DOES SHAPE MATTER? THE EVOLUTION OF THE PENIS BONE

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Thesis Declaration

I, Gonçalo Igreja André, certify that:

This thesis has been substantially accomplished during enrolment in the degree. This thesis does not contain material that has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution. In the future, no part of this work will be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Western Australia (UWA) and where applicable, any partner institution responsible for the joint-award of this degree.

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The research reported in this thesis involved the use of the house mice (*Mus musculus domesticus*) and was assessed and approved by The University of Western Australian Research Ethics Committee (RA/3/100/1456). The research involving animals reported in this thesis followed The University of Western Australia and national standards for the care and use of laboratory animals. The research reported in this thesis was supported by ARC Discovery grants awarded to Leigh Simmons (DP 35000300). Gonçalo Igreja André was supported by the Australian Government Postgraduate Research Training Program (RTP) and the UWA Ad Hoc Postgraduate Scholarship.

This thesis contains published work and/or work prepared for publication, some of which has been co-authored.

Gonçalo Igreja André
Abstract

Animal genitalia are recognised as being among the most highly variable and evolutionary divergent of all animal structures, and the evolutionary divergence of the mammalian baculum or penis bone is one of the most puzzling enigmas of mammalian morphology. Sexual selection is accepted as being responsible for the evolutionary divergence of genital morphology with cryptic female choice being thought to be an important mechanism of selection on the baculum via its stimulatory role during copulation. However, empirical studies that test this hypothesis are lacking, and those studies that are available have focused exclusively on male morphology, neglecting the likely female role in male genital evolution. I used the house mouse (*Mus musculus domesticus*) as a model species to investigate the selective mechanism acting on the mammalian baculum, considering female mediated processes as possible drivers for male genital evolution.

In my first chapter, I investigated phenotypic plasticity in baculum morphology in response to the level of sperm competition risk perceived by male house mice during development. By exposing male house mice to two different social environments during their sexual development and later measuring baculum shape and size, I found that males reared under the risk of future sperm competition developed a relatively wider and more distally extended baculum bulb compared with males reared under no sperm competition risk. This suggests that the house mouse baculum, like other sexual selected traits, has the ability to respond plastically to the competitive environment and adds to growing evidence of the role of sexual selection in the evolution of the mammalian baculum.

In Chapter Two, I established a paternal half-sibling design to explore the level of genetic variance and covariance in the house mouse baculum and female reproductive
tract. I measured baculum size and shape in males and the length of the vaginal tract and width of the vaginal cervix in females. Baculum morphology (shape and size) and female vaginal cervix width were found to harbour significant additive genetic variation and I found evidence of genetic covariation between male baculum size and shape and female vaginal cervix width. While it is expected that almost all phenotypic traits exhibit additive genetic variance to a certain degree, the genetic covariance between male and female genital traits provides novel insight into the potential for the coevolutionary divergence of male and female genital traits in mammals.

In Chapter Three, I assessed how variation in male and female genital morphology affects reproductive success. I selected males and females from families characterised by extremes of baculum shape (wide-narrow) and conducted non-competitive (monogamous) and competitive (polyandrous) mating trials from which I could examine paternity outcomes. Baculum shape had no effect on a male’s ability to sire offspring when females mated with just one male. However, when competing for fertilisation, baculum shape was found to influence a male’s paternity success, but the effect of baculum shape depended on the baculum shape characteristic of families from which females were drawn. This provides support for the role of cryptic female choice on the coevolution of the house mouse baculum and lends support to the hypothesis that cryptic female choice exerts selection on male genitalia by its stimulatory role during copulation.

In Chapter Four, I investigated how variation in baculum shape affects female physiological responses to copulation. Using males and females from families characterised by extremes of baculum shape, I conducted monogamous matings to quantify the extent to which male and female traits predict postcoital Prolactin hormonal levels in the blood (fifteen minutes and seventy-five minutes after ejaculation). Prolactin has been linked to the rewarding system of mammalian sexual
behaviour and to mediate embryo implantation. I found that the levels of Prolactin were significantly increased fifteen minutes after females received an ejaculation and that this increase was influenced by baculum shape and a male’s copulatory behaviour. Males with a relatively wide baculum induced higher levels of Prolactin when delivering less peri-copulatory behaviour, however for males with a relatively thin baculum, the amount of peri-copulatory behaviour did not affect the levels of circulating Prolactin.

