. Therefore selection on clasper morphology does not appear to be able to explain the significant pre-copulatory selection on processus length detected when rival males are present during mating trials. There was no significant difference in clasper size or shape between males that mated for less than or greater than 300 minutes. This suggests that once intromission has been achieved, and the claspers are locked in place, clasper shape is not important for maintaining intromission. This suggests that copulation duration may be primarily under behavioural control.

The ablation experiment shows clearly that males are unable to mate when both claspers have been removed. By recording the number of male mating attempts I have shown that manipulated males still attempt to mate with females, even after multiple

failed attempts. The fact that mating is not possible for these males means that they attempt to mate significantly more times than males with both claspers intact (**Figure 7.4b**). This also strongly suggests that the difference in mating success between males with and without claspers seen here is not an unwanted side-effect of manipulation, such as reduced sexual motivation due to injury.

However, though such ablation experiments can tell us that claspers are required for mating, they do not allow us to rule out potential additional clasper functions. For example, Moreno-García & Cordero (2008) list seven possible functions of male claspers in insects, which in most cases are not mutually exclusive. One problem with these studies is that in many cases it is very hard to separate out specific functions of genital structures, for example by distinguishing between whether a trait functions to overcome resistance prior to mating vs extending copulation duration or maintaining intromission. For example, Arnqvist & Rowe (2005) give an example of the water strider *Rheumatobates rileyi*, in which males possess modified antennae which grasp the female during copulatory struggles, but let go as soon as genital insertion is complete (Westlake *et al.*, 2000). Therefore this structure obviously functions only to prevent female resistance prior to copulation, and so influences pre-copulatory success, but is not able to influence post-copulatory success. This distinction may be difficult to make for genital structures which may be in contact with the female for the entire duration of copulation.

I found evidence for significant sexual selection acting on clasper shape: there was a small but significant difference in left clasper shape between mated and unmated males in no-choice trials. Mated males have a tooth section that is both straighter and thinner than in unmated males. This may allow a closer fit between the left clasper and the female external genitalia. One reason why left clasper shape influences male mating success whereas right clasper shape doesn't could be if the left clasper more closely contacts the female when the two claspers come together. This would be more likely if it is the fit between the underside of the clasper and the male genital region, as

it can be seen in Figure 6.3 that (at least for the mating pair used to for micro-CT scanning) the underside of the right clasper is not in contact with the female, but instead rests on the upper surface of the right clasper. However at present the female reproductive anatomy has not been studied in detail, so that the exact way in which the male claspers interact with the female prior to intromission remains unclear. The actual sequence of events leading to mating is typically difficult to observe in insect species, though detailed mechanical studies of genital interactions have been performed in some species (e.g. Eberhard & Ramirez, 2004; Jagadeeshan & Singh, 2006).

The fact that this difference was detected in left claspers but not right probably reflects the relatively large size and shape differences between the two. Length measurements show that right claspers are significantly bigger than left, and that asymmetry is directional, with almost all males being 'right handed', though the degree of asymmetry is small compared to many insect orders (e.g. Huber *et al.*, 2007; Holwell, 2010). Micro-CT shows that the right clasper actually folds underneath the left during mating (**Figure 5.2a**), and so may need to be fractionally larger to ensure correct placement.

A main aim of this chapter was to investigate whether pre-copulatory selection on processus length could be explained by correlated selection on male clasper morphology. However I found little evidence for this hypothesis. First, there was no obvious correlation between processus length and clasper size or shape. Processus length did correlate significantly with one aspect of clasper shape (the 3rd principal component of left clasper shape). However, I could detect no significant sexual selection on clasper morphology in female-choice mating trials. I thus remain unable to explain the pre-copulatory selection on processus length in *L. simulans* (see Chapter 8 for discussion of pre-copulatory selection in both species).

Sexual selection on clasper shape has been shown to be influenced by social context, but the pattern is the opposite of that seen for processus length. This firstly suggests that the morphological traits measured are not important in male-male competition, as if this were true then selection should be stronger in the female-choice condition. Why might there be stronger selection on clasper shape when no rival males are present? One potential explanation could be if copulatory struggles are more important in no-choice tests. This could arise if females are more likely to encounter an acceptable mate in choice tests, and so show less resistance to mating, whereas in no-choice tests females may be more likely to receive mating attempts from non-preferred males, in which case claspers may be more important for initiating coercive copulation.

One limitation of this study was that I was unable to accurately describe clasper shape variation in three dimensions. For example, the degree to which the tooth of each clasper is hooked could influence male mating success (see **Figure 7.2**). Although three-dimensional shape data of such small structures can be obtained using high-resolution scanning technology such as micro-CT (See Chapter 6), performing such scans on enough samples to be able to detect intraspecific variation is currently very costly in terms of both time and resources. However, at least some of this three-dimensional variation should have been captured in the two-dimensional shape measurements, especially given the large sample sizes used.

It remains unclear whether the differences in clasper morphology between *L. equestris* and *L. simulans* are due to differences in the strength or shape of sexual selection, or whether other selective processes are important. The clearest difference in clasper morphology between the two species is in the shape of the notch on the distal portion of the clasper. The shape of this notch in *L. simulans* was not apparent in this experiment, as a consequence of choosing a repeatable method of positioning each clasper before imaging. It is also important to note that these notches do not actually contact the female during mating, as only the distal 'tooth' extends outside of the

genital capsule (**Figure 7.1b**). The muscles used to control clasper movement appear to attach to the basal surface of the clasper below the notch, so that variation in the shape of the base could possibly reflect variation in the position or size of the attachment muscles used to move the claspers and hold them in place during mating. These muscles could be important for example if females are more resistant to mating in one of the species. In contrast, the notches do not seem to be muscle attachment points (*pers. obs*.). It is thus still unclear whether the shape of this notch has any functional significance.

7.5 Conclusion

I have shown firstly that male genital claspers appear to be necessary for intromission, so that their primary function is to facilitate normal mating. The ablation experiments presented in this chapter differ from those in Chapter 6 in that the normal function of the claspers is completely impaired following cutting, which was not the case for the processus manipulations. Such an approach used in isolation tells us little about how selection acts on clasper morphology (Simmons, 2014). However, combining both functional ablation studies with analyses of natural phenotypic variation overcomes the limitations of both approaches. Such analysis suggests that some aspects of clasper shape may influence the likelihood of males achieving mating. Once mating is achieved, clasper morphology does not seem to influence the duration of copulation. Studies that consider the functional morphology of genital claspers, and that quantify selection on clasper size and shape, are currently lacking. I suggest that these structures should not be overlooked, especially since they have the potential to influence both pre- and post-copulatory male reproductive success. Finally, selection on clasper morphology does not seem to account for the significant pre-copulatory selection on processus length in this species.

Chapter 8

Discussion

In this chapter I provide an overall discussion of the work presented in this thesis. I first present a brief overview of the main results from each chapter, and then consider how these results may contribute generally to the field of sexual selection. I then consider a question that has arisen during my work: should the post-copulatory selection measured in my experiments be considered sexual selection if females were only mated once? Finally, I consider the reproductive biology of *L. equestris* and *L. simulans* again in light of my results, and highlight key unanswered questions that require future study.

8.1 Results overview

I consider this thesis to have two main themes. The first is the functional morphology and evolution of male genitalia, focusing on two genital traits in the seed bugs *Lygaeus equestris* and *Lygaeus simulans*: processus length and clasper size and shape. I used experimental manipulation to test the function of both traits, and performed experiments in which I assess the strength of pre- and post-copulatory sexual selection acting on both traits. The second main theme is the effect of the social environment on the strength of pre-copulatory sexual selection and mating preferences. Specifically I performed several experiments in which I varied the number of mate options presented to males and females. I have considered how different choice designs affect the strength of sexual selection on overall body size, processus length and clasper size and shape in *L. equestris* and *L. simulans*. I also used meta-analysis to investigate the influence of choice design on the strength of mating preferences across 38 published studies. I will briefly recap the main results from my five data chapters in turn.

In Chapter 3 I investigated the strength of pre- and post-copulatory sexual selection on male and female body size in *L. equestris*, whilst varying the experimental choice design. I detected significant positive, linear pre-copulatory selection on female body length and stabilizing pre-copulatory selection on male overall size. Female fertility was significantly associated with copulation duration and female size, but not with male size. However, I found no significant effect of choice design on the patterns of sexual selection for males or females. I suggest that the method of mate assessment in *L. equestris* may preclude simultaneous comparison of mates.

In order to assess whether there is a general relationship between choice design and the strength of mating preference, I next performed a phylogenetically-controlled meta-analysis of published studies performing mate choice experiments using both a no-choice and a choice design, which I present in Chapter 4. I used only those studies for which the same preferences (the same species, sex and trait) were tested using

both designs. I found that, across 38 studies, mating preferences were significantly stronger when tested using a choice design. However, this effect was only seen in studies that tested for female, intraspecific preferences. I suggest that this difference may be due to the increased potential cost of rejecting a mate in no-choice tests.

In Chapter 5 I investigated sexual selection acting on male processus length in both *L. equestris* and *L. simulans*. I detected negative linear pre-copulatory selection on processus length in the *L. equestris* males used in Chapter 3. However, this effect was only seen in mating trials for which two males were present (in which male-male competition and/or female choice were possible). I suggest that this pre-copulatory selection arises via selection on a trait correlated with processus length. I also detected significant stabilising post-copulatory selection on male processus length. I then performed a similar experiment in *L. simulans*, but was unable to detect significant pre- or post-copulatory selection on processus length. However, by combining both published estimates of selection with results from Chapters 5 and 6, I show that there is significant stabilising post-copulatory selection acting on processus length in *L. simulans* across studies.

I then presented a series of experiments concerning the functional morphology of the male processus in *L. simulans*. I first used micro-CT scans of flash-frozen mating pairs to visualise the interaction between male and female genitalia inside the female during mating. Reconstructions clearly showed that a large proportion of the processus remains in the female bursa during mating. I then showed that the processus may break naturally if males are given many chances to mate during their lifetime. Finally, I then experimentally manipulated male processus length in order to directly confirm that processus length influences sperm transfer in *L. simulans*. Male fertilisation ability was reduced in relation to the proportion of processus removed. Importantly, I was able to show that this reduction was not due to the experimental manipulation itself.

Finally, in Chapter 7 I presented a series of experiments investigating pre-copulatory sexual selection on male genital clasper size and shape in *L. simulans*. I used geometric morphometrics to characterise clasper shape in two dimensions. I first showed by experimental ablation that male claspers are necessary for mating to be achieved. I then showed that there is consistent directional asymmetry between left and right claspers, with right claspers being significantly longer and less curved. I then present evidence for significant sexual selection on left clasper shape, but only in no-choice mating trials. However, clasper morphology did not significantly influence copulation duration. Finally, I also detected significant positive pre-copulatory selection on processus length in choice tests, in contrast to the negative selection seen in Chapter 5 for *L. equestris*. Sexual selection on clasper morphology is therefore unable to explain the pre-copulatory selection on processus length seen in both species.

8.2 General discussion

My hope is that the results presented here, as well as giving specific insights into the reproductive biology of *L. equestris* and *L. simulans*, may also contribute more generally to our knowledge of sexual selection. In this section I consider how my results give important insights into three key processes: the influence of the social environment on the strength and patterns of sexual selection, the effect of experimental design on the measurement of mate choice, and the evolution of animal genitalia.

8.2.1 Sexual selection and the social environment

A recurring result from these studies is that the strength and pattern of sexual selection acting on a given phenotypic trait may be highly context dependent. In this case I focus on the social environment; with Chapters 3, 4, 5 & 7 all considering how the number of males and females present during an experiment influences the

strength of sexual selection and mating preferences. This is especially pronounced when considering pre-copulatory selection on male processus length, with strong linear selection detected following choice trials in which two males were present, but no significant selection detected when only a single male was present (Chapters 5 & 7). I also found significant pre-copulatory selection on left clasper shape in no-choice trials but not in choice trials (Chapter 7). In other cases social environment had no effect on the strength of selection (Chapter 3).

More generally, a history of studies of natural selection in the wild has shown that the strength and direction of selection can vary strongly both spatially and temporally (Endler, 1986; Siepelski *et al.*, 2010; 2013; Morrissey & Hadfield, 2011; Sæther & Engen, 2015). This may be caused by variation in both biotic and abiotic factors (Endler, 1986; Kingsolver *et al.*, 2012). However only relatively recently have these phenomena begun to be investigated for sexually selected traits (e.g. Cornwallis & Uller, 2009; Ingleby *et al.*, 2010; Candolin & Vlieger, 2013; Apakupakul & Rubenstein, 2015). These insights naturally require selection to be measured in wild populations across multiple locations and times. Such studies tend to consider sexually-selected traits less commonly than naturally-selected traits (Siepelski *et al.*, 2010; 2013), despite the fact that sexual selection may be even more prone to fluctuations in strength than natural selection, given that the social environment may play such a strong role in the former (Siepelski *et al.*, 2010; Miller & Svensson, 2014). For example, empirical studies have shown that the strength and direction of sexual selection can vary due to social factors such as the presence or absence of members of the opposite sex (Hall *et al.*, 2008; Kasumovic & Andrade, 2008; Procter *et al.*, 2012), or population density (Conner, 1989; McLain, 1992; Arnqvist, 1992b; Blanckenhorn *et al.*, 2000; Gosden & Svensson, 2008; Punzalan *et al.*, 2010). Additionally, sexual selection may also be influenced by environmental factors such as climate (Robinson *et al.*, 2012), resource quality (Gillespie *et al.*, 2014) and temperature (Moya-Laraño *et al.*, 2007).

Though studies have begun to characterise changes in the strength of sexual selection over spatial and temporal scales, the general problem remains that fluctuations in the strength and direction of sexual selection may be larger than we think in natural populations. This is another reason why the patterns of sexual selection detected under experimental conditions, either in the lab or in the field, may not accurately reflect selection in the natural environment. Specifically, by taking 'snapshots' of selection in time and space we may be consistently overestimating its strength, as variation in selection is not detected (Cornwallis & Uller, 2009). This may have consequences for the interpretation of theoretical models of sexual selection, as most do not take into account the fact that selection may not be consistent (Bussiere et al, 2008; Cornwallis & Uller, 2009; Miller & Svensson, 2014; Sæther & Engen, 2015).

In response to such problems one may become overwhelmed by the daunting task of detecting any consistent selection on traits that may be subject to strongly fluctuating selection from multiple sources in natural populations. However such research is not hopeless. What is certainly needed though is more studies that obtain multiple measurements of selection in *ecologically relevant* scenarios, and preferably in natural populations (Cornwallis & Uller, 2009). Studies should also measure selection arising from multiple sources when possible. Clearly to fully understand the evolution of even relatively simple sexually-selected traits (such as the processus in *Lygaeus*), will require long-term studies of selection in the wild, across multiple environmental contexts.

8.2.2 Experimental design and mate choice

The results of the meta-analysis presented in Chapter 4 clearly show that the number of options a subject is presented with can have a large effect on the measurement of mate choice. As well as leading to variation in the strength of selection in the wild, this effect will also be important when considering how best to design choice experiments. Meta-analysis is well suited to testing the effects of such confounding factors. In fact, experimental design is commonly shown to have a significant effect on the results of

meta-analyses in ecology and evolution (e.g. South & Lewis, 2011; Santos *et al.*, 2011; Kelly & Jennions, 2011; Kamiya *et al.*, 2014; Arct *et al.*, 2015).

One way that this difference could be reduced is in the adoption of standardised experimental protocols. So why isn't experimental design more consistent in behavioural ecology? One key factor is that researchers in this field endeavour to find general processes that act across species. This requires testing the same hypotheses in different species, each of which brings their own technical and logistical problems that need to be solved. Nevertheless, in the absence of standardised experimental protocols, analyses such as this that quantify the effect of different experimental factors across many studies are very valuable. I suggest that such an approach would be very useful to determine large-scale variation in the strength of mating preferences in relation to multiple factors. For example, my analysis probably contained too few studies to be able to detect significant differences in mating preferences due to sex or trait type as predicted by theory (see Chapter 4). However the mate choice literature is very large and provides ample opportunity to test such questions.

Overall my analysis shows that mating preferences are significantly stronger when tested using a choice design compared to a no-choice design. Furthermore, this difference is consistent across a very wide range of species and traits. Though my study is unable to determine the reason for this difference, I suggest that the difference in the 'cost of rejection' between designs plays a major role. This arises primarily due to the difference in perceived mate encounter rate between the two choice designs (Werner & Lotem, 2006; Barry & Kokko, 2010; Booksmythe *et al.*, 2011). This gives clear predictions that may be tested using a suitable study system. For example, if we can control for perceived mate encounter rate, perhaps by giving subjects experience of the same number of mates before choice tests (Barrett *et al.*, 2014), then I predict that choice should be high in both choice designs in high mate encounter conditions, and that choice in no-choice tests should be more strongly affected by varying the mate encounter rate (**Figure 8.1a**). However, a recent study in the wolf spider *Schizocosa*

ocreata found an unexpected result: mate encounter rate significantly influenced female preference for male leg tufts in choice tests, but not in no-choice tests (Stoffer & Uetz, 2015). Further studies are needed to tests whether this is a general effect.

Additionally, varying the cost of rejection in other ways, for example by varying the sampling costs, should influence the strength of preference observed in no-choice tests (as is seen for example in sequential choice experiments: e.g. Milinski and Bakker 1992) but should have no effect on the strength of preference in choice tests (**Figure 8.1b**). Finally, I also predict that the difference in the strength of preference between designs should decrease as the costs of mating and/or reproduction increase (for example in species in which females are harmed during mating, or in which females invest heavily in offspring; Halliday, 1983): if this cost is sufficiently high it will outweigh the cost of rejection and so subjects should remain choosy even in the no-choice situation (**Figure 8.1c**).

The large difference between choice designs found here makes it all the more surprising that such an effect has seemingly been ignored in the past. Though several authors have previously suggested that the two designs may give different results (e.g. Wagner, 1998), results from either design tend to be treated as equivalent. This study shows clearly that results from studies or experiments testing mating preferences or mate choice outcomes using different choice designs are not directly comparable, and may even give conflicting results. The same may also be true for other aspects of experimental design not considered here. At the very least I suggest that authors should explicitly indicate which choice design was used when referring to previous studies.

These results also show that mating preferences are highly context dependent (see section 8.4.1 above). The two choice designs can be seen to represent the different patterns of mate encounter in natural populations, and thus we can predict that the

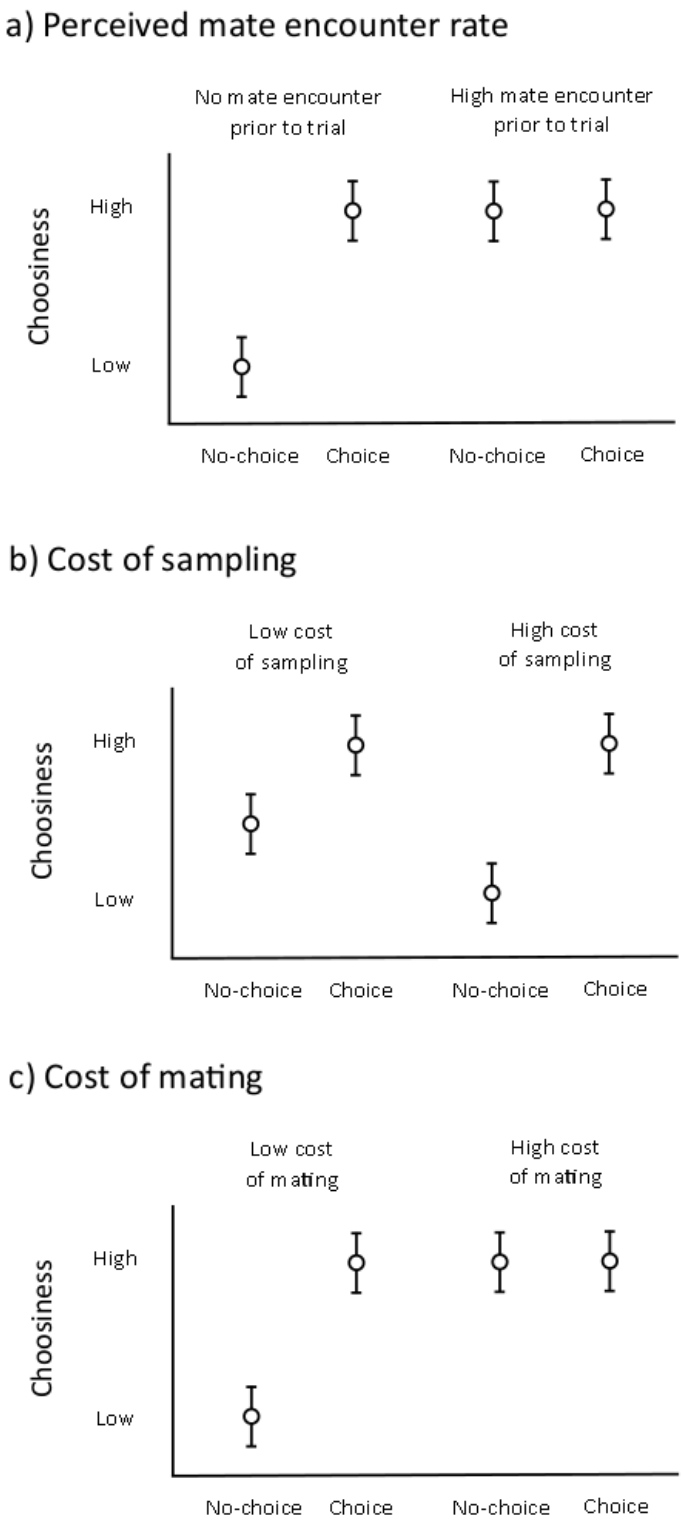

Figure 8.1. Predictions arising from the cost of rejection hypothesis concerning the differences in choosiness between subjects tested using no-choice and choice experiments. Specifically, predicted differences following changes in **a)** the perceived mate encounter rate, **b)** the cost of mate sampling, and **c)** the cost of mating.

strength of mating preferences, and sexual selection arising from these preferences, may vary in the wild. Finally, and perhaps most importantly, this means that if a given experimental choice design is not ecologically realistic (in that it doesn't represent the pattern of mate encounter in the wild for a given species), then we may be significantly under- or overestimating the strength of selection in natural populations. The extent of this problem is unclear. I predict that mating preferences in many species are much more likely to be overestimated than underestimated in lab experiments, due to a combination of preferences being most commonly tested using a choice design (e.g. see Owen *et al.*, 2012), and sequential assessment of mates being the predominant mechanism of choice in many animal species, even for those in which simultaneous assessment is possible (e.g. Gibson, 1996). However I think we currently have insufficient data on the patterns of mate encounter in the wild to be able to test this idea.

8.2.3 The evolution of genitalia

My survey of the literature considering the evolution of elongate genitalia reveals that there have been few empirical studies of either the functional morphology of these traits, or the selection acting on such traits in current populations. In many cases selection acting on elongate genitalia may seem obvious, but nevertheless explicit tests are needed to confirm that our assumptions are correct. For example, despite its extreme length, post-copulatory selection on the processus in *L. equestris* and *L. simulans* is strongly stabilising, so that 'bigger is not always better'. This is probably very common for male genital traits, which need to be stored internally before mating, and then everted and fitted into the female tract during mating. Any linear, open-ended selection (for example arising through sperm competition) will likely be quickly counter-balanced by selection for maintaining normal function (Eberhard *et al.*, 1998; Bonduriansky, 2007). This is clearly less of a problem for secondary sexual traits which have no other functions, such as ornaments or weapons (Andersson, 1994).

I have also shown how male genital traits may be subject to selection from multiple sources. For example, in addition to the post-copulatory selection already described (Tadler, 2000), I also detected significant pre-copulatory sexual selection on male processus length in *L. equestris* and *L. simulans* (Chapters 5 and 7). Few studies present estimates for the strength of pre-copulatory sexual selection on intromittent organs that are stored internally before mating (but see Simmons *et al.*, 2009; Xu & Wang, 2010). This is perhaps unsurprising given that these traits do not interact with females prior to copulation and so are not predicted to influence mating success directly. However I suggest that indirect selection on such traits may be more common than currently realised. It must also be noted that although pre-copulatory selection on processus length was relatively strong in *L. equestris*, stabilising post-copulatory selection was much stronger, leading to stabilising selection overall. Studies measuring selection on genital traits from multiple sources are still lacking (see Chapter 5), as are studies conducted in natural populations (e.g. Arnqvist, 1989; 1992; Koshio *et al.*, 2007).

In the face of such complex patterns of selection the need to understand the functional morphology of genital traits in more detail is as great as ever (Jagadeeshan & Singh, 2006; Werner & Simmons, 2008b; Simmons, 2014). Such studies will require the application of complementary experimental techniques, which will require interdisciplinary research combining behavioural ecology, molecular biology, anatomy, physiology and biochemistry (Birkhead, 2010). In Chapter 6 I show how two techniques: experimental manipulation and micro-CT scanning, can give important insights into the functional morphology of genital traits. I suggest that the potential insights that could be gained from micro-CT in particular have been underappreciated, especially given the ability to record the interactions between male and female genitalia during mating in high detail.

On the other hand, experimental ablation studies such as those presented in Chapters 6 & 7 will likely not work for most genital traits, as the negative effects due to injury

are usually too great (Arnqvist & Rowe, 2005). However, it is possible to manipulate tough, non-vascularised structures without harming the individual (Moreno-García & Cordero, 2008; Briceño & Eberhard, 2009; Hotzy *et al.*, 2012; Grieshop & Polak, 2012; Chapter 7). Additionally, it may be possible to investigate functional morphology using other types of manipulation that do not damage males or females, for example by experimentally lengthening genital traits (Arnqvist & Rowe, 1995), blocking male or female sensory structures (Eberhard, 1996; Briceño & Eberhard, 2009; Aisenberg *et al*., 2015) or allowing populations to evolve experimentally over several generations (Hotzy *et al.*, 2012). I suggest that these methods are still underutilised, and this is especially so for the experimental evolution approach. This is illustrated by the fact that studies employing experimental evolution of genital traits have begun to be published only in the last six years (Simmons *et al.*, 2009; Cayetano *et al.*, 2011; Hotzy *et al.*, 2012; Simmons & Firman, 2013).

Finally, I conclude by highlighting the common problem that female genitalia in general are still studied much less than male genitalia (Simmons, 2014; Arnqvist, 2014). This has been suggested to arise due to the cultural biases of researchers in the field (Ah-King *et al.*, 2014). However, though such biases have undoubtedly influenced the development of the field historically (Birkhead, 2010), I suggest that this effect is more strongly driven by the difficulties of describing internal compared to external reproductive traits (Eberhard, 1985). Nevertheless, this remains a significant problem for the study of post-copulatory sexual selection. How can we hope to understand the evolution of male traits that function inside the female reproductive tract during mating, without studying the associated female anatomy?

8.3 Can there be post-copulatory sexual selection if a female only mates once?

In this section I address a question that I have considered during my PhD: is the post-copulatory selection observed in my experiments sexual selection if females only mate once? Influential definitions of post-copulatory sexual selection tend to rule this out. Here I suggest that such definitions are overly strict, and try to give examples of cases were sexual selection is clearly happening even when females mate once.

8.3.1 What is post-copulatory sexual selection?

Post-copulatory sexual selection (hereafter referred to as PCSS for short) arises via traits that are expressed during and after mating that increase the likelihood of an individual gaining fertilisations, relative to other members of the same sex. It can be seen as the combined selection arising from sperm competition and cryptic female choice (Birkhead & Pizzari, 2002; Pitnick & Hosken, 2010; see Chapter 1). However, as with many concepts in the field of behavioural ecology, the exact definitions of these two processes (sperm competition and cryptic female choice), and hence post-copulatory sexual selection overall, vary slightly according to different authors. Critically, many definitions of PCSS suggest that it arises due to, or is a consequence of, multiple mating by females (Birkhead & Pizzari, 2002). This has been stated in various ways in the literature (**Table 8.1**). This has led to the view that PCSS can only be measured by performing experiments in which females are mated to different males in succession, so that male ejaculates compete simultaneously within the female tract (e.g. Parker & Birkhead, 2013). This emphasis on competing ejaculates may result from an historic focus on the evolution of sperm traits when considering post-copulatory processes.

In this thesis I have presented the results of several experiments in which females were mated to a single male (Chapters 5-7), and selection on male genital morphology was inferred by correlating male fitness with the target trait. Under the traditional definition described above, the post-copulatory selection that I detect

Table 8.1. Descriptions of post-copulatory sexual selection (or cryptic female choice) in the literature for which multiple mating by females is deemed necessary.

Source	Quote
Eberhard (1996)	"*Sexual selection by cryptic female choice can result from a female-controlled process or structure that selectively favors paternity by conspecific males with a particular trait over that of others that lack the trait* ***when the female has copulated with both types***"
Birkhead & Pizzari, (2002)	"*Female promiscuity, or* ***polyandry, has important biological implications****: it means that sexual selection persists after copulation…"* *"Post-copulatory sexual selection,* ***arising from sexual promiscuity****…*"
Garcia-Gonzalez, (2008)	"***Polyandry enables*** *intra- and inter-sexual selection to continue after mating in the form of sperm competition and/or cryptic female choice*"
Pitnick & Hosken, (2010)	*"****Female remating*** *(or multiple males participating in a spawn)* ***is an obvious prerequisite*** *for sperm competition and cryptic female choice to occur*"
Birkhead, (2010)	"*Thornhill (1983) referred to this process as cryptic female choice – cryptic because it took place out of sight inside the female's body – and proposed that under certain circumstances, it might pay females* ***that had been inseminated by more than one male*** *to discriminate between their sperm*"
Parker & Birkhead, (2013)	" *Klug et al.* ***only considered cases where females mate once*** *(i.e. they* ***excluded post-copulatory sexual selection****)*"
Kvarnemo & Simmons, (2013)	"*Sperm competition…and cryptic female choice…are probably the most widely appreciated* ***consequences of polyandry*** *for sexual selection acting on males*"

(whether significant or not) cannot be considered a form of sexual selection, as females were only allowed a single mating in all cases, and so this would presumably be considered a form of natural selection instead. Below I discuss why I disagree with this interpretation. I suggest here that this is a misinterpretation of sexual selection theory, and that such definitions of PCSS are overly restrictive. My view is that in many cases PCSS, and more specifically cryptic female choice, may occur regardless of how many times females mate, and consequently regardless of the experimental design used to test for such selection. Below I give several examples of situations in which

PCSS could theoretically occur when a female mates once. I then consider more broadly the relationship between multiple mating and PCSS.

8.3.2 Cryptic female choice in the absence of simultaneously competing ejaculates

First, in many species females may actively control whether or not to allow insemination (e.g. Pizzari & Birkhead, 2000; Tallamy *et al.*, 2002; Bretman *et al.*, 2009). This is a clear example of cryptic female choice which by definition does not require sperm from two males to be present simultaneously in the female reproductive tract. For example, in red jungle fowl *Gallus gallus*, females are able to eject sperm from males immediately following mating, and do so more often when mating with subordinate males for example (Pizzari & Birkhead, 2000). Such a process could also be occurring in *L. equestris* and *L. simulans* if females have active control over the valve at the entrance to the spermatheca (Gschwenter & Tadler, 2000). So why should female control of insemination, when seen in a single-mating context, not be considered sexual selection? In the same way that the decision to mate or not is a form of choice (Kokko & Mappes, 2012; Edward, 2015; see Chapter 4), so too is the decision to allow insemination during or after mating.

Second, a distinction needs to be made between selective mechanisms that do require male ejaculates to be competing directly and those that do not. For example, sperm competition may drive the evolution of male traits such as sperm removal organs that prevent male ejaculates from directly interacting (Simmons, 2001; Córdoba-Aguilar *et al.*, 2003). These adaptations do not increase male fertilisation success directly, but instead indirectly reduce the likelihood of losing out to other males in sperm competition. Therefore we should be unable to detect selection acting on these traits using a single-mating design.

However, selection that arises due to an interaction between males and females (i.e. cryptic female choice) is not restricted in this way (Eberhard 1996; Arnqvist, 2014). If some aspect of female anatomy or physiology selects for males with a given genital or sperm morphology (e.g. Eberhard, 1996; Rönn *et al.*, 2007; García-Gonzaléz & Simmons, 2007), then this selection will be the same regardless of whether she has mated once, or twice in succession. Therefore the strength and shape of selection on a given trait may still be detected using a single-mating design. In fact, a multiple-mating design may be an unsuitable method of testing for selection on some traits. For example, selection on male genital morphology may be masked by selection for other male sperm traits when we use such a design. Nevertheless, calling the same process sexual selection if we perform a multiple-mating experiment, and natural selection if we perform a single-mating experiment, is logically inconsistent if that process does not require ejaculates to interact directly. Additionally, females may also have active control over how sperm are utilised following insemination, and is not clear why a female needs to be inseminated by more than one male before she can make such a decision, for example as suggested by Eberhard (1996).

8.3.3 Post-copulatory sexual selection and monandry

The fact that PCSS can occur in the absence of simultaneously competing ejaculates means that such selection is possible even when females are monandrous. This could happen in several ways. First, if a female mates with a succession of males but only allows successful insemination to occur with one of them then she is technically monandrous. This could also occur if males manipulate females in some way so as to inhibit remating (Hosken *et al.*, 2009), for example by using mating plugs (Simmons, 2001; Uhl *et al.*, 2010) or seminal products (Simmons, 2001; Chapman, 2001; Avila *et al.*, 2011).

Second, the number of matings an individual achieves is a discrete number, and the environment can be unpredictable, therefore mating success is stochastic to some

extent (Jennions *et al.*, 2012b; Kokko & Mappes, 2012). Therefore even for a population in which females on average mate with more than one male, some females may be monandrous by chance (Kokko & Mappes, 2012). A given post-copulatory mechanism of selection may have evolved in a polyandrous population, but that doesn't stop it from being expressed in females that are monandrous.

Finally, sexual selection can still act in strictly monandrous species if there is competition for access to high quality mates (Kirkpatrick *et al.*, 1990; Altmann, 1997; Kraaijeveld *et al.*, 2007; Fitzpatrick, 2015). This process can also be extended to PCSS, if post-copulatory choice leads to the utilisation of sperm from a single male. For example, females may only allow insemination by males that perform copulatory courtship (Eberhard, 1996; Edvardsson & Arnqvist, 2000; Tallamy *et al.*, 2002), or possess genitalia that stimulate the female (Eberhard, 1996; Briceño & Eberhard, 2009). Additionally, male traits that manipulate female fecundity following mating will also be sexually selected in this way (e.g. Simmons, 2001; Chapman, 2001; Avila *et al.*, 2011).

In summary, it is clear that the presence of multiple mating by females is not a prerequisite for PCSS to occur. Polyandry may make PCSS on some traits (e.g. sperm swimming speed) much stronger, but such selection may also occur to some extent in monandry as well.

8.3.4 Future definitions

This discussion is in some senses entirely a semantic issue: selection arises through many different processes, but all use the same overall currency- inclusive fitness (Shuker, 2010). Sexual selection can be seen as a subset of natural selection in the broad sense, so that the distinction between natural and sexual selection is therefore a dichotomy imposed by researchers in order to help us think about the process of selection. However, such a theoretical distinction has been shown to be incredibly

useful over the past century of research in behavioural ecology (Andersson, 1994; Davies *et al.*, 2012). Furthermore, there has been much work in the past few decades to generate a strong theory of sexual selection that is logically consistent and unbiased (Shuker, 2010; Kokko *et al.*, 2006; 2012; Ah-king & Ahnesjö, 2013). To this end, pre- and post-copulatory sexual selection should also be logically compatible with each other as well. I hope I have shown here that discounting PCSS when a female mates once is an error. To this end I suggest that the definition of PCSS in general, and cryptic female choice more specifically, need not explicitly require simultaneously competing ejaculates. At the very least, current definitions tend to be worded in a way that makes misinterpretation possible.

I consider the definition of cryptic female choice stated by Eberhard (1996; **Table 8.1**) to be overly restrictive. Specifically, the insistence that choice can only be shown to have occurred when a female mates with multiple males is too limiting, especially given that the same criteria is not needed to demonstrate pre-copulatory choice. In the same way that mate choice occurs both sequentially and simultaneously (Chapter 4), so can post-copulatory choice be said to occur when ejaculates are present sequentially or simultaneously. Furthermore, I suggest that female rejection of a male before, during or after copulation should all be considered mechanisms of mate choice. This fits with a definition of pre-copulatory mate choice which includes the decision to mate or not as a form of choice (Edward, 2015). If selection arises via cryptic female choice, then single-mating experiments are valid for quantifying selection. It is true that in many cases determining the selective mechanisms may be difficult. However, until we do PCSS cannot be ruled out as an explanatory factor following single-mating experiments.

8.4 Sexual selection in Lygaeus equestris and Lygaeus simulans revisited

In this section I discuss what my results can tell us about the reproductive biology of *L. equestris* and *L. simulans*. I also highlight what I consider to be key unanswered questions regarding these species (**Table 8.2**), and suggest future lines of research.

8.4.1 Pre-copulatory sexual selection

I first reconsider the extent to which pre-copulatory choice is important in both species. The results from Chapter 3 suggest that mating in both male and female *L. equestris* depends to some extent on body size. However body size only explains a small proportion of the variance in mating success for either sex. This is perhaps unsurprising given that sexual dimorphism is small. Behavioural observations suggest that pre-copulatory selection arises primarily via mating struggles between the sexes (Sillén-Tullberg, 1981). However, I was unable to detect selection on male or female leg lengths directly, even though the legs appear to be important during mating struggles. In Chapters 5 & 6 I also found evidence for linear pre-copulatory selection on male body length in *L. simulans*.

There was some evidence that male clasper shape influences male mating success in *L. simulans* (Chapter 7), and in both species I also detected significant pre-copulatory selection on male processus length in a competitive mating context (Chapters 5 & 7). Significantly, the pattern of pre-copulatory selection on processus length differed in the two species: being linear and negative for *L. equestris* and linear and positive for *L. simulans*. It is still unclear what is driving this selection, but in *L. simulans* I have shown that the selection is very likely indirect (Chapter 6). Interestingly, due to the differences in processus length between the two species males of both species with a processus length of around 7 mm appear to have the highest mating success, which is shorter than the mean length for *L. equestris* but longer than the mean length for *L. simulans*.

Table 8.2. Unanswered questions in the reproductive biology of *L. equestris* and *L. simulans*.

Pre-copulatory selection • Is there any active mate choice by females? • How are mates encountered in the wild? • What are the mechanisms driving the pre-copulatory selection on male processus length? • What mechanisms lead to reproductive isolation between the species? Post-copulatory selection • Do females have any control over copulation duration? • Can females actively prevent male insemination whilst in copula? • What factors influence the frequency of infertile matings? • What are the mechanisms driving the post-copulatory selection on male processus length? • Do females have specialised external structures that the male claspers fit into? • Is processus length related to female tract length across Lygaeid species? • What were the ancestral processes that drove the evolution of elongate genitalia in this clade?

One potentially important trait that I have not considered is the cuticular hydrocarbon (CHC) profile, which plays an important role in mate assessment in many insect species (Tregenza & Wedell, 1997; Chenoweth & Blows, 2005; Howard & Blomquist, 2005). Recent work has shown that CHC profiles are species- and sex-specific in several lygaeid species, including *L. equestris* and *L. simulans* (Burdfield-Steel, 2014). Additionally, disrupting these profiles appears to reduce the ability of *L. equestris* males to distinguish between females of *L. equestris* or *L. simulans* (Burdfield-Steel, 2014). Further work is needed to determine whether CHC profiles are used by either sex to assess mate quality and so influence choice. However if mate assessment is performed solely using contact cues such as CHCs, then this allows us to make predictions about the decision rules females (or males) may use in the wild. Specifically, I suggest that pre-copulatory choice is based on sequential assessment of

mates. This would mean that a no-choice design is valid when performing choice tests, despite the fact that a simultaneous choice scenario may be ecologically realistic. However note that the same may not apply for post-copulatory processes (Chapter 5).

It is probably unsurprising that males exhibit little pre-copulatory choice in this species, given that males are frequently observed attempting to mate with nymphs, other males, and even individuals of the wrong species (*pers. obs*.; Burdfield-Steel & Shuker, 2014). Indiscriminate mating is probably an adaptive strategy for males, if matings are relatively cheap. However, mating appears to be much more costly to females (Shuker *et al*., 2006). Given this, why is female pre-copulatory choice so weak? It may be simply that males are able to coerce matings without any female acceptance behaviour, so that females are unable to exercise pre-copulatory choice. In this situation the evolution of female post-copulatory choice mechanisms may be much more likely (Møller & Birkhead, 1998); this is discussed in the next section.

Alternatively, female choice seen in the lab may be weaker than that in natural populations. One reason for this could be the fact that all my choice experiments were performed using virgin females. Virgin females are predicted to be less choosy than mated females, in order to increase the chances of obtaining sufficient sperm to fertilize their eggs (Kokko & Mappes, 2005; Peretti & Carrera, 2005; Rhainds, 2010; **Table 1.1**). This may be especially important in *L. equestris*, where mating stimulates oogenesis, so that mated females produce more eggs than virgins (Kugelberg, 1973; Sillén-Tullberg, 1981). Alternatively, choosiness could be low regardless of mating status if population density in the wild is not as high as expected. For example, bug population dynamics are governed primarily by host plant distribution, which can vary greatly between years (Solbreck & Sillén-Tullberg, 1990). If mate encounter rate is unpredictable between years, then females may reduce their choosiness in order to ensure they achieve a mating.

In summary then, pre-copulatory sexual selection in *L. equestris* and *L. simulans* appears to be weak. This might make post-copulatory processes more important, especially for females (see below).

8.4.2 Post-copulatory sexual selection

In this section I first consider the patterns of post-copulatory selection measured in this thesis. I then consider two notable features of the reproductive biology *of L. equestris* and *L. simulans*: a high proportion of matings do not end in the production of fertile offspring (insemination failure), the function of long copulations, and the functional morphology of male genitalia.

Insemination success was significantly influenced by female size in both *L. equestris* and *L. simulans* (Chapters 3 & 5). This could be due to the fact that body size correlates with female fecundity in insects (Honěk, 1993), or because may allocate more or better quality sperm to large (high quality) females (Bonduriansky, 2001; Kelly & Jennions, 2011). In *L. simulans* insemination success was also influenced by male body length (Chapter 6) and processus length (Chapters 5, 6 & 7). The length of the processus in *L. simulans* also influenced the number of offspring produced following a fertile mating by in Chapter 6 (though not in Chapter 5). The mechanisms leading to stabilising post-copulatory selection on processus length also remain obscure. Importantly, it does not seem to be driven by a simple correspondence between the length of the processus and the length of the spermathecal duct. Instead, the female spermathecal duct is significantly shorter than the processus, so that a large proportion of the processus remains in the bursa during mating (Chapter 6). Perhaps the answer is that the spermathecal duct is not where we should be looking if we want to explain this selection. Instead, perhaps it is the complicated manoeuvres the male has to perform once the entire intromittent organ is in the female bursa which are most important. Functional studies of copulations as they progress (as in Micholitsch *et al*., 2000), but focusing on the female bursa instead of the spermathecal duct, may help to elucidate

common reasons why matings fail. Indeed such investigations might be possible when used in conjunction with micro-CT scanning (Chapter 6).

It is especially clear from my experiments that insemination failure is very common in both *L. equestris* and *L. simulans*, with as many as half of all matings failing to lead to the production of any fertile eggs (**Figure 8.2**). Though similar estimates of the frequency of failure have been reported in studies of post-copulatory selection in *L. simulans* (Tadler, 1999; Tadler *et al.*, 1999), such estimates are not reported at all in most cases (e.g. Sillén-Tullberg, 1981; Shuker *et al.*, 2006; Higgins, 2009). However as the presence of sperm in the spermatheca was not measured directly in my experiments it is unclear whether females did not produce fertile eggs because males failed to successfully transfer sperm, or whether sperm was present but not used for some reason. Therefore my measure of insemination success should be more correctly considered 'failure to produce offspring following mating'. Such failure could also conceivably arise due to the failure to fertilise eggs successfully, or early embryo mortality. However, previous studies in *L. simulans* did record the presence of sperm in the spermatheca, and estimated that males do fail to transfer any sperm to the spermatheca in about 40% of matings (Tadler, 1999; Tadler *et al.*, 1999). The most important determinant of insemination success in both species appears to be copulation duration: the proportion of matings that fail is reduced to between 10% and 25% for matings that lasted over six hours (**Figure 8.2**).

One potential reason for a high incidence of insemination failure would be if not all females were sexually mature during the experiments. I can test this by recording whether females lay infertile eggs following mating; if so, this suggests that ovary development is complete. Though I did not record this for all experiments, in cases where I did most females do in fact appear to be sexually mature. For example, in Chapter 5 only 2 of the 64 mated *L. simulans* females did not lay infertile eggs during the two week oviposition period. Furthermore, previous studies suggest that the majority of males and females are sexually mature after seven days (Evans, 2011).

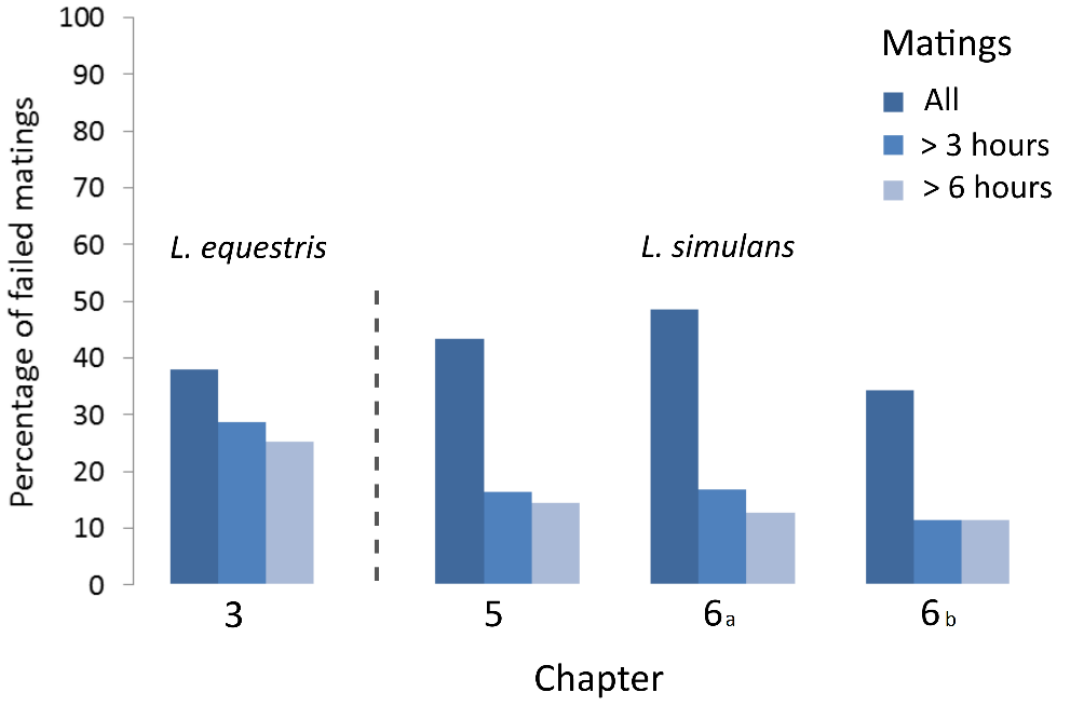

Figure 8.2. The frequency of mating failures in *L. equestris* and *L. simulans* observed in this thesis. Bars show the percentage of matings that did not lead to the production of offspring after two weeks; for all matings, matings that lasted over three hours (between 3 and 10 hours) and matings that lasted over six hours (between 6 and 10 hours). Data from chapter 6 are from sham males in experiments 6.1 (6a) and 6.3 (6b).

The fact that so many matings in both species fail to result in offspring may be surprising given that mating has been shown to be costly to females (Shuker *et al.*, 2006). Such failures may be a maladaptive consequence of other processes, for example the difficulty of manoeuvring the processus into the female spermathecal duct. Alternatively, insemination failures could represent the outcome of cryptic female choice (Eberhard, 1996). Females could exercise cryptic female choice after mating either by preventing access to the spermatheca, or by not utilising sperm once it has been transferred. Previous studies in *L. simulans* have described the valve at the entrance to the female spermathecal duct as a possible cryptic female choice mechanism, although it is still unclear whether females have active control over its function (Gschwentner & Tadler, 2000).

Previous work in *L. equestris* has suggested that long copulations function as a form of post-copulatory mate guarding. This was supported by the observation that sperm transfer is fastest in the first few hours of copulation (Sillén-Tullberg, 1981). My results show that insemination success plateaus after around three hours in *L. simulans* during no-choice mating trials (**Figure 8.3a**). Additionally, there is no significant relationship between copulation duration and offspring production (Linear regression: $F_{1,64}$= 2.93, *N*= 99, *P*= 0.092; **Figure 8.3b**). Therefore long copulations do not directly benefit females in terms of fecundity. This supports the idea that matings over three hours have additional functions apart from sperm transfer, of which mate guarding is one possible explanation, and that males are primarily in control of copulation duration (Sillén-Tullberg, 1981). This is also supported by the observation that copulation duration is longer when rival males are present (Sillén-Tullberg, 1981), and when mating with larger females (Chapter 3).