Our data are consistent with the hypothesis that vagino-cervical stimulation by the baculum generates a release of Prolactin in females following copulation, and thereby provide novel support for the stimulation hypothesis for the evolution of the mammalian baculum.

Together, the results of this thesis provide support for the role of post-copulatory sexual selection in the coevolution of mammalian genitalia and provide novel insight into the stimulatory role of the mammalian baculum.
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This thesis is for my Parents.
Authorship Declaration

The data chapters of this thesis are presented as a series of manuscripts. Chapters One have been published, and Chapter Two is under consideration for publication at the time of thesis submission.

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Lead author, overall responsibility for planning experiment, executed this design and collected data, statistical analysis and writing. Overall contribution over 85%.

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Student signature

I, Leigh Simmons certify that the student’s statements regarding their contribution to each of the works listed above are correct.

Coordinating supervisor signature
PROLOGUE

“Science knows no country, because knowledge belongs to humanity,

and is the torch which illuminates the world”

Louis Pasteur
A Brief introduction to sexual selection and sexual conflict

Sexual selection was described by Charles Darwin as arising from ‘the advantages that certain individuals have over others of the same sex and species in exclusive relation to reproduction’ (Darwin 1871). In his observations, Darwin noticed that males with more elaborated ornaments or bigger weapons had a reproductive advantage, through their success in intrasexual competition for access to females or by being preferred by females as mates (Darwin 1871). Mate choice is considered to occur when the choosy sex (commonly females) has a preference to reproduce with individuals of the opposite sex that have particular characteristics (commonly males) (Andersson and Iwasa 1996; Andersson and Simmons 2006). There are a number of different, but non-exclusive, mechanisms that have been proposed for the evolution of mate choice. Mate choice may evolve due to the direct and/or indirect benefits to the choosing individual (Møller and Jennions 2001; Andersson and Simmons 2006). Direct benefits may include protection and care of the female and her offspring, leading to a higher rate of survival and fecundity (Møller and Jennions 2001; Andersson and Simmons 2006). Indirect benefits accrue in offspring that have a higher breeding value as a consequence of inherited, genetically based traits of the chosen male (Møller and Jennions 2001; Kuijper et al. 2012).

Fisher (1930) championed the evolution of mate choice by sexual selection, proposing two mechanisms that differ in the nature of the indirect benefits that females receive from their choice (Fisher 1930; Kokko et al. 2002; Andersson and Simmons 2006). His name became associated primarily with Runaway coevolution of secondary sexual traits and female preferences, also known as the ‘sexy sons’ hypothesis (Fisher 1930; Andersson and Simmons 2006). Fisherian runaway argues that females increase the reproductive success of their offspring by choosing males with heritable attractiveness (Andersson and Iwasa 1996; Kokko et al. 2002; Andersson and Simmons 2006). Under
the runaway model of preference evolution, females choose males that possess traits which give a reproductive advantage to their male offspring because of the attractiveness of those traits and produce female offspring that exhibit the same preference (Andersson and Iwasa 1996; Andersson and Simmons 2006). Evolution of trait and preference are self-reinforcing, hence the term “runaway selection”. The second mechanism proposed by Fisher (1930) argues that mating preferences evolve through the indirect benefits that females receive when choosing a mate over and above male attractiveness (Møller and Jennions 2001; Kokko et al. 2002; Andersson and Simmons 2006). Known as the good genes hypotheses, mate choice is thought to evolve when attractive secondary sexual traits in males also serve as indicators of the overall genetic quality of individuals (Kokko et al. 2002; Andersson and Simmons 2006).

Female choice then leads to higher reproductive success, both through the mating success of their sons and the general viability of both sons and daughters (Iwasa et al. 1991; Møller and Alatalo 1999). The reliability of attractive traits as cues to genetic quality is thought to arise because of the costs of secondary sexual trait expression – the handicap hypothesis (Zahavi 1975).