Very long copulations as a form of copulatory mate guarding are seen in several Heteroptera species (McLain, 1980; McLain, 1989; Carroll & Loye, 1990; Carroll, 1991; Hosokawa & Suzuki, 2001; Schofl & Taborsky, 2002), as well as in some Phasmatodea and Coleoptera species (Sivinski, 1980; Snead & Alcock, 1985). Interestingly, many of these species are aposematic (Sillen-Tullberg, 1981; Snead & Alcock, 1985; McLain, 1989; Schofl & Taborsky, 2002), cryptic (Sivinski, 1980), or possess chemical defences (Sivinski, 1980; Hosokawa & Suzuki, 2001) which are predicted to reduce the predation risk associated with remaining in copula (Alcock, 1994). Additionally, in some species mating may even protect individuals from predation, for example because an individual searching for mates is more likely to encounter an ambush predator (McCauley & Lawson, 1986), or because the defences of two individuals are more effective then when in isolation (Sivinski, 1980). In *L. equestris* and *L. simulans* several factors have probably contribute to the evolution and maintenance of prolonged copulation as an adaptive male strategy. These include: high male density, multiple mating by females, strong last-male sperm precedence, a short copulation-oviposition

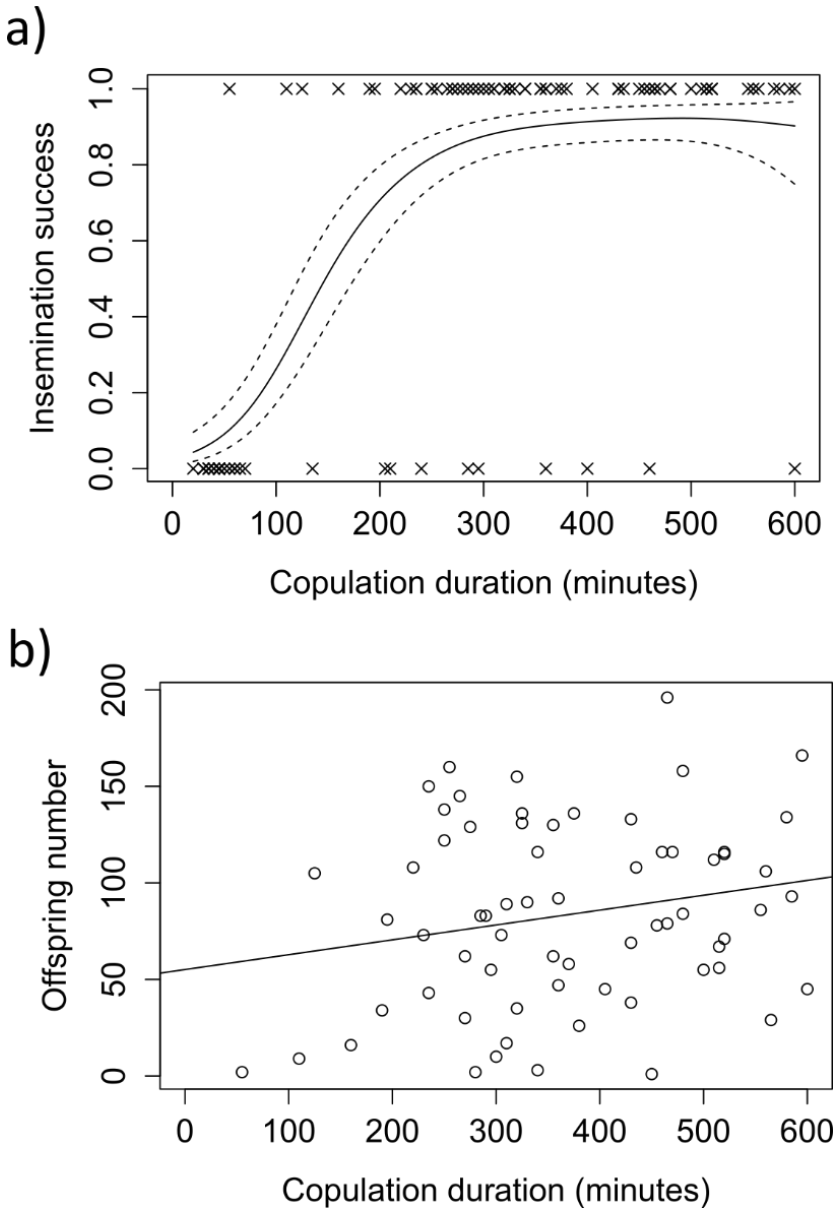

Figure 8.3. The relationship between copulation duration and **a)** insemination success and **b)** offspring number, for *Lygaeus simulans* mated pairs during no-choice mating trials. Data are from Chapter 6 (sham and tip-removed males from the small reduction experiment, *N*= 99).

interval, low risk of takeovers by other males, low predation risk and the ability to feed during mating (Alcock, 1994).

8.5 Conclusion

Overall, my results illustrate that the strength of pre-copulatory sexual selection acting on morphological traits can be strongly affected by social context. This has been shown to apply to multiple genital traits in *L. equestris* and *L. simulans*, and also seems to be a general trend across many animal species. These results also suggest that sexual selection in the wild may vary both spatially and temporally depending on the social environment. Additionally, these effects highlight how small differences in experimental design may lead to significantly different results. Our results in the lab may be especially misleading if they do not accurately reflect the social context in natural populations.

I have also shown that male genital traits may be subject to selection arising from different stages of mating. However, detailed studies of functional morphology may be needed in order to determine how selection arises, both in these species and more generally.

Finally, I believe I have shown that *L. equestris* and *L. simulans* will be incredibly useful in the future as model organisms for the study of post-copulatory reproductive processes, for several reasons. First both species exhibit high intraspecific variation in the duration of copulation and the likelihood of successful insemination, and as such may exhibit both male and female post-copulatory choice. Second, the male intromittent organ is relatively simple but greatly exaggerated, and has been shown to be subject to selection from multiple sources, which varies according to the stage of mating or the social context. Finally, the length of the male processus can be manipulated with high precision and without impairing its sperm transfer ability. Future studies will thus be able further investigate the complex behavioural, physiological and morphological interactions between males and females that occur during mating.

Literature cited

Agnarsson, I., Coddington, J. A. & Knoflach, B. 2007. Morphology and evolution of cobweb spider male genitalia (Araneae, Theridiidae). *Journal of Arachnology,* **35**, 334–395.

Ah-King, M. & Ahnesjö, I. 2013. The "sex role" concept: an overview and evaluation. *Evolutionary biology*, **40**, 461-470.

Ah-King, M., Barron, A. B. & Herberstein, M. E. 2014. Genital evolution: why are females still understudied? *Plos Biology*, **12**, e1001851.

Aisenberg, A., Barrantes, G. & Eberhard, W. G. 2015. Hairy kisses: tactile cheliceral courtship affects female mating decisions in *Leucauge mariana* (Araneae, Tetragnathidae). *Behavioral Ecology and Sociobiology,* **69**, 313–323.

Alcock, J. 1994. Postinsemination associations between males and females in insects: the mate-guarding hypothesis. *Annual Review of Entomology*, **39**, 1-21.

Aldrich, J. R. 1988. Chemical ecology of Heteroptera. *Annual Review of Entomology*, **33**, 211-238.

Aldrich, J. R. 1995. Chemical communication in the true bugs and parasitoid exploitation. In: *Chemical Ecology of Insects 2* (Ed. by R. T. Carde & W. J. Bell), pp. 318-363. New York: Chapman & Hall.

Aldrich, J. R., Leal, W. S., Nishida, R., Khrimian, A. P., Lee, C. J. & Sakuratani, Y. 1997. Semiochemistry of aposematic seed bugs. *Entomologia Experimentalis et Applicata*, **84**, 127-135.

Aldrich, J. R., Oliver, J. E., Taghizadeh, T., Ferreira, J. T. B. & Liewehr, D. 1999. Pheromones and colonization: reassessment of the milkweed bug migration model (Heteroptera: Lygaeidae: Lygaeinae). *Chemoecology*, **9**, 63-71.

Ali, J. G. & Tallamy, D. W. 2010. Female spotted cucumber beetles use own cuticular hydrocarbon signature to choose immunocompatible mates. *Animal Behaviour*, **80**, 9-12.

Allison, J. D. & Cardé, R. T. 2008. Male pheromone blend preference function measured in choice and no-choice wind tunnel trials with almond moths, *Cadra cautella*. *Animal Behaviour*, **75**, 259-266.

Altmann, J. 1997. Mate choice and intrasexual reproductive competition: contributions to reproduction that go beyond acquiring more mates. In: *Feminism and evolutionary biology*, pp. 320-333: Springer.

Andersson, M. B. 1994. *Sexual selection*. Princeton: Princeton University Press.

Apakupakul, K. & Rubenstein, D. R. 2015. Bateman's principle is reversed in a cooperatively breeding bird. *Biology Letters*, **11**, In press.

Arbuthnott, D., Elliot, M., McPeek, M. & Crespi, B. 2010. Divergent patterns of diversification in courtship and genitalic characters of Timema walking-sticks. *Journal of Evolutionary Biology*, **23**, 1399-1411.

Arct, A., Drobniak, S. M. & Cichoń, M. 2015. Genetic similarity between mates predicts extrapair paternity—a meta-analysis of bird studies. *Behavioral Ecology*, In press.

Arnold, S. J. & Wade, M. J. 1984. On the measurement of natural and sexual selection: applications. *Evolution*, **38**, 720-734.
Arnqvist, G. 1989. Sexual selection in a water strider: the function, mechanism of selection and heritability of a male grasping apparatus. *Oikos*, **56**, 344-350.
Arnqvist, G. 1992a. Pre-copulatory fighting in a water strider: inter-sexual conflict or mate assessment? *Animal Behaviour*, **43**, 559-567.
Arnqvist, G. 1992b. Spatial variation in selective regimes: sexual selection in the water strider, *Gerris odontogaster*. *Evolution*, **46**, 914-929.
Arnqvist, G. 1997. The evolution of animal genitalia: distinguishing between hypotheses by single species studies. *Biological Journal of the Linnean Society*, **60**, 365-379.
Arnqvist, G. 1998. Comparative evidence for the evolution of genitalia by sexual selection. *Nature*, **393**, 784-786.
Arnqvist, G. 2014. Cryptic female choice. In: *The evolution of insect mating systems* (Ed. by D. M. Shuker & L. W. Simmons), pp. 204- 220. Oxford: Oxford University Press.
Arnqvist, G. & Danielsson, I. 1999. Copulatory behavior, genital morphology, and male fertilization success in water striders. *Evolution*, **53**, 147-156.
Arnqvist, G. & Nilsson, T. 2000. The evolution of polyandry: multiple mating and female fitness in insects. *Animal Behaviour*, **60**, 145-164.
Arnqvist, G. & Rowe, L. 1995. Sexual conflict and arms races between the sexes: a morphological adaptation for control of mating in a female insect. *Proceedings of the Royal Society B: Biological Sciences*, **261**, 123-127.
Arnqvist, G. & Rowe, L. 2005. *Sexual conflict*: Princeton University Press.
Arnqvist, G. & Wooster, D. 1995. Meta-analysis: synthesizing research findings in ecology and evolution. *Trends in Ecology & Evolution*, **10**, 236-240.
Ashlock, P. D. 1957. An investigation of the taxonomic value of the phallus in the Lygaeidae (Hemiptera-Heteroptera). *Annals of the Entomological Society of America*, **50**, 407-426.
Aspbury, A. S. & Gabor, C. R. 2004. Discriminating males alter sperm production between species. *Proceedings of the National Academy of Sciences*, **101**, 15970-15973.
Aspöck, U. & Aspöck, H. 2008. Phylogenetic relevance of the genital sclerites of Neuropterida (Insecta: Holometabola). *Systematic Entomology*, **33**, 97-127.
Avila, F. W., Sirot, L. K., LaFlamme, B. A., Rubinstein, C. D. & Wolfner, M. F. 2011. Insect seminal fluid proteins: identification and function. *Annual Review of Entomology*, **56**, 21-40.
Bailey, N. W. 2008. Love will tear you apart: different components of female choice exert contrasting selection pressures on male field crickets. *Behavioral Ecology*, **19**, 960-966.
Bailey, N. W. & Zuk, M. 2008. Acoustic experience shapes female mate choice in field crickets. *Proceedings of the Royal Society B: Biological Sciences*, **275**, 2645-2650.
Bakker, T. C. & Milinski, M. 1991. Sequential female choice and the previous male effect in sticklebacks. *Behavioral Ecology and Sociobiology*, **29**, 205-210.
Bangham, J., Chapman, T. & Partridge, L. 2002. Effects of body size, accessory gland and testis size on pre-and postcopulatory success in *Drosophila melanogaster*. *Animal Behaviour*, **64**, 915-921.

Barrett, L. T., Evans, J. P. & Gasparini, C. 2014. The effects of perceived mating opportunities on patterns of reproductive investment by male guppies. *Plos One*, **9**, e93780.
Barry, K. L., Holwell, G. I. & Herberstein, M. E. 2010. Multimodal mate assessment by male praying mantids in a sexually cannibalistic mating system. *Animal Behaviour*, **79**, 1165-1172.
Barry, K. L. & Kokko, H. 2010. Male mate choice: why sequential choice can make its evolution difficult. *Animal Behaviour*, **80**, 163-169.
Basolo, A. L. 1995. Phylogenetic evidence for the role of a pre-existing bias in sexual selection. *Proceedings of the Royal Society B: Biological Sciences*, **259**, 307-311.
Basolo, A. L. 2002. Female discrimination against sworded males in a poeciliid fish. *Animal Behaviour*, **63**, 463-468.
Bateman, A. J. 1948. Intra-sexual selection in Drosophila. *Heredity*, **2**, 349-368.
Bateman, P. W. & Fleming, P. A. 2006. Males are selective too: mating, but not courtship, with sequential females influences choosiness in male field crickets (*Gryllus bimaculatus*). *Behavioral Ecology and Sociobiology*, **59**, 577-581.
Bateson, M. & Healy, S. D. 2005. Comparative evaluation and its implications for mate choice. *Trends in Ecology & Evolution*, **20**, 659-664.
Beatty, C. D. & Franks, D. W. 2012. Discriminative predation: simultaneous and sequential encounter experiments. *Current Zoology*, **58**, 649-657.
Beckers, O. M. & Wagner Jr, W. E. 2011. Mate sampling strategy in a field cricket: evidence for a fixed threshold strategy with last chance option. *Animal Behaviour*, **81**, 519-527.
Beckers, O. M. & Wagner Jr, W. E. 2013. Parasitoid infestation changes female mating preferences. *Animal Behaviour*, **85**, 791–796.
Bee, M. A. & Schwartz, J. J. 2009. Behavioral measures of signal recognition thresholds in frogs in the presence and absence of chorus-shaped noise. *Journal of the Acoustical Society of America*, **126**, 2788-2801.
Begg, C. B. & Mazumdar, M. 1994. Operating characteristics of a rank correlation test for publication bias. *Biometrics*, **50**, 1088-1101.
Belles-Isles, J. C., Cloutier, D. & FitzGerald, G. J. 1990. Female cannibalism and male courtship tactics in threespine sticklebacks. *Behavioral Ecology and Sociobiology*, **26**, 363-368.
Benton, T. & Evans, M. R. 1998. Measuring mate choice using correlation: the effect of female sampling behaviour. *Behavioral Ecology and Sociobiology*, **44**, 91-98.
Bentsen, C. L., Hunt, J., Jennions, M. D. & Brooks, R. 2006. Complex multivariate sexual selection on male acoustic signaling in a wild population of *Teleogryllus commodus*. *The American Naturalist*, **167**, E102-E116.
Berglund, A. 1994. The operational sex ratio influences choosiness in a pipefish. *Behavioral Ecology*, **5**, 254-258.
Berglund, A. 1995. Many mates make male pipefish choosy. *Behaviour*, **132**, 3-4.
Bertin, A. & Fairbairn, D. 2005. One tool, many uses: precopulatory sexual selection on genital morphology in *Aquarius remigis*. *Journal of Evolutionary Biology*, **18**, 949-961.
Birkhead, T. 1998. Cryptic female choice: criteria for establishing female sperm choice. *Evolution*, **52**, 1212-1218.

Birkhead, T. 2010. How stupid not to have thought of that: post-copulatory sexual selection. *Journal of Zoology*, **281**, 78-93.
Birkhead, T. R. & Møller, A. P. 1998. *Sperm Competition and Sexual Selection*. London: Academic Press.
Birkhead, T. R. & Pizzari, T. 2002. Postcopulatory sexual selection. *Nature Reviews Genetics*, **3**, 262-273.
Bishop, P. J., Jennions, M. D. & Passmore, N. I. 1995. Chorus size and call intensity: female choice in the painted reed frog, *Hyperolius marmoratus*. *Behaviour*, **132**, 721-731.
Bissell, A. N. & Martins, E. P. 2006. Male approach and female avoidance as mechanisms of population discrimination in sagebrush lizards. *Behavioral Ecology and Sociobiology*, **60**, 655-662.
Björklund, M. 1997. Are 'comparative methods' always necessary? *Oikos*, **80**, 607-612.
Blanckenhorn, W., Kraushaar, U., Teuschl, Y. & Reim, C. 2004. Sexual selection on morphological and physiological traits and fluctuating asymmetry in the black scavenger fly *Sepsis cynipsea*. *Journal of Evolutionary Biology*, **17**, 629-641.
Blomberg, S. P., Garland, T. & Ives, A. R. 2003. Testing for phylogenetic signal in comparative data: behavioral traits are more labile. *Evolution*, **57**, 717-745.
Boake, C. 1989. Repeatability: Its role in evolutionary studies of mating behavior. *Evolutionary Ecology*, **3**, 173-182.
Boake, C. R. B. & Poulsen, T. 1997. Correlates versus predictors of courtship success: courtship song in *Drosophila silvestris* and *D.heteroneura*. *Animal Behaviour*, **54**, 699-704.
Bonduriansky, R. 2001. The evolution of male mate choice in insects: a synthesis of ideas and evidence. *Biological Reviews*, **76**, 305-339.
Bonduriansky, R. 2007. Sexual selection and allometry: a critical reappraisal of the evidence and ideas. *Evolution*, **61**, 838-849.
Bonduriansky, R. & Rowe, L. 2003. Interactions among mechanisms of sexual selection on male body size and head shape in a sexually dimorphic fly. *Evolution*, **57**, 2046-2053.
Bonhag, P. F. & Wick, J. R. 1953. The functional anatomy of the male and female reproductive systems of the milkweed bug, *Oncopeltus fasciatus* (Dallas) (Heteroptera: Lygaeidae). *Journal of Morphology*, **93**, 177-283.
Booksmythe, I., Detto, T. & Backwell, P. R. 2008. Female fiddler crabs settle for less: the travel costs of mate choice. *Animal Behaviour*, **76**, 1775-1781.
Booksmythe, I., Jennions, M. D. & Backwell, P. R. Y. 2011. Male fiddler crabs prefer conspecific females during simultaneous, but not sequential, mate choice. *Animal Behaviour*, **81**, 775-778.
Brennan, P. L., Clark, C. J. & Prum, R. O. 2010. Explosive eversion and functional morphology of the duck penis supports sexual conflict in waterfowl genitalia. *Proceedings of the Royal Society B: Biological Sciences*, **277**, 1309-1314.
Brennan, P. L., Prum, R. O., McCracken, K. G., Sorenson, M. D., Wilson, R. E. & Birkhead, T. R. 2007. Coevolution of male and female genital morphology in waterfowl. *Plos One*, **2**, e418.

Bretman, A., Newcombe, D. & Tregenza, T. 2009. Promiscuous females avoid inbreeding by controlling sperm storage. *Molecular Ecology*, **18**, 3340-3345.
Briceño, R. & Eberhard, W. 2009. Experimental modifications imply a stimulatory function for male tsetse fly genitalia, supporting cryptic female choice theory. *Journal of Evolutionary Biology*, **22**, 1516-1525.
Briskie, J. V. & Montgomerie, R. 1997. Sexual selection and the intromittent organ of birds. *Journal of Avian Biology*, **28**, 73-86.
Burdfield-Steel, E. R. 2014. The causes and consequences of reproductive interference in the Lygaeidae, Doctoral thesis, University of St Andrews.
Burdfield-Steel, E. R., Dougherty, L. R., Smith, L. A., Collins, L. A. & Shuker, D. M. 2013. Variation in social and sexual behaviour in four species of aposematic seed bugs (Hemiptera: Lygaeidae): the role of toxic and non-toxic food. *Behavioural processes*, **99**, 52-61.
Burdfield-Steel, E. R. & Shuker, D. M. 2014. The evolutionary ecology of the Lygaeidae. *Ecology and Evolution*, **4**, 2278-2301.
Bussiere, L. F., Hunt, J., Stölting, K. N., Jennions, M. D. & Brooks, R. 2008. Mate choice for genetic quality when environments vary: suggestions for empirical progress. *Genetica*, **134**, 69-78.
Callander, S., Hayes, C. L., Jennions, M. D. & Backwell, P. R. 2012. Experimental evidence that immediate neighbors affect male attractiveness. *Behavioral Ecology*, **24**, 730-733.
Candolin, U. & Vlieger, L. 2013. Estimating the dynamics of sexual selection in changing environments. *Evolutionary biology*, **40**, 589-600.
Carroll, S. P. 1991. The adaptive significance of mate guarding in the soapberry bug, *Jadera haematoloma* (Hemiptera: Rhopalidae). *Journal of Insect Behavior,* **4**, 509–530.
Carroll, S. P. & Loye, J. E. 1990. Male-biased sex ratios, female promiscuity, and copulatory mate guarding in an aggregating tropical bug, *Dysdercus bimaculatus*. *Journal of Insect Behavior,* **3**, 33–48.
Casares, P., Carracedo, M. C., del Rio, B., Piñeiro, R., Garcia-Florez, L. & Barros, A. R. 1998. Disentangling the effects of mating propensity and mating choice in Drosophila. *Evolution*, **52**, 126-133.
Cayetano, L., Maklakov, A. A., Brooks, R. C. & Bonduriansky, R. 2011. Evolution of male and female genitalia following release from sexual selection. *Evolution*, **65**, 2171-2183.
Chamberlain, S. A., Hovick, S. M., Dibble, C. J., Rasmussen, N. L., Van Allen, B. G., Maitner, B. S., Ahern, J. R., Bell-Dereske, L. P., Roy, C. L. & Meza-Lopez, M. 2012. Does phylogeny matter? Assessing the impact of phylogenetic information in ecological meta-analysis. *Ecology Letters*, **15**, 627-636.
Chapman, T. 2001. Seminal fluid-mediated fitness traits in Drosophila. *Heredity*, **87**, 511-521.
Chenoweth, S. F. & Blows, M. W. 2005. Contrasting mutual sexual selection on homologous signal traits in *Drosophila serrata*. *The American Naturalist*, **165**, 281-289.
Chiang, R. G. 2010. A newly discovered sperm transport system in the female of Lygaeidae bugs. *Physiological Entomology*, **35**, 87-92.

Cocchiararo-Bastias, L. M., Mijailovsky, S. J., Calderon-Fernández, G. M., Figueiras, A. N. L. & Juárez, M. P. 2011. Epicuticle lipids mediate mate recognition in *Triatoma infestans*. *Journal of Chemical Ecology*, **37**, 246-252.
Cohen, J. 1992. A power primer. *Psychological bulletin*, **112**, 155.
Coker, C. R., McKinney, F., Hays, H., Briggs, S. V., Cheng, K. M. & Hohman, W. 2002. Intromittent organ morphology and testis size in relation to mating system in waterfowl. *The Auk*, **119**, 403-413.
Conner, J. 1989. Density-dependent sexual selection in the fungus beetle, *Bolitotherus cornutus*. *Evolution*, **43**, 1378-1386.
Cook, S. E., Vernon, J. G., Bateson, M. & Guilford, T. 1994. Mate choice in the polymorphic African swallowtail butterfly, *Papilio dardanus*- male-like females may avoid sexual harassment. *Animal Behaviour*, **47**, 389-397.
Córdoba-Aguilar, A., Salamanca-Ocaña, J. C. & Lopezaraiza, M. 2003. Female reproductive decisions and parasite burden in a calopterygid damselfly (Insecta: Odonata). *Animal Behaviour*, **66**, 81-87.
Córdoba-Aguilar, A., Uhía, E. & Rivera, A. C. 2003. Sperm competition in Odonata (Insecta): the evolution of female sperm storage and rivals' sperm displacement. *Journal of Zoology*, **261**, 381-398.
Cornwallis, C. K. & Uller, T. 2010. Towards an evolutionary ecology of sexual traits. *Trends in Ecology & Evolution*, **25**, 145-152.
Coyne, J. A., Elwyn, S. & Rolán-Alvarez, E. 2005. Impact of experimental design on *Drosophila* sexual isolation studies: direct effects and comparison to field hybridization data. *Evolution*, **59**, 2588-2601.
Cunningham, J. A., Rahman, I. A., Lautenschlager, S., Rayfield, E. J. & Donoghue, P. C. 2014. A virtual world of paleontology. *Trends in Ecology & Evolution*, **29**, 347-357.
Dallai, R. & Afzelius, B. A. 1980. Characteristics of the sperm structure in Heteroptera (Hemiptera, Insecta). *Journal of Morphology*, **164**, 301-309.
Danielsson, I. 2001. Antagonistic pre–and post–copulatory sexual selection on male body size in a water strider (*Gerris lacustris*). *Proceedings of the Royal Society B: Biological Sciences*, **268**, 77-81.
Darwin, C. R. 1854. *The Balanidae (or Sessile Cirripedes); the Verrucidae. A Monograph of the Sub-class Cirripedia, with figures of all the species.* UK: The Ray Society.
Darwin, C. R. 1871. *The descent of man, and selection in relation to sex.* London: John Murray.
David, M. & Cezilly, F. 2011. Personality may confound common measures of mate-choice. *Plos One*, **6**.
Davies, N. B., Krebs, J. R. & West, S. A. 2012. *An introduction to behavioural ecology*: John Wiley & Sons.
Deb, R., Bhattacharya, M. & Balakrishnan, R. 2012. Females of a tree cricket prefer larger males but not the lower frequency male calls that indicate large body size. *Animal Behaviour*, **84**, 137-149.
Deckert, J. 1985. Über *Lygaeus simulans* spec. nov. und *L. equestris* (LINNAEUS, 1758), zwei nahe verwandte paläarktische Lygaeinae (Heteroptera, Lygaeidae). *Mitteilungen aus dem Museum für Naturkunde in Berlin. Zoologisches Museum und Institut für Spezielle Zoologie (Berlin)*, **61**, 273-278.

Deckert, J. 1990. Zum Bau von Parameren, Phallus und Pygophore der Lygaeinae und Bemerkungen zur Systematik der Unterfamilie (Heteroptera, Lygaeidae). *Mitteilungen aus dem Museum für Naturkunde in Berlin. Zoologisches Museum und Institut für Spezielle Zoologie (Berlin)*, **66**, 91-119.
DelBarco-Trillo, J. 2011. Adjustment of sperm allocation under high risk of sperm competition across taxa: a meta-analysis. *Journal of Evolutionary Biology*, **24**, 1706-1714.
Doherty, J. A. 1985. Phonotaxis in the cricket, *Gryllus bimaculatus* De Geer: comparisons of choice and no-choice paradigms. *Journal of Comparative Physiology A*, **157**, 279-289.
Dougherty, L. R. & Shuker, D. M. 2014. Pre-copulatory sexual selection in the seed bug *Lygaeus equestris*: a comparison of choice and no-choice paradigms. *Animal Behaviour*, **89**, 207-214.
Dougherty, L. R. & Shuker, D. M. 2015. The effect of experimental design on the measurement of mate choice: a meta-analysis. *Behavioral Ecology*, **26**, 311-319.
Dougherty, L. R., Rahman, I. A., Burdfield-Steel, E. R., Greenway, E. V. & Shuker, D. M. 2015. Experimental reduction of intromittent organ length reduces male reproductive success in a bug. *Proceedings of the Royal Society B: Biological Sciences*, **282**, 201507824.
Drijfhout, F. P. & Groot, A. T. 2001. Close-Range Attraction in *Lygocoris pabulinus* (L.). *Journal of Chemical Ecology*, **27**, 1133-1149.
Duffey, S. & Scudder, G. 1972. Cardiac glycosides in North American Asclepiadaceae, a basis for unpalatability in brightly coloured Hemiptera and Coleoptera. *Journal of Insect Physiology*, **18**, 63-78.
Dufour, L. 1844. Anatomie Generale des Dipteres. *Annales des Sciences Naturelles*, **1**, 244-264.
Duval, S. & Tweedie, R. 2000. Trim and fill: a simple funnel-plot–based method of testing and adjusting for publication bias in meta-analysis. *Biometrics*, **56**, 455-463.
Eberhard, W. 1996. *Female control: sexual selection by cryptic female choice*. Princeton: Princeton University Press.
Eberhard, W. G. 1985. *Sexual selection and animal genitalia*: Harvard University Press.
Eberhard, W. G. 2001. The functional morphology of species-specific clasping structures on the front legs of male Archisepsis and Palaeosepsis flies (Diptera, Sepsidae). *Zoological Journal of the Linnean Society*, **133**, 335-368.
Eberhard, W. G. 2009. Static allometry and animal genitalia. *Evolution,* **63**, 48–66.
Eberhard, W. G. 2010. Evolution of genitalia: theories, evidence, and new directions. *Genetica*, **138**, 5-18.
Eberhard, W. G. & Huber, B. A. 2010. Spider genitalia: precise maneuvres with a numb structure in a complex lock. In: *The evolution of primary sexual characters in animals* (Ed. by J. L. Leonard & A. Cordoba-Aguilar). New York: Oxford University Press.
Eberhard, W. G., Huber, B. A., S., R. L. R., Briceño, R. D., Salas, I. & Rodriguez, V. 1998. One size fits all? Relationships between the size and degree of variation in genitalia and other body parts in twenty species of insects and spiders. *Evolution*, **52**, 415-431.

Eberhard, W. G. & Ramirez, N. 2004. Functional morphology of the male genitalia of four species of Drosophila: failure to confirm both lock and key and male-female conflict predictions. *Annals of the Entomological Society of America*, **97**, 1007-1017.
Economopoulos, A. & Gordon, H. 1972. Sperm replacement and depletion in the spermatheca of the s and cs strains of *Oncopeltus fasciatus*. *Entomologia Experimentalis et Applicata*, **15**, 1-12.
Edvardsson, M. & Arnqvist, G. 2000. Copulatory courtship and cryptic female choice in red flour beetles *Tribolium castaneum*. *Proceedings of the Royal Society B: Biological Sciences*, **267**, 559-563.
Edward, D. A. 2015. The description of mate choice. *Behavioral Ecology*, **26**, 301-310.
Edward, D. A. & Chapman, T. 2011. The evolution and significance of male mate choice. *Trends in Ecology & Evolution*, **26**, 647-654.
Emlen, D. J. 2008. The evolution of animal weapons. *Annual Review of Ecology, Evolution, and Systematics*, **39**, 387-413.
Endler, J. A. 1986. *Natural selection in the wild*: Princeton University Press.
Evans, G. 2011. Sexual conflict over mating in *Lygaeus* seed bugs, Doctoral thesis, University of Edinburgh.
Fairbairn, D. J., Vermette, R., Kapoor, N. N. & Zahiri, N. 2003. Functional morphology of sexually selected gentalia in the water strider *Aquarius remigis*. *Canadian Journal of Zoology*, **81**, 400-413.
Fitzpatrick, C. L. 2015. Expanding sexual selection gradients; a synthetic refinement of sexual selection theory. *Ethology*, **121**, 207-217.
Forrest, T. 1994. From sender to receiver: propagation and environmental effects on acoustic signals. *American Zoologist*, **34**, 644-654.
Fowler-Finn, K. D. & Rodríguez, R. L. 2012. The evolution of experience-mediated plasticity in mate preferences. *Journal of Evolutionary Biology*, **25**, 1855-1863.
Fowler-Finn, K. D. & Rodríguez, R. L. 2013. Repeatability of mate preference functions in *Enchenopa* treehoppers (Hemiptera: Membracidae). *Animal Behaviour*, **85**, 493-499.
Friedrich, F., Matsumura, Y., Pohl, H., Bai, M., Hörnschemeyer, T. & Beutel, R. G. 2014. Insect morphology in the age of phylogenomics: innovative techniques and its future role in systematics. *Entomological science*, **17**, 1-24.
Friedrich, F., Pohl, H. W., Hünefeld, F., Beckmann, F., Herzen, J. & Beutel, R. G. 2008. SRμCT-based study of external and internal structures of adults and larvae of Endopterygota (Hexapoda). *Hasylab Annual Report*, **2007**, 1527-1528.
Gabor, C. 1999. Association patterns of sailfin mollies (*Poecilia latipinna*): alternative hypotheses. *Behavioral Ecology and Sociobiology*, **46**, 333-340.
Gabor, C. R., Krenz, J. D. & Jaeger, R. G. 2000. Female choice, male interference, and sperm precedence in the red-spotted newt. *Behavioral Ecology*, **11**, 115-124.
Gack, C. & Peschke, K. 2005. 'Shouldering'exaggerated genitalia: a unique behavioural adaptation for the retraction of the elongate intromittent organ by the male rove beetle (*Aleochara tristis* Gravenhorst). *Biological Journal of the Linnean Society*, **84**, 307-312.
Gage, A. R. & Barnard, C. J. 1996. Male crickets increase sperm number in relation to competition and female size. *Behavioral Ecology and Sociobiology*, **38**, 349-353.

Gage, M. J. G. 1998. Influences of sex, size, and symmetry on ejaculate expenditure in a moth. *Behavioral Ecology*, **9**, 592-597.
Galef, B. G. & White, D. J. 1998. Mate-choice copying in Japanese quail, *Coturnix coturnix japonica*. *Animal Behaviour*, **55**, 545-552.
Garamszegi, L. Z., Markó, G. & Herczeg, G. 2012. A meta-analysis of correlated behaviours with implications for behavioural syndromes: mean effect size, publication bias, phylogenetic effects and the role of mediator variables. *Evolutionary Ecology*, **26**, 1213-1235.
García-González, F. 2004. Infertile matings and sperm competition: the effect of "nonsperm representation" on intraspecific variation in sperm precedence patterns. *The American Naturalist*, **164**, 457-472.
García-González, F. 2008. Male genetic quality and the inequality between paternity success and fertilization success: consequences for studies of sperm competition and the evolution of polyandry. *Evolution*, **62**, 1653-1665.
García-González, F. & Gomendio, M. 2004. Adjustment of copula duration and ejaculate size according to the risk of sperm competition in the golden egg bug (*Phyllomorpha laciniata*). *Behavioral Ecology*, **15**, 23-30.
García-González, F. & Simmons, L. W. 2007. Shorter sperm confer higher competitive fertilization success. *Evolution*, **61**, 816-824.
Gaunt, M. W. & Miles, M. A. 2002. An insect molecular clock dates the origin of the insects and accords with palaeontological and biogeographic landmarks. *Molecular Biology and Evolution*, **19**, 748-761.
Gershman, S. N. & Sakaluk, S. K. 2009. No Coolidge effect in decorated crickets. *Ethology*, **115**, 774-780.
Ghedotti, M. J. 2000. Phylogenetic analysis and taxonomy of the poecilioid fishes (Teleostei: Cyprinodontiformes). *Zoological Journal of the Linnean Society*, **130**, 1-53.
Ghiselin, M., T. 2010. The distinction between primary and secondary sexual characters. In: *The evolution of primary sexual characters in animals* (Ed. by J. L. C.-A. Leonard, A.). New York: Oxford University Press.
Gibson, R. M. 1996. Female choice in sage grouse: the roles of attraction and active comparison. *Behavioral Ecology and Sociobiology*, **39**, 55-59.
Gibson, R. M. & Langen, T. A. 1996. How do animals choose their mates? *Trends in Ecology & Evolution*, **11**, 468-470.
Gillespie, S. R., Scarlett Tudor, M., Moore, A. J. & Miller, C. W. 2014. Sexual selection is influenced by both developmental and adult environments. *Evolution*, **68**, 3421-3432.
Gillingham, M. A. F., Richardson, D. S., Løvlie, H., Moynihan, A., Worley, K. & Pizzari, T. 2009. Cryptic preference for MHC-dissimilar females in male red junglefowl, *Gallus gallus*. *Proceedings of the Royal Society B: Biological Sciences*, **276**, 1083-1092.
Giribet, G., Edgecombe, G. D. & Wheeler, W. C. 2001. Arthropod phylogeny based on eight molecular loci and morphology. *Nature*, **413**, 157-161.
Glenner, H., Hansen, A. J., Sørensen, M. V., Ronquist, F., Huelsenbeck, J. P. & Willerslev, E. 2004. Bayesian inference of the metazoan phylogeny: a combined molecular and morphological approach. *Current Biology*, **14**, 1644-1649.

Godin, J.-G. J. & Dugatkin, L. A. 1995. Variability and repeatability of female mating preference in the guppy. *Animal Behaviour*, **49**, 1427-1433.
Godin, J. G. J. & Auld, H. L. 2013. Covariation and repeatability of male mating effort and mating preferences in a promiscuous fish. *Ecology and Evolution*, **3**, 2020-2029.
Gong, A. & Gibson, R. M. 1996. Reversal of a female preference after visual exposure to a predator in the guppy, *Poecilia reticulata*. *Animal Behaviour*, **52**, 1007-1015.
Goodall, C. 1991. Procrustes methods in the statistical analysis of shape. *Journal of the Royal Statistical Society. Series B (Methodological)*, **53**, 285-339.
Gosden, T. P. & Svensson, E. I. 2008. Spatial and temporal dynamics in a sexual selection mosaic. *Evolution*, **62**, 845-856.
Grant, J. W. A., Bryant, M. J. & Soos, C. E. 1995. Operational sex ratio, mediated by synchrony of female arrival, alters the variance of male mating success in japanese medaka. *Animal Behaviour*, **49**, 367-375.
Greenfield, M. D. 2015. Signal interactions and interference in insect choruses: singing and listening in the social environment. *Journal of Comparative Physiology A*, **201**, 143-154.
Grieshop, K. & Polak, M. 2012. The precopulatory function of male genital spines in *Drosophila ananassae* [Doleschall] (Diptera: Drosophilidae) revealed by laser surgery. *Evolution*, **66**, 2637-2645.
Griffith, S. C., Owens, I. P. & Thuman, K. A. 2002. Extra pair paternity in birds: a review of interspecific variation and adaptive function. *Molecular Ecology*, **11**, 2195-2212.
Gschwentner, R. & Tadler, A. 2000. Functional anatomy of the spermatheca and its duct in the seed bug *Lygaeus simulans* (Heteroptera: Lygaeidae). *European Journal of Entomology*, **97**, 305-312.
Gubernick, D. J. & Addington, R. L. 1994. The stability of female social and mating preferences in the monogamous California mouse, *Peromyscus californicus*. *Animal Behaviour*, **47**, 559-567.
Gupta, J. & Sundaran, A. 1994. Some evidence of incipient speciation in *Drosophila kikkawai*. *Genome*, **37**, 1041-1044.
Gusev, R. & Tatarnikov, D. 1991. On the distinguishing characters and distribution of the bugs *Lygaeus simulans* Deckert and *L. equestris* (Linnaeus)(Heteroptera, Lygaeidae). *Entomologicheskoe Obozrenie*, **70**, 404-406.
Gwynne, D. T. 1991. Sexual competition among females: What causes courtship-role reversal? *Trends in Ecology & Evolution*, **6**, 118-121.
Hadfield, J. & Nakagawa, S. 2010. General quantitative genetic methods for comparative biology: phylogenies, taxonomies and multi-trait models for continuous and categorical characters. *Journal of Evolutionary Biology*, **23**, 494-508.
Hall, M. D., Bussière, L. F., Hunt, J. & Brooks, R. 2008. Experimental evidence that sexual conflict influences the opportunity, form and intensity of sexual selection. *Evolution*, **62**, 2305-2315.
Halliday, T. R. 1983. The study of mate choice. In: *Mate choice* (Ed. by P. Bateson), pp. 3-32. Cambridge: Cambridge University Press.
Harris, W. E. & Moore, P. J. 2005. Female mate preference and sexual conflict: females prefer males that have had fewer consorts. *The American Naturalist*, **165**, S64-S71.

Havens, J., Orzack, S. & Etges, W. 2011. Mate choice opportunity leads to shorter offspring development time in a desert insect. *Journal of Evolutionary Biology*, **24**, 1317-1324.
Havens, J. A. & Etges, W. J. 2013. Premating isolation is determined by larval rearing substrates in cactophilic *Drosophila mojavensis*. IX. Host plant and population specific epicuticular hydrocarbon expression influences mate choice and sexual selection. *Journal of Evolutionary Biology*, **26**, 562-576.
Head, M. L., Lindholm, A. K. & Brooks, R. 2008. Operational sex ratio and density do not affect directional selection on male sexual ornaments and behavior. *Evolution*, **62**, 135-144.
Hedrick, A. V. & Dill, L. M. 1993. Mate choice by female crickets is influenced by predation risk. *Animal Behaviour*, **46**, 193-196.
Herdina, A. N., Kelly, D. A., Jahelková, H., Lina, P. H., Horáček, I. & Metscher, B. D. 2015. Testing hypotheses of bat baculum function with 3D models derived from microCT. *Journal of Anatomy,* **226**, 229–235.
Higgins, J., Thompson, S. G., Deeks, J. J. & Altman, D. G. 2003. Measuring inconsistency in meta-analyses. *British Medical Journal*, **327**, 557-560.
Higgins, S. L. 2009. Sexual selection and insect genitalia, Doctoral thesis, University of Exeter.
Higgins, S. L., Hosken, D. J. & Wedell, N. 2009. Phenotypic and genetic variation in male genitalia in the seedbug, *Lygaeus equestris* (Heteroptera). *Biological Journal of the Linnean Society*, **98**, 400-405.
Himuro, C. & Fujisaki, K. 2008. Males of the seed bug *Togo hemipterus* (Heteroptera: Lygaeidae) use accessory gland substances to inhibit remating by females. *Journal of Insect Physiology*, **54**, 1538-1542.
Himuro, C. & Fujisaki, K. 2010. Mating experience weakens starvation tolerance in the seed bug *Togo hemipterus* (Heteroptera: Lygaeidae). *Physiological Entomology*, **35**, 128-133.
Himuro, C. & Fujisaki, K. 2011. Sexual size dimorphism and aggressive interactions under starvation conditions in the seed bug *Togo hemipterus* (Heteroptera: Lygaeidae). *Journal of Insect Behavior*, **25**, 1-12.
Himuro, C. & Fujisaki, K. 2012. The effects of male harassment on mating duration in the seed bug, *Togo hemipterus*. *Entomologia Experimentalis et Applicata*, **142**, 53-59.
Hingle, A., Fowler, K. & Pomiankowski, A. 2001. The effect of transient food stress on female mate preference in the stalk–eyed fly *Cyrtodiopsis dalmanni*. *Proceedings of the Royal Society B: Biological Sciences*, **268**, 1239-1244.
Hoare, D., Krause, J., Peuhkuri, N. & Godin, J. G. 2000. Body size and shoaling in fish. *Journal of Fish Biology*, **57**, 1351-1366.
Höglund, J. & Alatalo, R. V. 2005. *Leks*. Princeton: Princeton University Press.
Hoikkala, A. & Aspi, J. 1993. Criteria of female mate choice in *Drosophila littoralis, D. montana*, and *D. ezoana*. *Evolution*, **47**, 768-777.
Holdsworth, D. W. & Thornton, M. M. 2002. Micro-CT in small animal and specimen imaging. *Trends in Biotechnology*, **20**, S34-S39.
Holm, S. 1979. A simple sequentially rejective multiple test procedure. *Scandinavian journal of statistics*, **6**, 65-70.

Holveck, M. J. & Riebel, K. 2007. Preferred songs predict preferred males: consistency and repeatability of zebra finch females across three test contexts. *Animal Behaviour*, **74**, 297-309.
Holwell, G. 2008. Geographic variation in genital morphology of Ciulfina praying mantids. *Journal of Zoology*, **276**, 108-114.
Holwell, G. I. & Herberstein, M. E. 2010. Chirally dimorphic male genitalia in praying mantids (Ciulfina: Liturgusidae). *Journal of Morphology*, **271**, 1176-1184.
Holwell, G. I., Winnick, C., Tregenza, T. & Herberstein, M. E. 2010. Genital shape correlates with sperm transfer success in the praying mantis *Ciulfina klassi* (Insecta: Mantodea). *Behavioral Ecology and Sociobiology*, **64**, 617-625.
Honěk, A. 1993. Intraspecific variation in body size and fecundity in insects: a general relationship. *Oikos*, **66**, 483-492.
Hormiga, G. & Scharff, N. 2005. Monophyly and phylogenetic placement of the spider genus Labulla Simon, 1884 (Araneae, Linyphiidae) and description of the new genus Pecado. *Zoological Journal of the Linnean Society,* **143**, 359–404.
Horváth, Z. & Frank, J. 2002. Data to the biology of the red spotted bug (*Spilostethus* [= *Lygaeus*] *equestris* L., Het., Lygaeidae) causing the achene greening of alimentary cross-bred sunflower. *Cereal research Communications*, **30**, 351-358.
Hosken, D., Jones, K., Chipperfield, K. & Dixson, A. 2001. Is the bat os penis sexually selected? *Behavioral Ecology and Sociobiology*, **50**, 450-460.
Hosken, D., Meyer, E. & Ward, P. 2000. Internal female reproductive anatomy and genital interactions during copula in the yellow dung fly, *Scathophaga stercoraria* (Diptera: Scathophagidae). *Canadian Journal of Zoology*, **77**, 1975-1983.
Hosken, D., Stockley, P., Tregenza, T. & Wedell, N. 2009. Monogamy and the battle of the sexes. *Annual Review of Entomology*, **54**, 361-378.
Hosken, D. J. & Stockley, P. 2004. Sexual selection and genital evolution. *Trends in Ecology & Evolution*, **19**, 87-93.
Hosokawa, T. & Suzuki, N. 2001. Significance of prolonged copulation under the restriction of daily reproductive time in the stink bug *Megacopta punctatissima* (Heteroptera: Plataspidae). *Annals of the Entomological Society of America,* **94**, 750–754.
Hothorn, T., Bretz, F. & Westfall, P. 2008. Simultaneous inference in general parametric models. *Biometrical Journal*, **50**, 346-363.
Hotová Svádová, K., Exnerová, A., Kopeckova, M. & Stys, P. 2010. Predator dependent mimetic complexes: Do passerine birds avoid Central European red-and-black Heteroptera? *European Journal of Entomology*, **107**, 349-355.
Hotová Svádová, K., Exnerová, A., Kopečková, M. & Štys, P. 2013. How do predators learn to recognize a mimetic complex: experiments with naïve great tits and aposematic Heteroptera. *Ethology*, **119**, 814-830.
Hotzy, C., Polak, M., Rönn, J. L. & Arnqvist, G. 2012. Phenotypic engineering unveils the function of genital morphology. *Current Biology*, **22**, 2258-2261.
Houde, A. E. 1987. Mate choice based upon naturally occurring color-pattern variation in a guppy population. *Evolution*, **41**, 1-10.

House, C. M., Lewis, Z., Hodgson, D. J., Wedell, N., Sharma, M. D., Hunt, J. & Hosken, D. J. 2013. Sexual and natural selection both influence male genital evolution. *Plos One*, **8**, e63807.
House, C. M. & Simmons, L. W. 2003. Genital morphology and fertilization success in the dung beetle *Onthophagus taurus*: an example of sexually selected male genitalia. *Proceedings of the Royal Society B: Biological Sciences*, **270**, 447-455.
Howard, R. W. & Blomquist, G. J. 2005. Ecological, behavioral and biochemical aspects of insect hydrocarbons. *Annual Review of Entomology*, **50**, 371-393.
Huang, Y., Ortí, G., Sutherlin, M., Duhachek, A. & Zera, A. 2000. Phylogenetic relationships of North American field crickets inferred from mitochondrial DNA data. *Molecular phylogenetics and evolution*, **17**, 48-57.
Huber, B. A. 2002. Functional morphology of the genitalia in the spider *Spermophora senoculata* (Pholcidae, Araneae). *Zoologischer Anzeiger-A Journal of Comparative Zoology*, **241**, 105-116.
Huber, B. A., Sinclair, B. J. & Schmitt, M. 2007. The evolution of asymmetric genitalia in spiders and insects. *Biological Reviews*, **82**, 647-698.
Hunt, J., Breuker, C. J., Sadowski, J. A. & Moore, A. J. 2009. Male–male competition, female mate choice and their interaction: determining total sexual selection. *Journal of Evolutionary Biology*, **22**, 13-26.
Hunt, J., Brooks, R. & Jennions, M. D. 2005. Female mate choice as a condition-dependent life-history trait. *The American Naturalist*, **166**, 79-92.
Hurt, C. R., Stears-Ellis, S., Hughes, K. A. & Hedrick, P. W. 2004. Mating behaviour in the endangered Sonoran topminnow: speciation in action. *Animal Behaviour*, **67**, 343-351.
Hutchinson, J. 2005. Is more choice always desirable? Evidence and arguments from leks, food selection, and environmental enrichment. *Biological Reviews*, **80**, 73-92.
IBM Corp. 2010. *IBM SPSS Statistics for Windows, Version 19.0*. Armonk, NY: IBM Corp.
Ihle, M. & Forstmeier, W. 2013. Revisiting the evidence for inbreeding avoidance in zebra finches. *Behavioral Ecology*, **24**, 1356-1362.
Ilango, K. & Lane, R. 2000. Coadaptation of male aedeagal filaments and female spermathecal ducts of the old world Phlebotomine sand flies (Diptera: Psychodidae). *Journal of medical entomology*, **37**, 653-659.
Ingleby, F., Hunt, J. & Hosken, D. 2010. The role of genotype-by-environment interactions in sexual selection. *Journal of Evolutionary Biology*, **23**, 2031-2045.
Isvaran, K. & Ponkshe, A. 2013. How general is a female mating preference for clustered males in lekking species? A meta-analysis. *Animal Behaviour*, **86**, 417-425.
Itzkowitz, M., Draud, M. J., Barnes, J. L. & Haley, M. 1998. Does it matter that male beaugregory damselfish have a mate preference? *Behavioral Ecology and Sociobiology*, **42**, 149-155.
Iyengar, S. S. & Lepper, M. R. 2000. When choice is demotivating: Can one desire too much of a good thing? *Journal of Personality and Social Psychology*, **79**, 995.
Jackson, L. L. 1983. Cuticular hydrocarbons of the milkweed bug, *Oncopeltus fasciatus* by age and sex. *Insect Biochemistry*, **13**, 19-25.
Jagadeeshan, S. & Singh, R. 2006. A time-sequence functional analysis of mating behaviour and genital coupling in Drosophila: role of cryptic female choice and male

sex-drive in the evolution of male genitalia. *Journal of Evolutionary Biology*, **19**, 1058-1070.
Jäger, P. 2006. Lengthening of embolus and copulatory duct: a review of an evolutionary trend in the spider family Sparassidae (Araneae). In *Acta Zoologica Bulgarica, Supplementum 1 (Proceedings of the 22nd European Colloquium of Arachnology, Blagoevgrad 2005)*, pp. 49-62.
Jamieson, I. G. & Colgan, P. W. 1989. Eggs in the nests of males and their effect on mate choice in the three-spined stickleback. *Animal Behaviour*, **38**, 859-865.
Janetos, A. C. 1980. Strategies of female mate choice: a theoretical analysis. *Behavioral Ecology and Sociobiology*, **7**, 107-112.
Jang, Y. & Gerhardt, H. C. 2006. Divergence in female calling song discrimination between sympatric and allopatric populations of the southern wood cricket *Gryllus fultoni* (Orthoptera: Gryllidae). *Behavioral Ecology and Sociobiology*, **60**, 150-158.
Jennings, J. H., Mazzi, D., Ritchie, M. G. & Hoikkala, A. 2011. Sexual and postmating reproductive isolation between allopatric *Drosophila montana* populations suggest speciation potential. *Bmc Evolutionary Biology*, **11**.
Jennions, M., Kokko, H. & Klug, H. 2012a. The opportunity to be misled in studies of sexual selection. *Journal of Evolutionary Biology*, **25**, 591-598.
Jennions, M. D. 1998. The effect of leg band symmetry on female–male association in zebra finches. *Animal Behaviour*, **55**, 61-67.
Jennions, M. D., Backwell, P. R. Y. & Passmore, N. I. 1995. Repeatability of mate choice: the effect of size in the African painted reed frog, *Hyperolius marmoratus*. *Animal Behaviour*, **49**, 181-186.
Jennions, M. D., Kahn, A. T., Kelly, C. D. & Kokko, H. 2012b. Meta-analysis and sexual selection: past studies and future possibilities. *Evolutionary Ecology*, **26**, 1119-1151.
Jennions, M. D. & Møller, A. P. 2002. Relationships fade with time: a meta-analysis of temporal trends in publication in ecology and evolution. *Proceedings of the Royal Society B: Biological Sciences*, **269**, 43-48.
Jennions, M. D. & Petrie, M. 1997. Variation in mate choice and mating preferences: a review of causes and consequences. *Biological Reviews*, **72**, 283-327.
Jennions, M. D. & Petrie, M. 2000. Why do females mate multiply? A review of the genetic benefits. *Biological Reviews of the Cambridge Philosophical Society*, **75**, 21-64.
Jeswiet, S. B. & Godin, J. G. J. 2011. Validation of a method for quantifying male mating preferences in the guppy (*Poecilia reticulata*). *Ethology*, **117**, 422-429.
Jiang, Y., Bolnick, D. I. & Kirkpatrick, M. 2013. Assortative Mating in Animals. *The American Naturalist*, **181**, E125-E138.
Jirotkul, M. 1999. Operational sex ratio influences female preference and male–male competition in guppies. *Animal Behaviour*, **58**, 287-294.
Johnson, J. B. & Basolo, A. L. 2003. Predator exposure alters female mate choice in the green swordtail. *Behavioral Ecology*, **14**, 619-625.
Jordan, L. A. & Brooks, R. C. 2012. Recent social history alters male courtship preferences. *Evolution*, **66**, 280-287.
Jost, M. & Shaw, K. 2006. Phylogeny of Ensifera (Hexapoda: Orthoptera) using three ribosomal loci, with implications for the evolution of acoustic communication. *Molecular phylogenetics and evolution*, **38**, 510-530.