Mate choice may also arise as a result of pleiotropic effects of, naturally selected preference traits in females (Endler and Basolo 1998; Boughman 2002; Andersson and Simmons 2006). For example, female preference for a colourful trait in males may arise because of a sensory bias towards a trait colour that it is favoured in the context of foraging without any direct or indirect advantage to the female in the context of reproduction (Endler and Basolo 1998; Boughman 2002; Andersson and Simmons 2006). Under pleiotropic selection, male sexual traits arise to exploit the pre-existing sensory bias in females. Finally, mate choice can also be based on genetic compatibility (Tregenza and Wedell 2000; Mays and Hill 2004). As each individual is genetically unique, a potential mating partner may be suitable for some individuals but not others,
so that in order to optimise offspring survival females will select the male that is optimal for promoting offspring viability given her own genotype (Compatible genes hypothesis) (Mays and Hill 2004; Andersson and Simmons 2006). Unlike Fisherian and good-genes mate choice, mate choice for genetic compatibility is not expected to impose directional selection on the traits that signal mate value.

The mechanisms proposed for the evolution of mate choice assume that assortative mating brings direct and/or indirect reproductive advantages to an individual. However, for a wide number of species, male and female reproductive fitness optima differ, creating a conflict between sexes (Parker 1979; Chapman et al. 2003; Arnqvist and Rowe 2005). Sexual conflict, can arise in the context of both natural and sexual selection (Arnqvist and Rowe 2005; Kokko and Jennions 2014). In the context of sexual selection, sexual conflict arises from a divergence in the fitness interests of males and females over reproductive decisions and/or outcomes (Parker 1979; Chapman et al. 2003; Arnqvist and Rowe 2005). Two main forms of conflict occur, intralocus and interlocus conflict (Chapman et al. 2003; Arnqvist and Rowe 2005). Intralocus sexual conflict refers to situations when selection leads to a divergence in the optimal expression of a trait shared by males and females (Chapman et al. 2003; Arnqvist and Rowe 2005). In contrast, interlocus selection occurs when a trait favoured in one sex, say a secondary sexual trait, reduces an aspect of fitness in the opposite sex, such as lifespan (Chapman et al. 2003; Arnqvist and Rowe 2005). Interlocus sexual conflict can occur over traits such as mating rate, female remating behaviour, and parental care (Chapman et al. 2003; Arnqvist and Rowe 2005). As mentioned previously, male secondary sexual traits can have their evolutionary origin due to pleiotropic selection imposed by a pre-existent sensory bias in females (Endler and Basolo 1998; Boughman 2002; Andersson and Simmons 2006). Once established, males capable of exploiting the pre-existent sensory bias (e.g. sounds, colours, behaviours) will be favoured by
selection because they will be able to induce females to mate beyond their naturally selected optimum, leading to counter-adaptations in females to resist the sensory stimulation of males (Endler and Basolo 1998; Boughman 2002; Arnqvist and Rowe 2005; Andersson and Simmons 2006; Arnqvist 2006). Under sexual selection therefore, the coevolution of male trait and female preference is more appropriately viewed as a chase away process in which male trait and female sensory response coevolve under sexually antagonistic selection (Holland and Rice 1998).

Secondary sexual characters that confer an advantage in contest competition, mate choice and sexual conflict arise as a result of pre-copulatory sexual selection, affecting an individual’s mating success (Clutton-Brock 2007). However, for most sexually reproducing species females’ mate with many males before or during offspring production (polyandry), Parker (1970) recognised that sexual selection will continue after copulation. Thus, post-copulatory sexual selection can act via variation in fertilisation success affected during or after mating and includes mechanisms of cryptic female choice, sperm competition, and sexual conflict (Parker 1979; Andersson and Simmons 2006; Eberhard 2009a).