Judge, K. A., Tran, K.-C. & Gwynne, D. T. 2010. The relative effects of mating status and age on the mating behaviour of female field crickets. *Canadian Journal of Zoology*, **88**, 219-223.
Kacelnik, A., Vasconcelos, M., Monteiro, T. & Aw, J. 2011. Darwin's "tug-of-war" vs. starlings'"horse-racing": how adaptations for sequential encounters drive simultaneous choice. *Behavioral Ecology and Sociobiology*, **65**, 547-558.
Kahn, A. T., Mautz, B. & Jennions, M. D. 2009. Females prefer to associate with males with longer intromittent organs in mosquitofish. *Biology Letters*, rsbl20090637.
Kamimura, Y. 2003. Effects of broken male intromittent organs on the sperm storage capacity of female earwigs, *Euborellia plebeja*. *Journal of Ethology*, **21**, 29-35.
Kamimura, Y. 2005. Last-male paternity of *Euborellia plebeja*, an earwig with elongated genitalia and sperm-removal behavior. *Journal of Ethology*, **23**, 35-41.
Kamiya, T., O'Dwyer, K., Westerdahl, H., Senior, A. & Nakagawa, S. 2014. A quantitative review of MHC-based mating preference: the role of diversity and dissimilarity. *Molecular Ecology*, **23**, 5151-5163.
Kasumovic, M. & Andrade, M. 2009. A change in competitive context reverses sexual selection on male size. *Journal of Evolutionary Biology*, **22**, 324-333.
Kawano, K. 2002. Character displacement in giant rhinoceros beetles. *The American Naturalist*, **159**, 255-271.
Kawano, K. 2003. Character displacement in stag beetles (Coleoptera: Lucanidae). *Annals of the Entomological Society of America*, **96**, 503-511.
Kelly, C. D. & Jennions, M. D. 2011. Sexual selection and sperm quantity: meta-analyses of strategic ejaculation. *Biological Reviews*, **86**, 863-884.
King, B., Saporito, K., Ellison, J. & Bratzke, R. 2005. Unattractiveness of mated females to males in the parasitoid wasp *Spalangia endius*. *Behavioral Ecology and Sociobiology*, **57**, 350-356.
Kingsolver, J. G., Diamond, S. E., Siepielski, A. M. & Carlson, S. M. 2012. Synthetic analyses of phenotypic selection in natural populations: lessons, limitations and future directions. *Evolutionary Ecology*, **26**, 1101-1118.
Kingsolver, J. G., Hoekstra, H. E., Hoekstra, J. M., Berrigan, D., Vignieri, S. N., Hill, C., Hoang, A., Gibert, P. & Beerli, P. 2001. The strength of phenotypic selection in natural populations. *The American Naturalist*, **157**, 245-261.
Kirkpatrick, M., Price, T. & Arnold, S. J. 1990. The Darwin-Fisher theory of sexual selection in monogamous birds. *Evolution*, 180-193.
Kjer, K. M., Carle, F. L., Litman, J. & Ware, J. 2006. A molecular phylogeny of Hexapoda. *Arthropod Systematics & Phylogeny*, **64**, 35-44.
Klingenberg, C. P. 2010. Evolution and development of shape: integrating quantitative approaches. *Nature Reviews Genetics*, **11**, 623-635.
Klingenberg, C. P. 2011. MorphoJ: an integrated software package for geometric morphometrics. *Molecular Ecology Resources*, **11**, 353-357.
Klingenberg, C. P., Barluenga, M. & Meyer, A. 2002. Shape analysis of symmetric structures: quantifying variation among individuals and asymmetry. *Evolution*, **56**, 1909-1920.

Klingenberg, C. P. & McIntyre, G. S. 1998. Geometric morphometrics of developmental instability: analyzing patterns of fluctuating asymmetry with Procrustes methods. *Evolution*, **52**, 1363-1375.
Klug, H., Heuschele, J., Jennions, M. & Kokko, H. 2010. The mismeasurement of sexual selection. *Journal of Evolutionary Biology*, **23**, 447-462.
Knoflach, B. & van Harten, A. 2001. *Tidarren argo* sp nov (Araneae : Theridiidae) and its exceptional copulatory behaviour: emasculation, male palpal organ as a mating plug and sexual cannibalism. *Journal of Zoology*, **254**, 449-459.
Kokko, H., Brooks, R., Jennions, M. D. & Morley, J. 2003. The evolution of mate choice and mating biases. *Proceedings of the Royal Society B: Biological Sciences*, **270**, 653-664.
Kokko, H. & Jennions, M. D. 2008. Parental investment, sexual selection and sex ratios. *Journal of Evolutionary Biology*, **21**, 919-948.
Kokko, H. & Jennions, M. D. 2015. Describing mate choice in a biased world. *Behavioral Ecology*, **26**, 320-321.
Kokko, H., Jennions, M. D. & Brooks, R. 2006. Unifying and testing models of sexual selection. *Annual Review of Ecology, Evolution, and Systematics*, 43-66.
Kokko, H. & Johnstone, R. A. 2002. Why is mutual mate choice not the norm? Operational sex ratios, sex roles and the evolution of sexually dimorphic and monomorphic signalling. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **357**, 319-330.
Kokko, H., Klug, H. & Jennions, M. D. 2012. Unifying cornerstones of sexual selection: operational sex ratio, Bateman gradient and the scope for competitive investment. *Ecology Letters*, **15**, 1340-1351.
Kokko, H. & Mappes, J. 2005. Sexual selection when fertilization is not guaranteed. *Evolution*, **59**, 1876-1885.
Koricheva, J. 2003. Non-significant results in ecology: a burden or a blessing in disguise? *Oikos*, **102**, 397-401.
Koricheva, J., Gurevitch, J. & Mengeresen, K. 2013. *Handbook of meta-analysis in ecology and evolution*. Princeton: Princeton University Press.
Koshio, C., Muraji, M., Tatsuta, H. & Kudo, S.-i. 2007. Sexual selection in a moth: effect of symmetry on male mating success in the wild. *Behavioral Ecology*, **18**, 571-578.
Kostarakos, K., Hartbauer, M. & Römer, H. 2008. Matched filters, mate choice and the evolution of sexually selected traits. *Plos One*, **3**.
Kozak, G. M., Head, M. L. & Boughman, J. W. 2011. Sexual imprinting on ecologically divergent traits leads to sexual isolation in sticklebacks. *Proceedings of the Royal Society B: Biological Sciences*, **278**, 2604-2610.
Kozak, G. M., Reisland, M. & Boughmann, J. W. 2009. Sex differences in mate recognition and conspecific preference in species with mutual mate choice. *Evolution*, **63**, 353-365.
Kraaijeveld, K., Kraaijeveld-Smit, F. J. L. & Komdeur, J. 2007. The evolution of mutual ornamentation. *Animal Behaviour*, **74**, 657-677.
Kraak, S. B. M. & Bakker, T. C. M. 1998. Mutual mate choice in sticklebacks: attractive males choose big females, which lay big eggs. *Animal Behaviour*, **56**, 859-866.

Kugelberg, O. 1973. Laboratory studies on the effects of different natural foods on the reproductive biology of *Lygaeus equestris* (L.) (Het. Lygaeidae). *Entomologica Scandinavica*, **4**, 181-190.
Kugelberg, O. 1974. Laboratory studies on the feeding preference and feeding behaviour in *Lygaeus equestris* (L.) (Het. Lygaeidae) 1. *Insect Systematics & Evolution*, **5**, 49-55.
Kugelberg, O. 1977. Distribution, feeding habits and dispersal of *Lygaeus equestris* (Heteroptera) larvae in relation to food supply. *Oikos*, **29**, 398-406.
Kullmann, H. & Klemme, B. 2007. Female mating preference for own males on species and population level in Chromaphyosemion killifishes (Cyprinodontiformes, Nothobranchiidae). *Zoology*, **110**, 377-386.
Kumano, N., Haraguchi, D. & Kohama, T. 2009. Female mating status does not affect male mating behavior in the West Indian sweetpotato weevil, *Euscepes postfasciatus*. *Entomologia Experimentalis et Applicata*, **131**, 39-45.
Kumaran, N., Balagawi, S., Schutze, M. K. & Clarke, A. R. 2013. Evolution of lure response in tephritid fruit flies: phytochemicals as drivers of sexual selection. *Animal Behaviour*, **85**, 781-789.
Kvarnemo, C. & Simmons, L. W. 1999. Variance in female quality, operational sex ratio and male mate choice in a bushcricket. *Behavioral Ecology and Sociobiology*, **45**, 245-252.
Kvarnemo, C. & Simmons, L. W. 2013. Polyandry as a mediator of sexual selection before and after mating. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **368**.
Kyogoku, D. & Sota, T. 2015. Exaggerated male genitalia intensifies interspecific reproductive interference by damaging heterospecific female genitalia. *Journal of Evolutionary Biology*, In press.
Lachaise, D., Harry, M., Solignac, M., Lemeunier, F., Benassi, V. & Cariou, M.-L. 2000. Evolutionary novelties in islands: *Drosophila santomea*, a new melanogaster sister species from Sao Tome. *Proceedings of the Royal Society B: Biological Sciences*, **267**, 1487-1495.
Lande, R. & Arnold, S. J. 1983. The measurement of selection on correlated characters. *Evolution*, **37**, 1210-1226.
Langerhans, R. B., Layman, C. A. & DeWitt, T. J. 2005. Male genital size reflects a tradeoff between attracting mates and avoiding predators in two live-bearing fish species. *Proceedings of the National Academy of Sciences*, **102**, 7618-7623.
Lawing, A. M. & Polly, P. D. 2010. Geometric morphometrics: recent applications to the study of evolution and development. *Journal of Zoology*, **280**, 1-7.
Lehmann, G. U. C. 2007. Density-dependent plasticity of sequential mate choice in a bushcricket (Orthoptera: Tettigoniidae). *Australian Journal of Zoology*, **55**, 123-130.
Lehmann, G. U. C. & Lehmann, A. W. 2008. Bushcricket song as a clue for spermatophore size? *Behavioral Ecology and Sociobiology*, **62**, 569-578.
Lenton, A. P., Fasolo, B. & Todd, P. M. 2009. The relationship between number of potential mates and mating skew in humans. *Animal Behaviour*, **77**, 55-60.
Lenton, A. P. & Francesconi, M. 2011. Too much of a good thing? Variety is confusing in mate choice. *Biology Letters*, rsbl20110098.

Lessells, C. & Boag, P. T. 1987. Unrepeatable repeatabilities: a common mistake. *The Auk*, **104**, 116-121.
Lowe, T., Garwood, R. J., Simonsen, T. J., Bradley, R. S. & Withers, P. J. 2013. Metamorphosis revealed: time-lapse three-dimensional imaging inside a live chrysalis. *Journal of The Royal Society Interface*, **10**.
Ludwig, W. 1926. Untersuchungen über den Copulationsapparat der Baumwanzen. *Zoomorphology*, **5**, 291-380.
MacLaren, R. D. & Rowland, W. J. 2006. Differences in female preference for male body size in *Poecilia latipinna* using simultaneous versus sequential stimulus presentation designs. *Behaviour*, **143**, 273-292.
Martel, V. & Boivin, G. 2011. Do choice tests really test choice? *Journal of Insect Behavior*, **24**, 329-336.
Masly, J. P. 2011. 170 years of 'lock-and-key': genital morphology and reproductive isolation. *International Journal of Evolutionary Biology,* **2012**, 247352.
Matsumura, Y. & Akimoto, S.-i. 2009. Mating behavior and genital damage during copulation in the leaf beetle *Lema coronata* (Chrysomelidae: Criocerinae). *Entomological science*, **12**, 215-217.
Matsumura, Y. & Yoshizawa, K. 2010. Insertion and withdrawal of extremely elongated genitalia: a simple mechanism with a highly modified morphology in the leaf beetle, *Lema coronata*. *Biological Journal of the Linnean Society*, **99**, 512-520.
Matsumura, Y., Yoshizawa, K., Machida, R., Mashimo, Y., Dallai, R., Gottardo, M., Kleinteich, T., Michels, J., Gorb, S. N. & Beutel, R. G. 2014. Two intromittent organs in *Zorotypus caudelli* (Insecta, Zoraptera): the paradoxical coexistence of an extremely long tube and a large spermatophore. *Biological Journal of the Linnean Society*, **112**, 40-54.
Mattei, A. L., Riccio, M. L., Avila, F. W. & Wolfner, M. F. 2015. Integrated 3D view of postmating responses by the *Drosophila melanogaster* female reproductive tract, obtained by micro-computed tomography scanning. *Proceedings of the National Academy of Sciences*, 201505797.
Mautz, B. S. & Sakaluk, S. K. 2008. The effects of age and previous mating experience on pre-and post-copulatory mate choice in female house crickets (*Acheta domesticus* L.). *Journal of Insect Behavior*, **21**, 203-212.
Mautz, B. S., Wong, B. B., Peters, R. A. & Jennions, M. D. 2013. Penis size interacts with body shape and height to influence male attractiveness. *Proceedings of the National Academy of Sciences*, **110**, 6925-6930.
Mayr, E. 1963. *Animal species and evolution*. Cambridge, Mass: Belknap.
Mazzi, D. 2004. Parasites make male pipefish careless. *Journal of Evolutionary Biology*, **17**, 519-527.
McCauley, D. E. & Lawson, E. C. 1986. Mating reduces predation on male milkweed beetles. *American Naturalist*, **127**, 112–117.
McCracken, K. G., Wilson, R. E., McCracken, P. J. & Johnson, K. P. 2001. Are ducks impressed by drakes' display? *Nature*, **413**, 128.
McLain, D. K. 1980. Female choice and the adaptive significance of prolonged copulation in *Nezara viridula* (Hemiptera: Pentatomidae). *Psyche,* **87**, 325–336.

McLain, D. K. 1984. Host plant density and territorial behavior of the seed bug, *Neacoryphus bicrucis* (Hemiptera: Lygaeidae). *Behavioral Ecology and Sociobiology*, **14**, 181-187.
McLain, D. K. 1986. Resource patchiness and variation in the intensity of sexual selection in a resource defending polygynous insect species. *Oikos*, **47**, 19-25.
Mclain, D., K. 1989. Prolonged copulation as a post-insemination guarding tactic in a natural population of the ragwort seed bug. *Animal Behaviour*, **38**, 659-664.
McLain, D. K. 1992. Population density and the intensity of sexual selection on body length in spatially or temporally restricted natural populations of a seed bug. *Behavioral Ecology and Sociobiology*, **30**, 347-356.
McLain, D. K. & Pratt, A. E. 1999. The cost of sexual coercion and heterospecific sexual harassment on the fecundity of a host-specific, seed-eating insect (*Neacoryphus bicrucis*). *Behavioral Ecology and Sociobiology*, **46**, 164-170.
McNamara, K. B., Jones, T. M. & Elgar, M. A. 2004. Female reproductive status and mate choice in the hide beetle, *Dermestes maculatus*. *Journal of Insect Behavior*, **17**, 337-352.
McPeek, M. A., Shen, L. & Farid, H. 2009. The correlated evolution of three-dimensional reproductive structures between male and female damselflies. *Evolution*, **63**, 73-83.
McPeek, M. A., Shen, L., Torrey, J. Z. & Farid, H. 2008. The tempo and mode of three-dimensional morphological evolution in male reproductive structures. *The American Naturalist*, **171**, E158-E178.
Meffert, L. M. & Regan, J. L. 2002. A test of speciation via sexual selection on female preferences. *Animal Behaviour*, **64**, 955-965.
Mendelson, T. C. & Shaw, K. L. 2012. The (mis) concept of species recognition. *Trends in Ecology & Evolution*, **27**, 421-427.
Mery, F., Varela, S. A., Danchin, É., Blanchet, S., Parejo, D., Coolen, I. & Wagner, R. H. 2009. Public versus personal information for mate copying in an invertebrate. *Current Biology*, **19**, 730-734.
Metscher, B. D. 2009. MicroCT for comparative morphology: simple staining methods allow high-contrast 3D imaging of diverse non-mineralized animal tissues. *BMC physiology*, **9**.
Meyer, A. & Lydeard, C. 1993. The evolution of copulatory organs, internal fertilization, placentae and viviparity in killifishes (Cyprinodontiformes) inferred from a DNA phylogeny of the tyrosine kinase gene X-src. *Proceedings of the Royal Society B: Biological Sciences*, **254**, 153-162.
Micholitsch, T., Krugel, P. & Pass, G. 2000. Insemination and fertilization in the seed bug *Lygaeus simulans* (Heteroptera: Lygaeidae). *European Journal of Entomology*, **97**, 13-18.
Milinski, M. & Bakker, T. C. 1992. Costs influence sequential mate choice in sticklebacks, *Gasterosteus aculeatus*. *Proceedings of the Royal Society B: Biological Sciences*, **250**, 229-233.
Miller, C. W. & Svensson, E. I. 2014. Sexual selection in complex environments. *Annual Review of Entomology*, **59**, 427-445.

Miller, P. 1991. The structure and function of the genitalia in the Libellulidae (Odonata). *Zoological Journal of the Linnean Society*, **102**, 43-73.
Milner, R. N., Detto, T., Jennions, M. D. & Backwell, P. R. 2010. Experimental evidence for a seasonal shift in the strength of a female mating preference. *Behavioral Ecology*, **21**, 311-316.
Mitchell-Olds, T. & Shaw, R. G. 1987. Regression analysis of natural selection: statistical inference and biological interpretation. *Evolution*, **41**, 1149-1161.
Miya, M., Takeshima, H., Endo, H., Ishiguro, N. B., Inoue, J. G., Mukai, T., Satoh, T. P., Yamaguchi, M., Kawaguchi, A. & Mabuchi, K. 2003. Major patterns of higher teleostean phylogenies: a new perspective based on 100 complete mitochondrial DNA sequences. *Molecular phylogenetics and evolution*, **26**, 121-138.
Moher, D., Liberati, A., Tetzlaff, J. & Altman, D. G. 2009. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *Annals of internal medicine*, **151**, 264-269.
Møller, A. P. 1994. Repeatability of female choice in a monogamous swallow. *Animal Behaviour*, **47**, 643-648.
Moore, A. J. & Moore, P. J. 1988. Female strategy during mate choice: threshold assessment. *Evolution*, **42**, 387-391.
Moore, P. J. & Moore, A. J. 2001. Reproductive aging and mating: the ticking of the biological clock in female cockroaches. *Proceedings of the National Academy of Sciences*, **98**, 9171-9176.
Moreno-García, M. & Cordero, C. 2008. On the function of male genital claspers in *Stenomacra marginella* (Heteroptera: Largidae). *Journal of Ethology*, **26**, 255-260.
Morrissey, M. B. & Hadfield, J. D. 2011. Directional selection in temporally replicated studies is remarkably consistent. *Evolution*, **66**, 435-442.
Morrissey, M. B. & Sakrejda, K. 2013. Unification of regression-based methods for the analysis of natural selection. *Evolution*, **67**, 2094-2100.
Moya-Laraño, J., El-Sayyid, M. E. T. & Fox, C. W. 2007. Smaller beetles are better scramble competitors at cooler temperatures. *Biology Letters*, **3**, 475-478.
Muller, P. & Robert, D. 2002. Death comes suddenly to the unprepared: singing crickets, call fragmentation, and parasitoid flies. *Behavioral Ecology*, **13**, 598-606.
Mundry, R. & Nunn, C. L. 2009. Stepwise model fitting and statistical inference: turning noise into signal pollution. *The American Naturalist*, **173**, 119-123.
Murata, A., Imafuku, M. & Abe, N. 2001. Copulation by the barnacle *Tetraclita japonica* under natural conditions. *Journal of Zoology*, **253**, 275-280.
Mutanen, M., Kaitala, A. & Mönkkönen, M. 2006. Genital variation within and between three closely related Euxoa moth species: testing the lock-and-key hypothesis. *Journal of Zoology*, **268**, 109-119.
Mutanen, M., Wahlberg, N. & Kaila, L. 2010. Comprehensive gene and taxon coverage elucidates radiation patterns in moths and butterflies. *Proceedings of the Royal Society B: Biological Sciences*, **277**, 2839-2848.
Nakagawa, S. & Poulin, R. 2012. Meta-analytic insights into evolutionary ecology: an introduction and synthesis. *Evolutionary Ecology*, **26**, 1085-1099.
Nakagawa, S. & Santos, E. S. 2012. Methodological issues and advances in biological meta-analysis. *Evolutionary Ecology*, **26**, 1253-1274.

Nakagawa, S. & Schielzeth, H. 2010. Repeatability for Gaussian and non-Gaussian data: a practical guide for biologists. *Biological Reviews*, **85**, 935-956.
Near, T. J., Eytan, R. I., Dornburg, A., Kuhn, K. L., Moore, J. A., Davis, M. P., Wainwright, P. C., Friedman, M. & Smith, W. L. 2012. Resolution of ray-finned fish phylogeny and timing of diversification. *Proceedings of the National Academy of Sciences*, **109**, 13698-13703.
Neufeld, C. J. & Palmer, A. R. 2008. Precisely proportioned: intertidal barnacles alter penis form to suit coastal wave action. *Proceedings of the Royal Society B: Biological Sciences*, **275**, 1081-1087.
Orr, T. J. & Brennan, P. L. 2015. Sperm storage: distinguishing selective processes and evaluating criteria. *Trends in Ecology & Evolution*, In press.
Otronen, M. 1998. Male asymmetry and postcopulatory sexual selection in the fly *Dryomyza anilis*. *Behavioral Ecology and Sociobiology*, **42**, 185-191.
Owen, M. A., Rohrer, K. & Howard, R. D. 2012. Mate choice for a novel male phenotype in zebrafish, *Danio rerio*. *Animal Behaviour*, **83**, 811-820.
Page Jr, R. E. 1986. Sperm utilization in social insects. *Annual Review of Entomology*, **31**, 297-320.
Paradis, E., Claude, J. & Strimmer, K. 2004. APE: analyses of phylogenetics and evolution in R language. *Bioinformatics*, **20**, 289-290.
Parker, G. A. 1970. Sperm competition and its evolutionary consequences in the insects. *Biological Reviews*, **45**, 525-567.
Parker, G. A. & Birkhead, T. R. 2013. Polyandry: the history of a revolution. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **368**, 20120335.
Parri, S., Alatalo, R. V., Kotiaho, J. & Mappes, J. 1997. Female choice for male drumming in the wolf spider *Hygrolycosa rubrofasciata*. *Animal Behaviour*, **53**, 305-312.
Pélissié, B., Jarne, P., Sarda, V. & David, P. 2014. Disentangling precopulatory and postcopulatory sexual selection in polyandrous species. *Evolution*, **68**, 1320-1331.
Peretti, A. V. & Carrera, P. 2005. Female control of mating sequences in the mountain scorpion *Zabius fuscus*: males do not use coercion as a response to unreceptive females. *Animal Behaviour*, **69**, 453-462.
Perreau, M. & Tafforeau, P. 2011. Virtual dissection using phase-contrast X-ray synchrotron microtomography: reducing the gap between fossils and extant species. *Systematic Entomology*, **36**, 573-580.
Perry, J. C., Sirot, L. & Wigby, S. 2013. The seminal symphony: how to compose an ejaculate. *Trends in Ecology & Evolution*, **28**, 414-422.
Phelps, S. M., Rand, A. S. & Ryan, M. J. 2006. A cognitive framework for mate choice and species recognition. *The American Naturalist*, **167**, 28-42.
Pischedda, A. & Rice, W. R. 2012. Partitioning sexual selection into its mating success and fertilization success components. *Proceedings of the National Academy of Sciences*, **109**, 2049-2053.
Pitcher, T. E., Neff, B. D., Rodd, F. H. & Rowe, L. 2003. Multiple mating and sequential mate choice in guppies: females trade up. *Proceedings of the Royal Society B: Biological Sciences*, **270**, 1623-1629.

Pitnick, S. & Brown, W. D. 2000. Criteria for demonstrating female sperm choice. *Evolution*, **54**, 1052-1056.
Pitnick, S. & Hosken, D. J. 2010. Postcopulatory sexual selection. In: *Evolutionary behavioral ecology* (Ed. by D. Westneat & C. Fox), pp. 379-399. New York: Oxford University Press.
Plath, M., Seggel, U., Burmeister, H., Heubel, K. U. & Schlupp, I. 2006. Choosy males from the underground: male mating preferences in surface-and cave-dwelling Atlantic mollies (*Poecilia mexicana*). *Naturwissenschaften*, **93**, 103-109.
Priklopil, T., Kisdi, E. & Gyllenberg, M. 2015. Evolutionarily stable mating decisions for sequentially searching females and the stability of reproductive isolation by assortative mating. *Evolution*, In press.
Procter, D., Moore, A. & Miller, C. 2012. The form of sexual selection arising from male–male competition depends on the presence of females in the social environment. *Journal of Evolutionary Biology*, **25**, 803-812.
Punzalan, D., Rodd, F. H. & Rowe, L. 2010. Temporally variable multivariate sexual selection on sexually dimorphic traits in a wild insect population. *The American Naturalist*, **175**, 401-414.
R Development Core Team. 2012. *R: A language and environment for statistical computing.* Vienna, Austria: R Foundation for Statistical Computing.
R Development Core Team. 2014. *R: A language and environment for statistical computing.* Vienna, Austria: R Foundation for Statistical Computing.
Rabitsch W & J, D. 2007. Die Ritterwanze *Lygaeus equestris* LINNAEUS, 1758 (Heteroptera: Lygaeidae)- das Insekt des Jahres 2007. *Betrage zur Entomofaunistik*, **8**, 212-218.
Reading, K. L. & Backwell, P. R. Y. 2007. Can beggars be choosers? Male mate choice in a fiddler crab. *Animal Behaviour*, **74**, 867-872.
Regier, J. C. & Shultz, J. W. 1998. Molecular phylogeny of arthropods and the significance of the Cambrian “explosion” for molecular systematics. *American Zoologist*, **38**, 918-928.
Regier, J. C., Zwick, A., Cummings, M. P., Kawahara, A. Y., Cho, S., Weller, S., Roe, A., Baixeras, J., Brown, J. W. & Parr, C. 2009. Toward reconstructing the evolution of advanced moths and butterflies (Lepidoptera: Ditrysia): an initial molecular study. *Bmc Evolutionary Biology*, **9**.
Rhainds, M. 2010. Female mating failures in insects. *Entomologia Experimentalis et Applicata*, **136**, 211-226.
Ritchie, M. G. 1996. The shape of female mating preferences. *Proceedings of the National Academy of Sciences*, **93**, 14628-14631.
Robertson, H. M. & Paterson, H. E. 1982. Mate recognition and mechanical isolation in Enallagma damselflies (Odonata: Coenagrionidae). *Evolution*, **36**, 243-250.
Robinson, M. R., Sander van Doorn, G., Gustafsson, L. & Qvarnström, A. 2012. Environment-dependent selection on mate choice in a natural population of birds. *Ecology Letters*, **15**, 611-618.
Rodriguez, S. 2000. On the fore legs of seed bugs (Heteroptera: Lygaeidae): aggression and allometric scaling in *Scolopostethus affinis* Schilling. *Journal of the Kansas Entomological Society*, **73**, 6-10.

Rodriguez, V. 1995. Relation of flagellum length to reproductive success in male *Chelymorpha alternans* Boheman (Coleoptera: Chrysomelidae: Cassidinae). *The Coleopterists' Bulletin*, **49**, 201-205.
Rodriguez, V., Windsor, D. & Eberhard, W. 2004. Tortoise beetle genitalia and demonstrations of a sexually selected advantage for flagellum length in *Chelymorpha alternans* (Chrysomelidae, Cassidini, Stolaini). In: *New developments in the biology of Chrysomelidae* (Ed. by P. Jolivet, Santiago-Blay, J. A. & Schmitt, M.), pp. 739-748. The Hague, The Netherlands: SPB Academic Publishing.
Römer, H. & Krusch, M. 2000. A gain-control mechanism for processing of chorus sounds in the afferent auditory pathway of the bushcricket *Tettigonia viridissima* (Orthoptera; Tettigoniidae). *Journal of Comparative Physiology A*, **186**, 181-191.
Rönn, J., Katvala, M. & Arnqvist, G. 2007. Coevolution between harmful male genitalia and female resistance in seed beetles. *Proceedings of the National Academy of Sciences*, **104**, 10921-10925.
Rose, E., Paczolt, K. A. & Jones, A. G. 2013. The contributions of premating and postmating selection episodes to total selection in sex-role-reversed Gulf pipefish. *The American Naturalist*, **182**, 410-420.
Rosenberg, M. S., Adams, D. C. & Gurevitch, J. 2000. *MetaWin: statistical software for meta-analysis*: Sinauer Associates Sunderland, Massachusetts, USA.
Rosenblum, E. B. 2008. Preference for local mates in a recently diverged population of the Lesser Earless Lizard (*Holbrookia maculata*) at White Sands. *Journal of Herpetology*, **42**, 572-583.
Rosvall, K. A. 2011. Intrasexual competition in females: evidence for sexual selection? *Behavioral Ecology*, **22**, 1131-1140.
Rowe, L. & Arnqvist, G. 2012. Sexual selection and the evolution of genital shape and complexity in water striders. *Evolution*, **66**, 40-54.
Rowland, W. J. 1982. Mate choice by male sticklebacks, *Gasterosteus aculeatus*. *Animal Behaviour*, **30**, 1093-1098.
Rutstein, A. N., Brazill-Boast, J. & Griffith, S. C. 2007. Evaluating mate choice in the zebra finch. *Animal Behaviour*, **74**, 1277-1284.
Ryan, M. J. 1985. *The túngara frog: a study in sexual selection and communication*: University of Chicago Press.
Ryan, M. J. & Keddy-Hector, A. 1992. Directional patterns of female mate choice and the role of sensory biases. *The American Naturalist*, **139**, S4-S35.
Ryan, M. J. & Rand, A. S. 1993. Species recognition and sexual selection as a unitary problem in animal communication. *Evolution*, **47**, 647-657.
Sæther, B.-E. & Engen, S. 2015. The concept of fitness in fluctuating environments. *Trends in Ecology & Evolution*, In press.
Sakaluk, S. K., Bangert, P. J., Eggert, A.-K., Gack, C. & Swanson, L. V. 1995. The gin trap as a device facilitating coercive mating in sagebrush crickets. *Proceedings of the Royal Society B: Biological Sciences*, **261**, 65-71.
Santos, E. S., Scheck, D. & Nakagawa, S. 2011. Dominance and plumage traits: meta-analysis and metaregression analysis. *Animal Behaviour*, **82**, 3-19.

Scheibehenne, B., Greifeneder, R. & Todd, P. M. 2010. Can there ever be too many options? A meta-analytic review of choice overload. *Journal of Consumer Research*, **37**, 409-425.
Schluter, D. 1988. Estimating the form of natural selection on a quantitative trait. *Evolution*, **42**, 849-861.
Schluter, D. & Nychka, D. 1994. Exploring fitness surfaces. *The American Naturalist*, **143**, 597-616.
Schofl, G., Dill, A., Heckel, D. G. & Groot, A. T. 2011. Allochronic separation versus mate choice: nonrandom patterns of mating between fall armyworm host strains. *The American Naturalist*, **177**, 470-485.
Schöfl, G. & Taborsky, M. 2002. Prolonged tandem formation in firebugs (*Pyrrhocoris apterus*) serves mate-guarding. *Behavioral Ecology and Sociobiology,* **52**, 426–433.
Schuh, R. T. & Slater, J. A. 1995. *True bugs of the world (Hemiptera: Heteroptera): classification and natural history*: Cornell University Press.
Schwartz, J. J., Buchanan, B. W. & Gerhardt, H. C. 2001. Female mate choice in the gray treefrog (*Hyla versicolor*) in three experimental environments. *Behavioral Ecology and Sociobiology*, **49**, 443-455.
Scudder, G. 1971. Comparative morphology of insect genitalia. *Annual Review of Entomology*, **16**, 379-406.
Seehausen, O., Van Alphen, J. J. & Witte, F. 1997. Cichlid fish diversity threatened by eutrophication that curbs sexual selection. *Science*, **277**, 1808-1811.
Shackleton, M. A., Jennions, M. D. & Hunt, J. 2005. Fighting success and attractiveness as predictors of male mating success in the black field cricket, *Teleogryllus commodus*: the effectiveness of no-choice tests. *Behavioral Ecology and Sociobiology*, **58**, 1-8.
Shapiro, A. M. & Porter, A. H. 1989. The lock-and-key hypothesis: evolutionary and biosystematic interpretation of insect genitalia. *Annual Review of Entomology*, **34**, 231-245.
Sharma, M. D., Tregenza, T. & Hosken, D. J. 2010. Female mate preferences in *Drosophila simulans*: evolution and costs. *Journal of Evolutionary Biology*, **23**, 1672-1679.
Shelly, T. E. & Bailey, W. J. 1992. Experimental manipulation of mate choice by male katydids: the effect of female encounter rate. *Behavioral Ecology and Sociobiology*, **30**, 277-282.
Shuker, D. M. 2010. Sexual selection: endless forms or tangled bank? *Animal Behaviour*, **79**, e11-e17.
Shuker, D. M., Ballantyne, G. A. & Wedell, N. 2006. Variation in the cost to females of the sexual conflict over mating in the seed bug, *Lygaeus equestris*. *Animal Behaviour*, **72**, 313-321.
Shuker, D. M. & Day, T. H. 2001. The repeatability of a sexual conflict over mating. *Animal Behaviour*, **61**, 755-762.
Shuker, D. M. & Day, T. H. 2002. Mate sampling and the sexual conflict over mating in seaweed flies. *Behavioral Ecology*, **13**, 83-86.
Siepielski, A. M., DiBattista, J. D., Evans, J. A. & Carlson, S. M. 2010. Differences in the temporal dynamics of phenotypic selection among fitness components in the wild. *Proceedings of the Royal Society B: Biological Sciences*, **282**, rspb20101973.

Siepielski, A. M., Gotanda, K. M., Morrissey, M. B., Diamond, S. E., DiBattista, J. D. & Carlson, S. M. 2013. The spatial patterns of directional phenotypic selection. *Ecology Letters*, **16**, 1382-1392.
Sih, A. & Krupa, J. J. 1992. Predation risk, food deprivation and non-random mating by size in the stream water strider, *Aquarius remigis*. *Behavioral Ecology and Sociobiology*, **31**, 51-56.
Sillén-Tullberg, B. 1981. Prolonged copulation: A male 'postcopulatory' strategy in a promiscuous species, *Lygaeus equestris* (Heteroptera: Lygaeidae). *Behavioral Ecology and Sociobiology*, **9**, 283-289.
Sillén-Tullberg, B. 1985. Higher survival of an aposematic than of a cryptic form of a distasteful bug. *Oecologia*, **67**, 411-415.
Sillén-Tullberg, B., Gamberale-Stille, G. & Solbreck, C. 2000. Effects of food plant and group size on predator defence: differences between two co-occurring aposematic Lygaeinae bugs. *Ecological Entomology*, **25**, 220-225.
Sillén-Tullberg, B. & Solbreck, C. 1990. Population dynamics of a seed feeding bug, *Lygaeus equestris*. 2. Temporal dynamics. *Oikos*, **58**, 210-218.
Sillén-Tullberg, B., Wiklund, C. & Järvi, T. 1982. Aposematic coloration in adults and larvae of *Lygaeus equestris* and Its bearing on müllerian mimicry: an experimental study on predation on living bugs by the great tit *Parus major*. *Oikos*, **39**, 131-136.
Silva, K., Vieira, M. N., Almada, V. C. & Monteiro, N. M. 2007. The effect of temperature on mate preferences and female-female interactions in *Syngnathus abaster*. *Animal Behaviour*, **74**, 1525-1533.
Simmons, L. W. 2001. *Sperm competition and its evolutionary consequences in the insects*: Princeton University Press.
Simmons, L. W. 2014. Sexual selection and genital evolution. *Austral Entomology*, **53**, 1-17.
Simmons, L. W. & Firman, R. C. 2014. Experimental evidence for the evolution of the mammalian baculum by sexual selection. *Evolution*, **68**, 276-283.
Simmons, L. W. & García-González, F. 2011. Experimental coevolution of male and female genital morphology. *Nature communications*, **2**.
Simmons, L. W., House, C. M., Hunt, J. & García-González, F. 2009. Evolutionary response to sexual selection in male genital morphology. *Current Biology*, **19**, 1442-1446.
Simons, M. J. & Verhulst, S. 2011. Zebra finch females prefer males with redder bills independent of song rate—a meta-analysis. *Behavioral Ecology*, **22**, 755-762.
Simonsen, T. J. & Kitching, I. J. 2014. Virtual dissections through micro-CT scanning: a method for non-destructive genitalia 'dissections' of valuable Lepidoptera material. *Systematic Entomology*, **39**, 606-618.
Singh, B. N. & Sisodia, S. 1999. Mating propensity in *Drosophila bipectinata* under different sex-ratios and choice situations. *Current Science*, **76**, 222-225.
Sivinski, J. 1980. The effects of mating on predation in the stick insect *Diapheromera veliei* Walsh (Phasmatodea: Heteronemiidae). *Annals of the Entomological Society of America,* **73**, 553–556.
Snead, J. & Alcock, J. 1985. Aggregation formation and assortative mating in two meloid beetles. *Evolution*, **39**, 1123–1131.

Snodgrass, R. E. 1935. *Principles of insect morphology*: McGraw-Hill Book Company, Inc.
Snow, L. S., Abdel-Mesih, A. & Andrade, M. C. 2006. Broken Copulatory Organs are Low-Cost Adaptations to Sperm Competition in Redback Spiders. *Ethology,* **112**, 379–389.
Solbreck, C. 1972. Sexual cycle, and changes in feeding activity and fat body size in relation to migration in *Lygaeus equestris* (L.) (Heteroptera., Lygaeidae). *Insect Systematics & Evolution*, **3**, 267-274.
Solbreck, C. & Kugelberg, O. 1972. Field observations on the seasonal occurrence of *Lygaeus eguestris* (L.) (Heteroptera., Lygaeidae) with special reference to food plant phenology. *Insect Systematics & Evolution*, **3**, 189-210.
Solbreck, C., Olsson, R., Anderson, D. B. & Förare, J. 1989. Size, life history and responses to food shortage in two geographical strains of a seed bug *Lygaeus equestris*. *Oikos*, **55**, 387-396.
Solbreck, C. & Sillén-Tullberg, B. 1981. Control of Diapause in a "Monovoltine" Insect, *Lygaeus equestris* (Heteroptera). *Oikos*, **36**, 68-74.
Solbreck, C. & Sillén-Tullberg, B. 1990. Population dynamics of a seed feeding bug, *Lygaeus equestris*. 1. Habitat patch structure and spatial dynamics. *Oikos*, **58**, 199-209.
South, A. & Lewis, S. M. 2011. The influence of male ejaculate quantity on female fitness: a meta-analysis. *Biological Reviews*, **86**, 299-309.
Spicer, G. S. & Bell, C. 2002. Molecular phylogeny of the *Drosophila virilis* species group (Diptera: Drosophilidae) inferred from mitochondrial 12S and 16S ribosomal RNA genes. *Annals of the Entomological Society of America*, **95**, 156-161.
Suk, H. Y. & Choe, J. C. 2002. Females prefer males with larger first dorsal fins in the common freshwater goby. *Journal of Fish Biology*, **61**, 899-914.
Sutton, M., Rahman, I. & Garwood, R. 2014. *Techniques for virtual palaeontology*: John Wiley & Sons.
Sutton, M. D., Garwood, R. J., Siveter, D. J. & Siveter, D. J. 2012. SPIERS and VAXML; A software toolkit for tomographic visualisation and a format for virtual specimen interchange. *Palaeontologia Electronica*, **15**, 1-14.
Svensson, P. A., Lehtonen, T. K. & Wong, B. B. M. 2010. The interval between sexual encounters affects male courtship tactics in a desert-dwelling fish. *Behavioral Ecology and Sociobiology*, **64**, 1967-1970.
Swaddle, J. P. & Cuthill, I. C. 1994. Preference for symmetric males by female zebra finches. *Nature*, **367**, 165-166.
Swaddle, J. P. & Page, L. C. 2007. High levels of environmental noise erode pair preferences in zebra finches: implications for noise pollution. *Animal Behaviour*, **74**, 363-368.
Sweet, M. H. 2000. Seed and Chinch bugs (Lygaeoidea). In: *Heteroptera of economic importance* (Ed. by C. W. Schaefer & A. P. Panizzi), pp. 143-264. Boca Raton, Fl: CRC Press.
Sziráki, G. 2002. Contribution to knowledge of female internal genitalia of Neuroptera. *Acta Zoologica Academiae Scientiarum Hungaricae*, **48**, 341-349.
Taborsky, B., Guyer, L. & Taborsky, M. 2009. Size-assortative mating in the absence of mate choice. *Animal Behaviour*, **77**, 439-448.

Tadler, A. 1996. Functional morphology of genitalia of four species of julidan millipedes (Diplopoda: Nemasomatidae; Julidae). *Zoological Journal of the Linnean Society*, **118**, 83-97.
Tadler, A. 1999. Selection of a conspicuous male genitalic trait in the seedbug *Lygaeus simulans*. *Proceedings of the Royal Society B: Biological Sciences*, **266**, 1773-1777.
Tadler, A., Nemeschkal, H. L. & Pass, G. 1999. Selection of male traits during and after copulation in the seedbug *Lygaeus simulans* (Heteroptera, Lygaeidae). *Biological Journal of the Linnean Society*, **68**, 471-483.
Takami, Y. & Sota, T. 2007. Rapid diversification of male genitalia and mating strategies in Ohomopterus ground beetles. *Journal of Evolutionary Biology*, **20**, 1385-1395.
Tallamy, D. W., Powell, B. E. & McClafferty, J. A. 2002. Male traits under cryptic female choice in the spotted cucumber beetle (Coleoptera: Chrysomelidae). *Behavioral Ecology*, **13**, 511-518.
Thornhill, R. 1980. Rape in Panorpa scorpionflies and a general rape hypothesis. *Animal Behaviour*, **28**, 52-59.
Thornhill, R. & Alcock, J. 1983. *The evolution of insect mating systems*: Harvard University Press.
Thornhill, R. & Sauer, K. P. 1991. The notal organ of the scorpionfly (*Panorpa vulgaris*): an adaptation to coerce mating duration. *Behavioral Ecology*, **2**, 156-164.
Timm, N. H. 2002. *Applied multivariate analysis*. New York: Springer.
Tomaru, M. & Oguma, Y. 2000. Mate choice in *Drosophila melanogaster* and *D. sechellia*: criteria and their variation depending on courtship song. *Animal Behaviour*, **60**, 797-804.
Tregenza, T. & Wedell, N. 1997. Definitive evidence for cuticular pheromones in a cricket. *Animal Behaviour*, **54**, 979-984.
Trivers, R. L. 1972. Parental investment and sexual selection. In: *Sexual Selection and the Descent of Man, 1871-1971* (Ed. by B. Campbell), pp. 795-838. Chicago: Aldine.
Tsuchiya, K. & Hayashi, F. 2008. Surgical examination of male genital function of calopterygid damselflies (Odonata). *Behavioral Ecology and Sociobiology*, **62**, 1417-1425.
Tuxen, S. L. 1970. *Taxonomist's glossary of genitalia in insects*. Copenhagen: Scandinavian University Press.
Tyler, F. & Tregenza, T. 2012. Why do so many flour beetle copulations fail? *Entomologia Experimentalis et Applicata*, **146**, 199-206.
Uetz, G. W. & Norton, S. 2007. Preference for male traits in female wolf spiders varies with the choice of available males, female age and reproductive state. *Behavioral Ecology and Sociobiology*, **61**, 631-641.
Uhl, G., Nessler, S. H. & Schneider, J. M. 2010. Securing paternity in spiders? A review on occurrence and effects of mating plugs and male genital mutilation. *Genetica*, **138**, 75-104.
Ureta-Vidal, A., Ettwiller, L. & Birney, E. 2003. Comparative genomics: genome-wide analysis in metazoan eukaryotes. *Nature Reviews Genetics*, **4**, 251-262.
Vahed, K. & Carron, G. 2008. Comparison of forced mating behaviour in four taxa of Anonconotus, the Alpine bushcricket. *Journal of Zoology*, **276**, 313-321.

Vahed, K., Lehmann, A., Gilbert, J. & Lehmann, G. 2011. Increased copulation duration before ejaculate transfer is associated with larger spermatophores, and male genital titillators, across bushcricket taxa. *Journal of Evolutionary Biology*, **24**, 1960-1968.
Valone, T. J., Nordell, S. E., Giraldeau, L.-A. & Templeton, J. J. 1996. The empirical question of thresholds and mechanisms of mate choice. *Evolutionary Ecology*, **10**, 447-455.
van der Linde, K. & Houle, D. 2008. A supertree analysis and literature review of the genus Drosophila and closely related genera (Diptera, Drosophilidae). *Insect Systematics & Evolution*, **39**, 241-267.
van Lieshout, E. & Elgar, M. A. 2011. Longer exaggerated male genitalia confer defensive sperm-competitive benefits in an earwig. *Evolutionary Ecology*, **25**, 351-362.
Verrell, P. A. 1995. Males choose larger females as mates in the salamander *Desmognathus santeelah*. *Ethology*, **99**, 162-171.
Viechtbauer, W. 2010. Conducting meta-analyses in R with the metafor package. *Journal of Statistical Software*, **36**, 1-48.
Von Euw, J., Reichstein, T. & Rothschild, M. 1971. Heart poisons (cardiac glycosides) in the lygaeid bugs *Caenocoris nerii* and *Spilostethus pandurus*. *Insect Biochemistry*, **1**, 373-384.
Wade, M. J., Chang, N. W. & McNaughton, M. 1995. Incipient speciation in the flour beetle, *Tribolium confusum*: premating isolation between natural populations. *Heredity*, **75**, 453-459.
Wagner Jr, W. E., Murray, A. & Cade, W. 1995. Phenotypic variation in the mating preferences of female field crickets, *Gryllus integer*. *Animal Behaviour*, **49**, 1269-1281.
Wagner, W. E. 1998. Measuring female mating preferences. *Animal Behaviour*, **55**, 1029-1042.
Walker, S. M., Schwyn, D. A., Mokso, R., Wicklein, M., Müller, T., Doube, M., Stampanoni, M., Krapp, H. G. & Taylor, G. K. 2014. In vivo time-resolved microtomography reveals the mechanics of the blowfly flight motor. *Plos Biology*, **12**, e1001823.
Walling, C. A., Royle, N. J., Lindström, J. & Metcalfe, N. B. 2010. Do female association preferences predict the likelihood of reproduction? *Behavioral Ecology and Sociobiology*, **64**, 541-548.
Wang, Q. & Davis, L. K. 2006. Females remate for sperm replenishment in a seed bug: evidence from offspring viability. *Journal of Insect Behavior*, **19**, 337-346.
Wang, Q., He, X. Z., Yang, L., Hedderley, D. & Davis, L. K. 2009. Biased sex ratio and low population density increase male mating success in the bug *Nysius huttoni* (Heteroptera: Lygaeidae). *Naturwissenschaften*, **96**, 87-91.
Wang, Q. & Shi, G. 2004. Mating frequency, duration, and circadian mating rhythm of New Zealand wheat bug *Nysius huttoni* White (Heteroptera: Lygaeidae). *New Zealand Entomologist*, **27**, 113-117.
Wang, Q., Yang, L. & Hedderley, D. 2008. Function of prolonged copulation in *Nysius huttoni* White (Heteroptera: Lygaeidae) under male-biased sex ratio and high population density. *Journal of Insect Behavior*, **21**, 89-99.
Ware, A. D. & Opell, B. D. 1989. A test of the mechanical isolation hypothesis in two similar spider species. *Journal of Arachnology*, **17**, 149-162.