Cryptic female choice is a mechanism of sexual selection that acts via the differential utilisation of sperm from prospective fathers after a female has mated multiply (Eberhard 1996, 2009a; Andersson and Simmons 2006). Females can covertly choose which males fertilise their ova by employing different physiological strategies, such as differential sperm ejection, transit and storage; moderating the performance of sperm within the tract; selective fertilisation; differential abortion of zygotes; and/or the suppression of oviposition (Eberhard 1996, 2009a; Birkhead and Møller 1998; Andersson and Simmons 2006; Firman et al. 2017). By mating with multiple males, females create an environment for male-male competition to continue in the form of sperm competition for the fertilisation of their oocytes (Eberhard 1996, 2009a; Birkhead
and Møller 1998). Males that are equipped with features that guarantee a higher chance of winning access to fertilisations will be favoured by selection (Eberhard 1996; Birkhead and Møller 1998). Male traits, including sperm motility, sperm number, testis size, sperm allocation per ejaculate, copulatory behaviour and genital morphology, have all been shown to be subject to selection from sperm competition (House and Simmons 2003; Firman and Simmons 2010, 2012; Wojcieszek and Simmons 2011; Stockley et al. 2013). Sperm competition however, can generate sexual conflict, especially if male adaptations to sperm competition are harmful to females (Chapman et al. 2003; Arnqvist and Rowe 2005; Parker 2006). Under sexual conflict, females are expected to evolve counter-adaptations to reduce the costs of male adaptations to future sperm competition (Chapman et al. 2003; Arnqvist and Rowe 2005; Parker 2006).

(ii) The Evolution of Animal Genitalia

In sexually reproducing species with internal insemination, male external genitalia are essential for the deposition of the ejaculate into the female reproductive tract (Frazee and Masly 2015). Male external genitalia are widely recognised as being among the most highly variable and evolutionary divergent of all animal structures (Eberhard 1985; Hosken and Stockley 2004). External genital morphology has long been one of the key features for insect taxonomists when classifying closely-related species (Tuxen 1970) and has been found to correlate strongly with mating systems, whereby male genitalia are typically more diverse in polygamous than in monogamous mating systems (Arnvist 1998; Dixson 2012). Variation in male genital morphology has been shown to influence mating, fertilisation and overall male reproductive success (Arnvist and Danielsson 1999; Danielsson and Askenmo 1999; House and Simmons 2003; Hotzy and Arnqvist 2009; Wojcieszek and Simmons 2011). Explaining the wide divergence in genital morphology has been a widely studied topic with three main hypotheses that
offer plausible scenarios for the evolution of animal genitalia (Eberhard 1985, 2010; Arnqvist 1997; Hosken and Stockley 2004; Simmons 2014).

The lock-and-key hypothesis was considered initially by Dufour (1844), who proposed that animal genitalia evolves via reproductive isolation (Arnqvist 1998; Simmons 2014). According to the lock-and-key hypothesis, in order to successfully copulate and avoid the costs of hybridisation, the male genitalia evolves to be species-specific (the key) to properly connect with the female genitalia (the lock) (Arnqvist 1998; Simmons 2014). The lock-and-key theory has mainly be supported by interspecific differences in genital morphology, however, most taxonomic studies have focused on male genitalia and have ignored female genital morphology (Eberhard 1985, 2010; Simmons 2014). Only recently have studies started to reveal the existence of interspecific variation in female reproductive tracts and their coevolution with male genitalia (Simmons 2014). An alternative hypothesis to the lock-and-key hypothesis argued that divergence in male genitalia was due to indirect/neutral selection - Pleiotropy selection theory (Eberhard 1985, 2010; Arnqvist 1997; Hosken and Stockley 2004; Simmons 2014). The pleiotropy hypothesis argues that the divergent evolution of genitalia is a by-product of selection on unrelated traits that confer ecological advantages to the individual (Arnqvist 1997; Hosken and Stockley 2004; Simmons 2014). However, neither the lock-and-key nor the pleiotropy hypothesis can account for the widely observed pattern of divergence in male genitalia that is due to mating system (Simmons 2014).