Wedell, N. & Hosken, D. 2010. The evolution of male and female reproductive organs in insects. In: *The evolution of primary sexual characters in animals* (Ed. by J. L. Leonard & A. Córdoba-Aguilar). New York: Oxford University Press.
Weirauch, C. & Schuh, R. T. 2011. Systematics and Evolution of Heteroptera: 25 Years of Progress. *Annual Review of Entomology*, **56**, 487-510.
Werner, M. & Simmons, L. W. 2008a. The evolution of male genitalia: functional integration of genital sclerites in the dung beetle *Onthophagus taurus*. *Biological Journal of the Linnean Society*, **93**, 257-266.
Werner, M. & Simmons, L. W. 2008b. Insect sperm motility. *Biological Reviews*, **83**, 191-208.
Werner, N. Y. & Lotem, A. 2006. Experimental evidence for male sequential mate preference in a lekking species. *Ethology*, **112**, 657-663.
Westlake, K. P., Rowe, L. & Currie, D. C. 2000. Phylogeny of the water strider genus Rheumatobates (Heteroptera: Gerridae). *Systematic Entomology*, **25**, 125-145.
White, D. J. & Galef, B. G. 1999. Affiliative preferences are stable and predict mate choices in both sexes of Japanese quail, *Coturnix japonica*. *Animal Behaviour*, **58**, 865-871.
Whittingham, M. J., Stephens, P. A., Bradbury, R. B. & Freckleton, R. P. 2006. Why do we still use stepwise modelling in ecology and behaviour? *Journal of Animal Ecology*, **75**, 1182-1189.
Wiegmann, D. D. 1999. Search behaviour and mate choice by female field crickets, *Gryllus integer*. *Animal Behaviour*, **58**, 1293-1298.
Wiley, R. H. & Poston, J. 1996. Perspective: indirect mate choice, competition for mates, and coevolution of the sexes. *Evolution*, **50**, 1371-1381.
Willis, P. M., Ryan, M. J. & Rosenthal, G. G. 2011. Encounter rates with conspecific males influence female mate choice in a naturally hybridizing fish. *Behavioral Ecology*, **22**, 1234-1240.
Witte, K. 2006. Time spent with a male is a good indicator of mate preference in female zebra finches. *Ethology Ecology & Evolution*, **18**, 195-204.
Witte, K. & Ryan, M. J. 2002. Mate choice copying in the sailfin molly, *Poecilia latipinna*, in the wild. *Animal Behaviour*, **63**, 943-949.
Wittenberger, J. F. 1983. Tactics of mate choice. In: *Mate choice* (Ed. by P. Bateson), pp. 435-447. Cambridge: Cambridge University Press.
Wojcieszek, J., Austin, P., Harvey, M. & Simmons, L. 2012. Micro-CT scanning provides insight into the functional morphology of millipede genitalia. *Journal of Zoology*, **287**, 91-95.
Wojcieszek, J. M. & Simmons, L. W. 2011. Male genital morphology influences paternity success in the millipede *Antichiropus variabilis*. *Behavioral Ecology and Sociobiology*, **65**, 1843-1856.
Wong, B. & Candolin, U. 2005. How is female mate choice affected by male competition? *Biological Reviews*, **80**, 559-571.
Wong, B. B. & Jennions, M. D. 2003. Costs influence male mate choice in a freshwater fish. *Proceedings of the Royal Society B: Biological Sciences*, **270**, S36-S38.

Wong, B. B., Jennions, M. D. & Keogh, J. S. 2004. Sequential male mate choice in a fish, the Pacific blue-eye *Pseudomugil signifer*. *Behavioral Ecology and Sociobiology*, **56**, 253-256.
Wong, B. B. & Svensson, P. A. 2009. Strategic male signalling effort in a desert-dwelling fish. *Behavioral Ecology and Sociobiology*, **63**, 543-549.
Wood, D. & Ringo, J. M. 1980. Male mating discrimination in *Drosophila melanogaster*, *D. simulans* and their hybrids. *Evolution*, **34**, 320-329.
Wu, C.-I., Hollocher, H., Begun, D. J., Aquadro, C. F., Xu, Y. & Wu, M.-L. 1995. Sexual isolation in *Drosophila melanogaster*: a possible case of incipient speciation. *Proceedings of the National Academy of Sciences*, **92**, 2519-2523.
Wulff, N. C., Lehmann, A. W., Hipsley, C. A. & Lehmann, G. U. 2015. Copulatory courtship by bushcricket genital titillators revealed by functional morphology, µCT scanning for 3D reconstruction and female sense structures. *Arthropod structure & Development,* in press.
Xia, X., Xie, Z. & Kjer, K. M. 2003. 18S ribosomal RNA and tetrapod phylogeny. *Systematic Biology*, **52**, 283-295.
Xu, J. & Wang, Q. 2009. A polyandrous female moth discriminates against previous mates to gain genetic diversity. *Animal Behaviour*, **78**, 1309-1315.
Xu, J. & Wang, Q. 2010. Form and nature of precopulatory sexual selection in both sexes of a moth. *Naturwissenschaften*, **97**, 617-625.
Yang, L. & Wang, Q. 2004. Precopulation sexual selection in *Nysius huttoni* White (Heteroptera: Lygaeidae) in relation to morphometric traits. *Journal of Insect Behavior*, **17**, 695-707.
Yoshizawa, K., Ferreira, R. L., Kamimura, Y. & Lienhard, C. 2014. Female penis, male vagina, and their correlated evolution in a cave insect. *Current Biology*, **24**, 1006-1010.
Yukilevich, R. & True, J. R. 2008. Incipient sexual isolation among cosmopolitan Drosophila melanogaster populations. *Evolution*, **62**, 2112-2121.
Zhao, X. C., Wu, K. M., Liang, G. M. & Guo, Y. Y. 2008. Altered mating behaviour in a Cry1Ac-resistant strain of *Helicoverpa armigera* (Lepidoptera : Noctuidae). *Journal of Applied Entomology*, **132**, 360-365.
Ziegler, A., Ogurreck, M., Steinke, T., Beckmann, F., Prohaska, S. & Ziegler, A. 2010. Opportunities and challenges for digital morphology. *Biology Direct*, **5**.

Appendix 1: statistical models

Chapter 3

Table S1. Determinants of male and female mating success in *L. equestris* (*N*= 303 males and 310 females), when body length was used as the morphological size measure, tested using a GLMM with mated status as a binary logistic response. Choice treatment is a categorical factor with four levels.

	Females				**Males**			
	F	*d.f.*1	*d.f.*2	*P*	*F*	*d.f.*1	*d.f.*2	*P*
Corrected model	10.03	4	305	<0.001	1.71	4	298	0.15
Choice treatment	12.09	3	305	<0.001	1.88	3	298	0.13
Body length	7.48	1	305	0.007	1.55	1	298	0.21

Table S2. Determinants of male and female mating success in *L. equestris* (*N*= 300 males and 305 females), when overall size was used as the morphological size measure, tested using a GLMM with mated status as a binary logistic response. Choice treatment is a categorical factor with four levels.

	Females				**Males**			
	F	*d.f.*1	*d.f.*2	*P*	*F*	*d.f.*1	*d.f.*2	*P*
Corrected model	9.04	4	300	<0.001	2.59	5	294	0.026
Choice treatment	11.75	3	300	<0.001	2.54	3	294	0.057
Overall size	1.75	1	300	0.19	3.67	1	294	0.056
Overall size^2	-	-	-	-	4.88	1	294	0.028

Table S3. Determinants of copulation duration in *L. equestris* mated pairs (*N*= 148 pairs), tested using a generalised linear model with copulation duration as a binary logistic response (whether copulation ended during the trial or not). Choice treatment is a categorical factor with four levels.

	Likelihood Ratio χ^2	***d.f.***	***P***
(Intercept)	3.07	1	0.08
Choice treatment	6.53	3	0.089
Male overall size	1.75	1	0.19
Female overall size	6.97	1	0.008

Table S4. Determinants of female fertility in *L. equestris* mated pairs (*N*= 148 pairs), tested using a generalised linear model with female offspring production (the presence or absence of fertile eggs after two weeks following mating) as a binary logistic response. Choice treatment is a categorical factor with four levels.

	Likelihood Ratio χ^2	***d.f.***	***P***
(Intercept)	5.9	1	0.015
Choice treatment	1.29	3	0.73
Copulation duration	40.99	1	0.001
Male overall size	1.78	1	0.18
Female overall size	8.86	1	0.003
Choice treatment * Female overall size	9.22	3	0.027
Copulation duration * Female overall size	12.06	1	0.001

Chapter 5

Table S5. Determinants of male mating success in *L. equestris* (*N*= 174), tested using a generalised linear model with male mated status as a binary logistic response (mated or non-mated). Choice treatment is a categorical factor with four levels.

	Likelihood Ratio χ^2	***d.f.***	***P***
Male body length	0.055	1	0.82
Processus length	1.39	1	0.24
Choice treatment	6.15	1	0.013
Processus length*choice treatment	6.1	1	0.014

Table S6. Determinants of insemination success for mated *L. equestris* males (*N*= 64), tested using a generalised linear model with female offspring production as a binary logistic response (the presence or absence of fertile eggs after two weeks following mating). Choice treatment is a categorical factor with four levels.

	Likelihood Ratio χ^2	***d.f.***	***P***
Male body length	10.81	1	0.001
Male body length^2	10.92	1	0.001
Female body length	3.26	1	0.07
Processus length	7.98	1	0.005
Processus length^2	7.84	1	0.005
Choice treatment	1.48	1	0.22
Copulation duration	5.68	1	0.02
Processus length * Choice treatment	1.52	1	0.22
Female body length * Copulation duration	6.08	1	0.013

Table S7. Determinants of insemination success for all *L. equestris* males (*N*= 174), tested using a generalised linear model with female offspring production as a binary logistic response (the presence or absence of fertile eggs after two weeks following mating). Choice treatment is a categorical factor with four levels.

	Likelihood Ratio χ^2	***d.f.***	***P***
Male body length	0.19	1	0.67
Processus length	7.48	1	0.006
Processus length^2	7.35	1	0.007
Choice treatment	4.21	1	0.04
Processus length * Choice treatment	4.3	1	0.038

Table S8. Determinants of male mating success in *L. simulans* (*N*= 140), tested using a generalised linear model with male mated status as a binary logistic response (mated or non-mated).

	Likelihood Ratio χ^2	***d.f.***	***P***
Male body length	5.89	1	0.015
Processus length	0.3	1	0.58

Table S9. Determinants of insemination success for mated *L. simulans* males (*N*= 86), tested using a generalised linear model with female offspring production as a binary logistic response (the presence or absence of fertile eggs after two weeks following mating).

	Likelihood Ratio χ^2	***d.f.***	***P***
Male body length	0.14	1	0.71
Processus length	1.48	1	0.22
Female body length	10.64	1	0.001
Copulation duration	36.02	1	<0.001

Table S10. Determinants of female offspring production in *L. simulans* following a fertile mating (*N*= 48).

	SS	***d.f.***	***F***	***P***
Male body length	7816	1	3.07	0.09
Processus length	31	1	0.01	0.91
Female body length	21927	1	8.61	0.005
Copulation duration	1226	1	0.48	0.49
Residuals	109529	43		

Chapter 6

Table S11. Determinants of male mating success in *L. simulans* in the large reduction experiment (*N*= 78), tested using a generalised linear model with male mated status as a binary logistic response (mated or non-mated). In the experimental treatment the male processus was shortened by 2mm, or manipulated but not cut.

	Likelihood Ratio χ^2	***d.f.***	***P***
Experimental treatment	0.6	1	0.44
Male body length	6.58	1	0.01

Table S12. Determinants of copulation duration in *L. simulans* mated pairs in the large reduction experiment (*N*= 59), tested using a generalised linear model with duration as a binary logistic response (whether copulation ended during the trial or not). In the experimental treatment the male processus was shortened by 2mm, or manipulated but not cut.

	SS	***d.f.***	***F***	***P***
(Intercept)	48351	1	2.31	0.13
Experimental treatment	147260	1	7.04	0.01
Male body length	88350	1	4.23	0.04
Residuals	1170654	56		

Table S13. Determinants of insemination success in *L. simulans* mated pairs in the large reduction experiment (*N*= 59), tested using a generalised linear model with female offspring production as a binary logistic response (the presence or absence of fertile eggs after two weeks following mating). In the experimental treatment the male processus was shortened by 2mm, or manipulated but not cut.

	Likelihood Ratio χ^2	***d.f.***	***P***
Experimental treatment	12.44	1	<0.001
Male body length	1.96	1	0.16

Table S14. Determinants of female offspring production following fertile matings in *L. simulans* in the large reduction experiment (*N*= 27). In the experimental treatment the male processus was shortened by 2mm, or manipulated but not cut.

	SS	***d.f.***	***F***	***P***
(Intercept)	8236.9	1	4.62	0.049
Experimental treatment	5747.1	1	3.22	0.09
Male body length	10762.6	1	6.03	0.028
Residuals	24972.4	14		

Table S15. Determinants of male mating frequency in *L. simulans* in the medium reduction experiment (*N*= 25). In the experimental treatment the male processus was shortened by 1mm, or manipulated but not cut.

	SS	***d.f.***	***F***	***P***
(Intercept)	3.24	1	44.03	<0.001
Experimental treatment	0.07	1	0.96	0.34
Residuals	1.69	23		

Table S16. Determinants of insemination success in *L. simulans* mated pairs in the medium reduction experiment (*N*= 25), tested using a generalised linear model with female offspring production as a binary logistic response (the presence or absence of fertile eggs after two weeks following mating). In the experimental treatment the male processus was shortened by 1mm, or manipulated but not cut.

	Likelihood Ratio χ^2	***d.f.***	***P***
Experimental treatment	2.59	1	0.11

Table S17. Determinants of *L. simulans* female offspring production in the medium reduction experiment, for all males (*N*= 17). In the experimental treatment the male processus was shortened by 1mm, or manipulated but not cut.

	SS	***d.f.***	***F***	***P***
(Intercept)	98406	1	29.93	<0.001
Experimental treatment	3755	1	1.14	0.3
Residuals	49324	15		

Table S18. Determinants of male mating success in *L. simulans* in the small reduction experiment (*N*= 165), tested using a generalised linear model with male mated status as a binary logistic response (mated or non-mated). In the experimental treatment the male processus was shortened by either 0.4 mm, 0.1mm, or manipulated but not cut.

	Likelihood Ratio χ^2	***d.f.***	***P***
Experimental treatment	0.13	2	0.94
Male body length	0.84	1	0.36

Table S19. Determinants of insemination success in *L. simulans* mated pairs in the small reduction experiment (*N*= 151), tested using a generalised linear model with female offspring production as a binary logistic response (the presence or absence of fertile eggs after two weeks following mating). In the experimental treatment the male processus was shortened by either 0.4 mm, 0.1mm, or manipulated but not cut.

	Likelihood Ratio χ^2	***d.f.***	***P***
Experimental treatment	0.03	2	0.99
Male body length	5.81	1	0.016

Table S20. Determinants of female offspring production following fertile matings in *L. simulans* in the small reduction experiment (*N*= 102). In the experimental treatment the male processus was shortened by either 0.4 mm, 0.1mm, or manipulated but not cut.

	SS	***d.f.***	***F***	***P***
(Intercept)	1895	1	0.82	0.37
Experimental treatment	21240	2	4.59	0.012
Male body length	4370	1	1.89	0.17
Residuals	226877	98		

Chapter 7

Table S21. Linear mixed model testing for difference in clasper length between left and right claspers, for *L. simulans* males from the no-choice and choice experiments (*N*= 152 left and 152 right claspers from 160 males). Male identity is included in the model as a random factor.

	Likelihood Ratio χ^2	***d.f.***	***P***
(Intercept)	200479.4	1	<0.001
Clasper side	235.58	1	<0.001

Table S22. Determinants of male mating success in no-choice mating trials in *L. simulans* (*N*= 80), tested using a generalised linear model with male mated status as a binary logistic response (mated or non-mated). Clasper length is the average of left and right clasper measurements.

	Likelihood Ratio χ^2	***d.f.***	***P***
Male body length	3.22	1	0.07
Male body length^2	3.22	1	0.07
Processus length	0.91	1	0.34
Clasper length	0.91	1	0.34

Table S23. Determinants of male mating frequency in no-choice mating trials in *L. simulans* (*N*= 80), tested using a generalised linear model with the number of matings as a Poisson response. Clasper length is the average of left and right clasper measurements.

	Likelihood Ratio χ^2	***d.f.***	***P***
Male body length	4.24	1	0.04
Male body length^2	4.27	1	0.04
Processus length	0.25	1	0.61
Clasper length	3.33	1	0.068
Clasper length^2	3.35	1	0.067

Table S24. Determinants of copulation duration for mated *L. simulans* males in no-choice mating trials (*N*= 64), tested using a generalised linear model with copulation duration as a binary logistic response (greater or less than 300 minutes). Clasper length is the average of left and right clasper measurements.

	Likelihood Ratio χ^2	***d.f.***	***P***
Male body length	1.1	1	0.3
Processus length	2.57	1	0.11
Clasper length	1.13	1	0.29

Table S25. Determinants of male mating success in choice mating trials in *L. simulans* (*N*= 132), tested using a generalised linear mixed model with male mated status as a binary logistic response (mated or non-mated). Female ID was included as a random factor to control for the non-independence of male mating success in each dish (*N*= 66 females). Clasper length is the average of left and right clasper measurements.

	Estimate	**Std. error**	**z value**	***P***
(Intercept)	-19.37	9.5	-2.04	0.04
Male body length	0.11	0.69	0.17	0.87
Processus length	3.4	1.2	2.81	0.005
Clasper length	-6.28	9.26	-0.68	0.5

Appendix 2: supplementary methods and results

In this section I present supplementary material not included in Chapters 3, 4, 5 & 7. For Chapter 3 I present several additional results concerning the relationships between the five morphological traits measured for males and females in *L. equestris*. For Chapter 4 I present a list of studies that were excluded from the final dataset after reading the full text. I also list the methods used to calculate statistics in cases were these were not presented in the original text. Finally, I present the results of meta-analysis models incorporating phylogenetic relatedness among species. For Chapter 5 I present standardised selection differentials estimating the strength of sexual selection acting on male processus length, male body length and female body length in *L. simulans* and *L. equestris*. For Chapter 7 I present a guide to the landmarks used in the geometric morphometric analysis of clasper shape. I also present the Eigenvalues for the 20 principal components extracted for each clasper, and the correlations between the first five principal components for each clasper and male body and processus length.

Chapter 3

Table S26. Pairwise correlations between all morphological traits measured, considered for males and females separately. Pearson correlation coefficients are above the diagonal. All correlations were highly significant, with $P < 0.001$. $N = 303$ males and 310 females, except for correlations including antenna length, where $N = 300$ males and 305 females.

Sex	Trait	Body length	Antenna length	1st leg length	2nd leg length	3rd leg length
Female	Body length	-	0.582	0.688	0.652	0.690
	Antenna length		-	0.675	0.698	0.700
	1st leg length			-	0.808	0.828
	2nd leg length				-	0.847
Male	Body length	-	0.536	0.656	0.597	0.632
	Antenna length		-	0.650	0.642	0.658
	1st leg length			-	0.833	0.792
	2nd leg length				-	0.839

Table S27. Factor loading of the single principal component extracted from the five morphological traits measured, for males and females. Component loads highly on all traits measured for both males and females. Loading is for rescaled components derived from a covariance matrix, with no rotation.

Trait	Factor loading	
	Females	Males
Body length	0.948	0.907
Antenna length	0.732	0.733
1st leg length	0.834	0.844
2nd leg length	0.827	0.829
3rd leg length	0.871	0.870

Allometry of morphological measures

Methods

To test for any differences in allometry I compared the relationships between body length and our other body measures (antennae and leg lengths for the two sexes) using analysis of covariance (ANCOVA). I tested for homogeneity of slopes using the sex*body length interaction term in each case: a significant interaction indicates that the regression lines for each sex have significantly different slopes. All interaction terms were non-significant and so were dropped from the models. Models were then repeated including only main effects (sex and body length).

Results

Females were slightly larger than males for three of the morphological traits measured: body length (t_{611}= 40.72, P< 0.001); prothoracic leg length (t_{611}= 5.28, P< 0.001); and metathoracic leg length (t_{611}= 17.58, P <0.001). There was no significant difference between males and females in antenna length (t_{603}= 1.05, P= 0.3) or mesothoracic leg length (t_{611}= 1.24, P= 0.21). There was no significant difference between males and females in the relationship between body length and the remaining four traits (ANCOVAs, interaction between body length and sex, all P> 0.05), but males do have relatively longer antennae and legs for their body length (ANCOVAs, main effect of sex after fitting body length as a covariate: antennae, $F_{1,602}$= 180.62, P< 0.001; prothoracic legs, $F_{1,610}$= 515.42, P< 0.001; mesothoracic legs, $F_{1,610}$= 257.48, P< 0.001; metathoracic legs, $F_{1,610}$= 41.69, P< 0.001).

Chapter 4

Table S28. List of relevant studies that were not included in our analysis, and the reasons for them not being included. In all these studies both no-choice and choice tests were used, however they did not meet all of our inclusion criteria. See below for full references.

Paper	Species	Reason for non-inclusion
Doherty, 1985	*Gryllus bimaculatus*	No data reported
Bissell & Martins, 2006	*Sceloporus graciosus*	No data reported
Kostarakos *et al*., 2008	*Gryllus bimaculatus*	No data reported
Allison & Cardé, 2008	*Cadra cautella*	No data reported
Yukilevich & True, 2008	*Drosophila melanogaster*	Do not report results of no-choice tests
Kozak *et al*., 2011	*Gasterosteus aculeatus*	Do not report results of no-choice tests
Meffert & Regan, 2012	*Musca domestica*	Do not report results of no-choice tests
Taborsky *et al*., 2009	*Eretmodus cyanostictus*	Do not report results of choice tests
Gupta & Sarandan, 1994	*Drosophila kikkawai*	Not true no-choice test, Do not report results of no-choice tests
Wu *et al*., 1995	*Drosophila melanogaster*	Not true no-choice test
Singh & Sisodia, 1999	*Drosophila bipectinata*	Not true no-choice test
Tomaru & Oguma, 2000	*Drosophila melanogaster*	Not true no-choice test
	Drosophila sechellia	Not true no-choice test
Zhao *et al*., 2008	*Helicoverpa armigera*	Not true no-choice test
Kumaran *et al*., 2013	*Bactrocera tryoni*	Not true no-choice test
Ryan & Rand, 1993	*Physalaemus pustulosus*	No conspecific calls tested in no-choice tests
Willis *et al*., 2011	*Xiphophorus birchmanni*	No conspecifics tested in no-choice trials
Bee & Schwartz, 2009	*Hyla chrysoscelis*	No use of heterospecific call in no-choice tests
Basolo, 1995	*Priapella olmecae*	Did not test for preference for no sword in no-choice trials
Basolo, 2002	*Heterandria bimaculata*	Did not test for preference for no sword in no-choice trials
Walling *et al*., 2010	*Xiphophorus helleri*	Females only tested with one type of male in no-choice trials
Havens & Etges, 2013	*Drosophila mojavensis*	No comparison between mated and unmated males in no-choice trials
Uetz & Norton, 2007	*Schizocosa ocreata*	Preference for different traits tested in choice an no-choice tests

Sharma *et al*., 2010	*Drosophila simulans*	Preference for different traits tested in choice an no-choice tests
Deb *et al*., 2012	*Oecanthus henryi*	Preference for different traits tested in choice an no-choice tests
Wade *et al*., 1995	*Tribolium confusum*	No mate choice: recorded offspring number from different matings
Grant *et al*., 1995	*Oryzias latipes*	No mate choice: measured male mating success
Wiegmann, 1999	*Gryllus integer*	No mate choice: ability of female to locate speakers
Muller & Robert, 2002	*Ormia ochracea*	No mate choice: ability of parasite to locate host
Silva *et al*., 2007	*Syngnathus abaster*	No mate choice in no-choice tests: female associations with females
Kozak & Boughamn, 2009	*Gasterosteus aculeatus*	Females tested with no-choice tests, males tested with choice tests
Havens *et al*., 2011	*Drosophila mojavensis*	No mate choice: recorded offspring fitness of matings

Data extraction methods

Table S29: Methods used for calculating effect sizes for papers in which statistics were not fully reported. Data were extracted from figures using the image analysis software Digitize It 2010.

Paper	Notes
Wagner *et al*., 1995	No-choice effect size calculated from data extracted from Figure 3, converted to eta-squared using sums of squares, then converted to *r*. Was unable to account for fact that data are from repeated-measures. Choice test: calculated Hedge's *d* using data from Table 1
Jang & Gerhardt, 2006	Performed t tests using data from Figure 3
Gershmann & Sakaluk, 2009	Repeated χ^2 test using data in text
Lehmann & Lehmann, 2008	Performed χ^2 test using data from Figure 2

Coyne *et al*., 2005	Statistics for both mating frequency and mating latency taken from data in table 1
Wood & Ringo, 1980	Mating frequency data: repeated analysis in order to obtain χ^2 statistics (Tables 1 & 2). Correlation of 1 for some measures, set at 2 when converted to *Zr*
Jennings *et al.*, 2011	Performed χ^2 tests using data from Table 1
Hoikkala & Aspi, 1993	Performed χ^2 test on proportion data extracted from Figure 3. Exact sample sizes not presented (only range), took largest sample size presented
Xu & Wang, 2009	Performed χ^2 tests on data from Figure 2
Schofl *et al.*, 2011	Performed χ^2 tests using data from Table 2 and Figure 3
Cook *et al.*, 1994	Performed χ^2 test on data in Table 1 & 2. Had to assume individuals were not encountered more than once
Barry *et al*., 2010	Performed χ^2 tests on data from text
King *et al*., 2005	Performed χ^2 tests on data from Tables 1 & 4
McNamara *et al*., 2004	Performed χ^2 test on mating frequency data from Figure 1
Dougherty & Shuker, 2013	Re-analysed own data, excluding mutual choice treatment
Jordan & Brooks, 2011	Performed *t* tests performed using data from Figures 1 & 2
Hurt *et al*., 2004	No-choice *t* statistic calculated from data extracted from Table 1. Choice test: calculated Hedge's *d* using data from Table 4
Rowland, 1982	Performed Wilcoxon test using data from Tables 1 & 2
Jamieson & Colgan, 1989	Performed χ^2 test from data in text
Belle-Isles, 1990	Performed χ^2 test using data from Table 2, combined results from all males
Owen *et al.*, 2012	Statistics obtained by contacting authors
Itzkowitz *et al.*, 1998	*z* scores back-converted from presented *P* values
Suk & Choe, 2002	*t* statistic for no-choice test calculated from *P* value and *N*
Phelps *et al*., 2006	Performed χ^2 tests on data from Figure 5
Verrell, 1995	Performed χ^2 test from data in text

Supplementary results

Multivariate meta-analysis revealed significant positive mating preferences after including study, species and phylogeny as random factors (*r*= 0.434, 95% CI: 0.323 to 0.533, n=214). The variance components associated with the three random factors were small (Study= 0.074, species= 0.079, phylogeny= 0.00). The addition of the three random factors leads to much wider confidence intervals and a much larger AIC score than the basic model (AIC= 832.88 for the multivariate model compared to 262.81 for the basic model).

Running the multivariate model separately for the two choice designs showed that mating preferences were larger in the choice design (*r*= 0.509, 95% CI: 0.391 to 0.61, n=107) compared to the no-choice design (*r*= 0.338, 95% CI: 0.221 to 0.446). Again the AIC score was increased after adding the three random factors (No-choice design: AIC= 105.45 for the basic model compared to 291.37 for the multivariate model, Choice design: AIC= 150.38 for the basic model compared to 516.35 for the multivariate model), as well as increasing the width of the confidence intervals slightly, but does not change the mean effect size estimates greatly. In fact, the difference in mean effect between the two designs is greater after adding the random factors. The variance components associated with the random factors were small for both no-choice tests (Study= 0.068, species= 0.068, phylogeny= 0.00) and choice tests (Study= 0.082, species= 0.11, phylogeny= 0.00).

Table S30 shows the mean effect size estimates for the subgroup models after incorporating the three random factors. Figure S2 shows comparisons between the mean effect sizes estimates and 95% CI's for the meta-analysis models with and without the inclusion of the three random factors, split by choice design and subgroup (sex and trait type).

Table S30. Mean effect size estimates resulting from multivariate meta-analysis models performed separately for effect sizes derived from no-choice and choice tests from each subgroup, after adding study, species and phylogeny as random factors. All analyses were performed using Fisher's z transform of the correlation coefficient (*Zr*), and then converted back to *r* for presentation. Mean effect size estimates and confidence intervals were calculated using a multivariate meta-analysis incorporating study, species and phylogeny as random factors. Confidence intervals for estimates were calculated by bootstrapping 1000 times.

			No-choice tests				Choice tests			
Group	Studies	Species	Mean *r*	Lower 95% CI	Upper 95% CI	Effect sizes	Mean *r*	Lower 95% CI	Upper 95% CI	Effect sizes
All	38	40	0.338	0.221	0.446	107	0.509	0.391	0.610	107
Sex										
Males	20	21	0.361	0.230	0.479	61	0.439	0.294	0.564	58
Females	21	25	0.330	0.149	0.489	46	0.540	0.393	0.660	49
Trait type										
Intra-species	29	29	0.366	0.239	0.481	68	0.530	0.411	0.631	65
Inter-population	4	4	0.232	0.065	0.385	9	0.372	0.084	0.602	9
Inter-species	7	11	0.419	0.243	0.569	30	0.505	0.258	0.690	33
Taxonomic group										
Arachnid	1	1	0.500	-	-	1	0.744	-	-	1
Crustacean	2	1	0.288	-0.214	0.670	5	0.430	0.308	0.538	6
Insect	17	21	0.262	0.032	0.466	55	0.467	0.247	0.642	55
Fish	12	11	0.466	0.350	0.568	33	0.595	0.461	0.703	34
Amphibian	3	3	0.368	-0.917	0.982	5	0.567	-0.886	0.991	4
Reptile	1	1	0.271	0.096	0.430	4	0.366	0.181	0.525	3
Bird	2	2	0.330	0.148	0.491	4	0.372	-0.271	0.785	4

Though phylogenetic history had no effect on mean effect size for the larger models, it did have a larger effect in some of the smaller subgroup models, for example for effect sizes considering inter-population choice (No-choice: phylogeny= 0.012, Choice: phylogeny= 0.049), amphibians (No-choice: phylogeny= 1.08, Choice: phylogeny= 1.18) and birds (No-choice: phylogeny= 0.00, Choice: phylogeny= 0.081). However this is likely driven by the fact that these models have much smaller sample sizes which will exaggerate any phylogenetic effects, especially within taxonomic groups.

Chapters 5 & 6

Methods

I calculated standardized linear (β) and quadratic (γ) selection gradients acting on male morphological traits (Morrissey & Sakrejda, 2013). The selection gradient is a common metric that represents the relationship of relative fitness to the variation in a quantitative trait measured in standard deviation units, and is standardized by setting the trait variance to 1 prior to measurement (Kingsolver *et al*., 2001). Note that stabilising selection on a trait results in a negative quadratic selection gradient (Mitchell-Olds & Shaw, 1987). For these models the morphological trait in question (processus length, male body length or female body length) was the only explanatory factor. These should therefore more corectly be termed *selection differentials*, as they record both direct selection acting on the trait and indirect selection due to correlated traits (Kingsolver *et al*., 2012). However for clarity I term them selection gradients throughout. I could control for additional covariates (such as body size) in an attempt to rule out this indirect selection, however it is this differential that reflects the total selection acting on a trait. I here present the standardised selection gradients for male processus length and male and female body length. Selection gradients were calculated separately for the three estimates of male reproductive success (pre-copulatory, post-copulatory, and a combination of the two).

Standardised selection gradients were calculated using the R package GSG (Morrissey & Sakrejda, 2013). The GSG package allows selection gradients to be derived non-parametrically using general additive models, and allows the calculation of standard errors and *P* values associated with estimated gradients via bootstrapping.

Chapter 5

Table S31. Standardised linear and quadratic selection gradients showing the strength of selection on male processus length in *L. equestris*, for the three measures of reproductive success.

Selection	*N*	Term	Estimate	*SE*	*P*
Pre-copulatory	174	Linear (β)	-0.25	0.088	<0.001
		Quadratic (γ)	-0.093	0.12	0.51
Post-copulatory	64	Linear (β)	0.086	0.1	0.33
		Quadratic (γ)	-0.41	0.098	0.012
Combined	174	Linear (β)	-0.19	0.11	0.09
		Quadratic (γ)	-0.38	0.11	0.006

Table S32. Standardised selection gradients derived from a general additive model considering pre-copulatory selection on male processus length in *L. equestris*, split by choice design.

Treatment	*N*	Term	Estimate	*SE*	*P*
1	39	Linear	0.126	0.216	0.574
		Quadratic	-0.352	0.295	0.184
2	54	Linear	-0.414	0.163	0.022
		Quadratic	-0.065	0.225	0.714
3	26	Linear	-0.246	0.267	0.394
		Quadratic	0.051	0.466	0.988
4	55	Linear	-0.424	0.133	0.006
		Quadratic	0.050	0.202	0.622

Table S33. Standardised linear and quadratic selection gradients showing the strength of selection on male body length in *L. equestris*, for the three measures of reproductive success.

Selection	*N*	Term	Estimate	*SE*	*P*
Pre-copulatory	174	Linear (β)	-0.09	0.1	0.35
		Quadratic (γ)	-0.12	0.11	0.31
Post-copulatory	64	Linear (β)	0.08	0.12	0.51
		Quadratic (γ)	0.37	0.16	0.04
Combined	174	Linear (β)	0.07	0.19	0.71
		Quadratic (γ)	0.16	0.25	0.52

Table S34. Standardised linear and quadratic selection gradients showing the strength of post-copulatory selection on female body length in *L. equestris*.

Selection	*N*	Term	Estimate	*SE*	*P*
Post-copulatory	174	Linear (β)	0.07	0.14	0.63
		Quadratic (γ)	0.01	0.19	0.95

Table S35. Standardised linear and quadratic selection gradients showing the strength of selection on male processus length in *L. simulans*, for the three measures of reproductive success.

Selection	*N*	Term	Estimate	*SE*	*P*
Pre-copulatory	139	Linear (β)	0.087	0.05	0.064
		Quadratic (γ)	-0.03	0.066	0.582
Post-copulatory	101	Linear (β)	-0.041	0.088	0.674
		Quadratic (γ)	-0.12	0.147	0.436
Combined	139	Linear (β)	-0.043	0.089	0.644
		Quadratic (γ)	-0.11	0.142	0.47

Table S36. Standardised linear and quadratic selection gradients showing the strength of selection on male body length in *L. simulans*, for the three measures of reproductive success.

Selection	*N*	Term	Estimate	*SE*	*P*
Pre-copulatory	139	Linear (β)	0.14	0.053	0.01
		Quadratic (γ)	-0.05	0.056	0.21
Post-copulatory	102	Linear (β)	-0.02	0.091	0.83
		Quadratic (γ)	-0.03	0.12	0.86
Combined	139	Linear (β)	0.12	0.11	0.27
		Quadratic (γ)	-0.09	0.13	0.42

Table S37. Standardised linear and quadratic selection gradients showing the strength of post-copulatory selection on female body length in *L. simulans*.

Selection	*N*	Term	Estimate	*SE*	*P*
Post-copulatory	102	Linear (β)	0.33	0.09	0
		Quadratic (γ)	0.83	0.12	0.46

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Table S38. Standardised linear and quadratic selection gradients showing the strength of post-copulatory selection on male processus length in *L. simulans* in terms of the likelihood of successful insemination, for unmanipulated males in the large and small reduction experiments.

Experiment	N	Term	Estimate	SE	P
Large reduction	31	Linear (β)	0.18	0.19	0.47
		Quadratic (γ)	-0.29	0.28	0.16
Small reduction	50	Linear (β)	-0.01	0.11	0.83
		Quadratic (γ)	-0.22	0.14	0.15

Chapter 7

Landmark descriptions

Landmark 1: Tip of the 'tooth'
Landmark 2: Point of minimum curvature (upper surface of tooth)
Landmark 3: Point of maximum curvature
Landmark 4: Point of inflection
Landmark 5: Point of maximum curvature
Landmark 6: Point of inflection
Landmark 7: Point where curved line reaches edge of clasper
Landmark 8: Point of minimum curvature
Landmark 9: Point of inflection
Landmark 10: Point of inflection
Landmark 11: Point of minimum curvature
Landmark 12: Point of maximum curvature (lower surface of tooth)

Table S39. The amount of variance explained by each of the 20 principal components of left clasper shape extracted using principal component analysis.

PC	Eigenvalues	% Variance	Cumulative %
1	0.00077	22.63	22.63
2	0.00050	14.77	37.40
3	0.00044	12.85	50.25
4	0.00034	9.88	60.13
5	0.00024	7.10	67.23
6	0.00021	6.11	73.34
7	0.00018	5.26	78.60
8	0.00015	4.35	82.95
9	0.00013	3.84	86.79
10	0.00009	2.74	89.53
11	0.00009	2.50	92.03
12	0.00006	1.74	93.77
13	0.00005	1.43	95.20
14	0.00004	1.21	96.41
15	0.00003	0.92	97.33
16	0.00003	0.75	98.08
17	0.00002	0.64	98.72
18	0.00002	0.56	99.28
19	0.00001	0.42	99.70
20	0.00001	0.30	100.00

Table S40. The amount of variance explained by each of the 20 principal components of right clasper shape extracted using principal component analysis.

PC	Eigenvalues	% Variance	Cumulative %
1	0.00094	27.39	27.39
2	0.00041	11.76	39.15
3	0.00032	9.41	48.55
4	0.00031	8.94	57.49
5	0.00029	8.42	65.91
6	0.00022	6.42	72.33
7	0.00019	5.46	77.78
8	0.00015	4.26	82.04
9	0.00013	3.78	85.82
10	0.00011	3.26	89.08
11	0.00009	2.67	91.76
12	0.00006	1.87	93.63
13	0.00005	1.41	95.04
14	0.00004	1.22	96.26
15	0.00004	1.06	97.32
16	0.00003	0.90	98.21
17	0.00002	0.61	98.83
18	0.00002	0.48	99.30
19	0.00001	0.39	99.70
20	0.00001	0.31	100.00

Table S41. Factor loadings for the principal component analysis of left clasper shape.

FACTOR	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9	PC10	PC11	PC12	PC13	PC14	PC15	PC16	PC17	PC18	PC19	PC20
X1	-0.115	0.170	-0.070	-0.201	0.078	0.041	0.029	0.487	0.252	0.048	-0.067	-0.348	-0.154	0.085	0.121	0.157	-0.006	-0.050	0.044	0.008
Y1	0.168	0.136	0.135	-0.395	0.197	0.288	0.341	-0.226	0.025	0.057	0.220	-0.075	0.041	0.085	-0.004	-0.100	-0.057	0.079	-0.047	0.003
X2	0.200	-0.020	0.005	-0.187	-0.199	-0.148	-0.173	-0.094	0.024	-0.033	0.058	0.350	0.024	-0.064	0.035	-0.183	-0.118	0.428	-0.492	-0.173
Y2	0.016	-0.232	-0.113	-0.202	0.109	-0.617	-0.427	-0.138	0.101	0.077	-0.038	0.007	0.045	0.007	-0.024	0.010	0.000	-0.157	0.248	0.059
X3	0.112	-0.109	0.127	0.041	-0.138	-0.024	0.047	-0.263	-0.190	-0.028	-0.040	-0.396	-0.058	0.093	0.067	-0.167	-0.414	0.327	0.475	0.027
Y3	-0.207	-0.803	-0.107	0.056	-0.198	0.286	0.177	0.065	-0.035	0.042	0.073	0.054	-0.012	-0.062	0.012	0.035	0.021	-0.038	-0.031	-0.006
X4	0.180	-0.130	-0.082	-0.083	0.268	-0.164	0.204	0.061	-0.031	-0.295	0.224	0.082	-0.530	-0.054	-0.461	0.123	0.036	-0.057	-0.012	0.054
Y4	-0.067	0.025	0.432	0.267	0.165	0.102	-0.027	-0.344	0.526	-0.074	-0.312	0.102	-0.202	0.099	-0.047	-0.021	0.071	-0.017	-0.031	0.047
X5	0.258	-0.165	-0.367	-0.126	0.303	0.098	0.115	0.015	0.308	0.022	-0.398	-0.056	0.366	-0.134	0.122	0.000	-0.045	0.003	-0.070	0.002
Y5	0.054	0.021	0.156	0.059	0.555	-0.056	0.030	0.106	-0.432	0.206	0.099	0.118	0.010	-0.137	0.327	-0.206	0.039	-0.110	-0.012	-0.014
X6	-0.019	-0.023	0.057	-0.030	-0.163	-0.049	-0.024	-0.100	0.133	-0.241	0.329	-0.021	-0.098	0.042	0.626	0.066	0.464	0.012	0.066	0.074
Y6	-0.193	0.153	0.001	0.061	0.050	-0.046	-0.001	0.038	0.173	-0.008	0.415	-0.068	0.463	-0.223	-0.296	0.231	0.107	0.362	0.117	0.154
X7	-0.091	-0.098	0.565	0.033	-0.122	-0.239	0.176	0.417	0.095	0.065	0.042	0.123	0.250	0.097	-0.051	-0.087	-0.232	-0.189	-0.046	-0.086
Y7	-0.098	0.142	-0.311	-0.026	-0.078	0.096	-0.138	-0.072	0.118	-0.096	0.309	-0.031	0.049	0.488	0.000	-0.154	-0.320	-0.355	-0.143	-0.164
X8	-0.079	-0.074	0.070	0.135	0.258	0.404	-0.586	0.165	-0.192	-0.027	-0.032	0.038	0.016	0.277	-0.149	0.058	0.114	0.218	0.028	-0.054
Y8	-0.014	0.119	-0.103	-0.190	-0.225	-0.075	0.164	0.121	-0.078	0.497	-0.235	0.228	-0.214	0.352	-0.031	0.148	0.205	0.263	0.119	0.069
X9	-0.388	0.161	-0.167	0.154	0.030	0.022	0.024	-0.160	-0.072	0.117	-0.017	0.241	-0.127	-0.185	0.167	0.209	-0.416	-0.056	-0.124	0.484
Y9	0.089	0.092	0.058	-0.120	-0.287	0.137	-0.248	0.187	-0.016	0.000	-0.092	-0.280	-0.155	-0.347	-0.123	-0.440	0.090	-0.106	-0.176	0.398
X10	-0.479	0.154	-0.221	0.119	0.010	-0.071	0.168	-0.119	-0.002	0.077	-0.075	0.006	-0.034	-0.174	-0.160	-0.471	0.253	-0.002	0.128	-0.406
Y10	0.059	0.161	0.065	-0.119	-0.168	0.178	-0.159	0.042	-0.027	-0.079	-0.079	0.107	-0.155	-0.454	0.102	0.371	-0.207	-0.097	0.185	-0.525
X11	0.030	-0.008	0.139	0.015	-0.101	-0.064	-0.010	-0.363	-0.259	0.285	-0.044	-0.400	0.118	0.009	-0.164	0.348	0.206	-0.282	-0.373	-0.101
Y11	-0.161	0.109	-0.008	-0.080	-0.063	-0.121	0.165	0.040	-0.370	-0.650	-0.402	0.011	0.205	0.165	0.001	0.092	0.080	0.034	-0.079	0.079
X12	0.390	0.143	-0.057	0.131	-0.224	0.192	0.030	-0.047	-0.066	0.010	0.020	0.381	0.226	0.008	-0.152	-0.053	0.157	-0.352	0.374	0.172
Y12	0.353	0.078	-0.206	0.689	-0.057	-0.172	0.122	0.180	0.015	0.027	0.041	-0.172	-0.076	0.028	0.083	0.034	-0.030	0.143	-0.150	-0.100

Table S42. Factor loadings for the principal component analysis of left clasper shape.

FACTOR	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9	PC10	PC11	PC12	PC13	PC14	PC15	PC16	PC17	PC18	PC19	PC20
X1	-0.080	-0.157	-0.194	-0.044	-0.144	0.112	0.277	-0.240	-0.168	-0.147	-0.188	0.111	-0.415	0.213	0.007	0.144	-0.133	0.007	-0.080	0.021
Y1	0.089	-0.183	-0.224	0.231	0.110	-0.219	-0.025	0.037	-0.432	0.348	-0.201	-0.108	0.067	0.008	-0.066	-0.087	0.029	-0.003	0.006	-0.120
X2	0.226	-0.196	0.091	0.166	0.064	0.215	-0.231	0.093	0.117	-0.152	0.063	-0.204	0.167	-0.033	-0.011	-0.113	0.306	-0.185	-0.030	-0.567
Y2	-0.017	-0.246	0.123	0.047	0.532	0.331	-0.162	0.135	0.276	-0.187	0.121	0.151	-0.126	0.020	0.004	0.054	-0.062	0.082	0.010	0.343
X3	0.089	0.110	0.147	0.098	0.021	-0.132	-0.120	0.159	-0.071	0.326	0.059	0.099	-0.219	0.256	-0.283	0.163	0.566	0.035	0.070	0.313
Y3	-0.283	-0.100	0.800	-0.104	-0.159	-0.163	-0.018	-0.101	-0.102	0.090	-0.142	0.076	0.102	-0.109	0.029	-0.028	-0.060	0.004	-0.021	-0.042
X4	0.065	0.060	0.102	-0.047	0.283	0.164	0.212	-0.063	-0.245	0.186	0.249	-0.116	-0.201	-0.503	-0.331	0.168	-0.270	-0.057	-0.039	-0.128
Y4	-0.090	0.226	-0.163	0.205	0.196	-0.478	-0.306	-0.479	0.240	-0.060	0.142	-0.114	-0.189	-0.104	-0.014	-0.093	-0.054	-0.012	0.011	-0.026
X5	0.213	-0.355	0.034	-0.286	0.279	-0.082	0.184	-0.425	0.111	0.186	-0.128	0.059	0.144	0.237	0.264	-0.127	0.042	-0.077	0.004	0.008
Y5	-0.122	0.158	-0.121	0.109	0.243	-0.054	0.473	0.408	0.115	0.110	-0.215	0.092	-0.003	-0.134	0.179	-0.367	0.074	-0.010	0.018	-0.026
X6	0.017	0.211	0.108	0.185	0.040	0.018	-0.160	0.035	-0.511	-0.374	0.133	0.158	-0.137	0.010	0.477	-0.199	0.035	0.094	-0.046	0.040
Y6	-0.068	0.079	-0.157	0.009	0.172	-0.006	0.027	-0.050	-0.213	-0.256	-0.169	0.055	0.511	0.006	-0.015	0.615	0.095	0.048	0.006	0.012
X7	-0.164	0.180	0.113	0.509	-0.118	0.228	0.173	-0.192	0.205	-0.140	-0.311	-0.155	0.136	0.172	-0.305	-0.131	-0.109	0.025	0.051	0.080
Y7	0.013	-0.125	-0.085	-0.363	-0.031	0.020	-0.257	0.181	-0.226	-0.220	-0.102	-0.231	-0.050	0.272	-0.399	-0.327	-0.220	0.081	0.096	0.029
X8	-0.098	-0.172	0.025	-0.114	-0.064	-0.516	0.270	0.316	0.205	-0.347	0.161	-0.268	-0.065	0.011	-0.024	0.172	0.048	0.057	-0.212	-0.028
Y8	0.070	-0.075	-0.021	0.072	-0.243	0.195	-0.214	0.126	0.145	0.202	-0.286	-0.360	-0.334	-0.088	0.370	0.302	-0.069	-0.071	-0.190	0.036
X9	-0.329	0.094	-0.132	-0.260	-0.050	0.020	-0.124	0.069	0.084	0.008	-0.095	0.007	-0.051	-0.149	0.125	0.075	0.050	-0.257	0.722	-0.055
Y9	0.093	-0.243	-0.095	0.091	-0.318	0.056	0.087	-0.072	0.129	-0.009	0.162	0.335	-0.099	-0.141	-0.060	0.022	0.108	0.589	0.245	-0.267
X10	-0.390	0.153	-0.230	-0.324	-0.040	0.169	-0.233	-0.053	0.086	0.159	-0.083	0.124	0.155	-0.186	-0.052	-0.155	0.177	0.226	-0.507	-0.014
Y10	0.069	-0.177	-0.135	0.054	-0.337	0.017	0.045	-0.053	-0.005	-0.144	0.153	0.340	0.048	-0.195	-0.150	-0.114	0.105	-0.636	-0.166	0.210
X11	0.023	0.049	-0.034	0.143	-0.048	-0.141	-0.212	0.290	0.138	0.266	0.137	0.379	0.160	0.291	0.035	0.127	-0.566	-0.078	-0.061	-0.157
Y11	-0.223	0.100	-0.043	-0.022	-0.134	0.243	0.222	-0.091	-0.059	0.204	0.621	-0.339	0.168	0.326	0.170	-0.027	0.028	-0.018	0.034	0.045
X12	0.427	0.022	-0.031	-0.028	-0.224	-0.054	-0.034	0.012	0.047	0.029	0.003	-0.194	0.325	-0.318	0.098	-0.124	-0.145	0.210	0.129	0.485
Y12	0.469	0.588	0.121	-0.328	-0.029	0.058	0.129	-0.040	0.133	-0.078	-0.084	0.103	-0.094	0.140	-0.046	0.051	0.025	-0.053	-0.049	-0.194

Table S43. Correlations between the first five principal components of clasper shape and male body length and processus length, performed separately for left and right claspers. Adjusted P values were calculated using the sequential Holm-Bonferroni method.

Factor	Side	PC	*r*	*t*	d.f.	*P*	Adjusted *P*
Body	Left	1	-0.12	-1.43	150	0.15	1
		2	-0.11	-1.33	150	0.19	1
		3	0.33	4.26	150	< 0.001	0.001
		4	0.15	1.90	150	0.06	0.84
		5	-0.14	-1.76	150	0.08	0.97
	Right	1	0.05	0.65	149	0.52	1
		2	0.24	2.98	149	0.0033	0.057
		3	0.16	2.03	149	0.044	0.66
		4	0.34	4.40	149	< 0.001	0.0004
		5	-0.09	-1.16	149	0.25	1
Processus	Left	1	0.06	0.78	149	0.44	1
		2	0.05	0.64	149	0.52	1
		3	0.29	3.75	149	< 0.001	0.005
		4	-0.03	-0.40	149	0.69	1
		5	0.07	0.86	149	0.39	1
	Right	1	-0.09	-1.06	148	0.29	1
		2	0.15	1.85	148	0.066	0.85
		3	-0.08	-0.96	148	0.34	1
		4	0.22	2.76	148	0.006	0.1
		5	0.01	0.10	148	0.92	1