Sexual selection and sexual conflict are now recognised as the most likely mechanisms driving the evolution of animal genitalia (Eberhard 1985, 2010; Arnqvist 1997; Hosken and Stockley 2004; Simmons 2014). A growing number of studies have provided evidence that male genitalia are subject to sexual selection during copulation (Eberhard 1985; Arnqvist 1998; Simmons et al. 2009). Sexual selection on genitalia via cryptic female choice may rely on the perception of stimulation received during copulation.
generating a bias in paternity outcome (Eberhard 1985, 1996; Hosken and Stockley 2004; Brennan and Prum 2015). Male stimulatory capabilities may be selected via a Fisherian mechanism (sexy sons) and/or if males of better genetic quality are better able to stimulate the female (good genes) (Eberhard 1985, 1996; Hosken and Stockley 2004; Brennan and Prum 2015). Furthermore, males equipped with genitalia that are able to remove and/or dislodge the ejaculate from previous males will have a reproductive advantage such that genital morphology will evolve in response to sperm competition (Eberhard 1985; Simmons 2001, 2014). Contrarily, male adaptation to male-male competition can be costly for females resulting in sexual conflict (Chapman et al. 2003; Hosken and Stockley 2004; Parker 2006; Brennan and Prum 2015). The sexual conflict hypothesis proposes that male genitalia develop structures that promote their fitness, often through success in sperm competition, at a cost to female fitness. In turn, selection on females counteracts male costs via adaptations in their genitalia that reduce the cost of mating (Arnvist and Rowe 2005; Brennan et al. 2007; Dougherty et al. 2017).

Compared to the evolution of the male genitalia, female genital diversity has been less well studied (Ah-King et al. 2014; Simmons 2014; Brennan and Prum 2015; Sloan and Simmons 2019). Nonetheless, female genitalia have been demonstrated to interact with male genitalia and to influence mating and fertilisation success (Arnvist and Danielsson 1999; Briceño and Eberhard 2009). Both theories of cryptic female choice and sexual conflict predict the coevolution of male and female genital morphology (Mead and Arnold 2004), either in response to sexual selection acting on interacting genital traits or to selection in response to costs that male genital traits impose. Empirical studies have offered support for both cryptic female choice and sexually antagonistic mechanisms of sexual selection. In dung beetles (Simmons and Holley 2011; García-Gonzalez et al. 2012; Simmons and Fitzpatrick 2019), bushcrickets (Wulff et al. 2015, 2017; Wulff and Lehmann 2016), tsetse fly (Briceño and Eberhard 2009).
and damselfly (Córdoba-Aguilar 1999, 2006) the coevolution of interacting male and female genital traits is consistent with a model of cryptic female choice. In contrast, the evolution of male and female genital traits in seed beetles (Rönn et al. 2007; Hotzy et al. 2012; Dougherty et al. 2017), water striders (Arnqvist and Danielsson 1999) and waterfowl (Brennan et al. 2007, 2010) appear consistent with a model of sexually antagonistic coevolution.

By far, the majority of studies that have examined sexual selection acting on genital evolution have used invertebrates as model systems. In contrast, much less research has explored these mechanisms among vertebrate species (Brennan et al. 2010; Gasparini et al. 2011; Friesen et al. 2013; Heinen-Kay and Langerhans 2013; Mautz et al. 2013; Orbach et al. 2017). For most mammalian species, cryptic female choice is believed to drive male genital evolution via its stimulatory role during copulation (Eberhard 1996; Brennan and Prum 2015; Firman et al. 2017), but there is currently little evidence in support of this hypothesis.

(iii) The Mammalian Baculum

The baculum, or penis bone, is a common feature of male genitalia across four orders of mammal: Carnivora, Chiroptera, Primates and Rodentia (Stockley 2012). The baculum is considered the most diverse bone across the mammals and yet, despite numerous theories, our understanding of the evolutionary divergence in baculum morphology remains one of the most challenging topics in mammalian morphology (Patterson and Thaeler 1982; Stockley 2012).

Different functional explanations have been offered to explain baculum evolution (Patterson and Thaeler 1982; Lariviere and Ferguson 2002; Stockley 2012). The baculum may be present in the intromitting organ to increase the level of rigidity and so facilitate intromission of the female reproductive tract (Long 1969). In species that
exhibit multiple intromissions during copulation, the baculum may facilitate repeated
intromissions by eliminating the problem of a narrower female reproductive tract (Ewer
1973). By being a rigid component of the penis, the baculum may have an impact on the
transmission of sperm to the female reproductive tract by promoting their delivery to
positions where they are more likely to fertilise ova (Ewer 1973). On the other hand, the
baculum may be present as a stimulatory device that facilitates sperm transport and
influences female sexual receptivity and pregnancy initiation (Greenwald 1956;
Eberhard 1996; Lariviere and Ferguson 2002).

For the most part, evidence that sexual selection is responsible for the evolution of the
mammalian baculum has been indirect. The majority of studies on baculum evolution
have looked for positive allometry (Lüpold et al. 2004; Ramm 2007; Miller and
Nagorsen 2008; Ramm et al. 2009; Schulte-Hostedde et al. 2011). Allometry refers to
the scaling relationship between different body traits. Positive allometry is found when
the sexual trait has a scaling relationship that exceeds one, meaning that the size of the
trait exceeds what would be expected for a trait that is under natural selection (Eberhard
2009b,a). Typically, male traits that are subject to sexual selection exhibit positive
allometry, with larger males having relatively larger sexual traits (Green 1992; Petrie
1992; Simmons and Tomkins 1996; Fromhage and Kokko 2014). Even though there is
some evidence for positive allometry of the baculum (Lüpold et al. 2004), not all studies
have found consistent patterns (Miller and Nagorsen 2008; Ramm et al. 2009; Schulte-
Hostedde et al. 2011). The lack of congruence among studies of baculum allometry
makes this measure an unreliable indication of sexual selection. Further, the
mammalian baculum has been shown to have been gained and lost multiple times
throughout the evolutionary history of the mammalian clade (Schultz et al. 2016b).
There have been nine independent origins of a baculum during mammalian evolution,
and an evolutionary loss of the baculum at least ten times. As such, the mammalian
baculum cannot be considered to be a homologous structure and is likely to have responded evolutionarily to species-specific challenges and to different selective pressures (Schultz et al. 2016b).

Perhaps our best understanding of contemporary baculum evolution comes from empirical studies of wild house mice (*Mus musculus domesticus*). Three populations of house mice known to be under different levels of post-copulatory sexual selection as estimated from the rates of mixed paternity per litters (Firman and Simmons 2008c), where brought to the laboratory and reared under common-garden conditions. After three generations, baculum morphology was assessed using geometric morphometric analyses. The authors found an association between the levels of post-copulatory sexual selection and the shape of the baculum; the males from populations with high levels of post-copulatory sexual selection were found to have a relatively wider baculum compared to populations with lower levels of post-copulatory sexual selection (Simmons and Firman 2014). Furthermore, the role of sexual selection in the evolution of the house mouse baculum was confirmed using experimental evolution. Thus, by establishing populations of house mice evolving under two treatments, with or without post-copulatory sexual selection, the authors demonstrated that after 2 and 27 generations of selection, post-copulatory sexual selection resulted in an evolutionary divergence in baculum shape that reflected the differences seen among natural populations of mice (Simmons and Firman 2014); specifically, males evolving under post-copulatory sexual selection evolved to have a relatively wider baculum when compared with males from populations evolving under enforced monogamy. These findings are consistent with an independent study of free-ranging house mice which found baculum width and not baculum length contributed toward a male’s reproductive success (Stockley et al. 2013). Despite this strong evidence for sexual selection’s role in
baculum evolution, there are currently no studies that have examined the functional significance of the baculum during reproduction.

By being an ossified structure in the mammalian penis (Glucksmann et al. 1976; Murakami and Mizuno 1984), the baculum is believed to evolve due to its stimulatory role during copulation (Eberhard 1996; Lariviere and Ferguson 2002; Dixson 2012; Brennan and Prum 2015). Mammal copulation includes behaviours that are typically required for a successful pregnancy, for example, by stimulating the female reproductive tract (Eberhard 1996; Pavličev and Wagner 2016). The mammalian vaginal tract is highly innervated (Hilliges et al. 1995; Barry et al. 2017; van Helden et al. 2017) and regulated by the neuroendocrine system (Brenner and West 1975) making it a candidate structure for the perception of stimulation that imposes sexual selection on male genitalia (Eberhard 1996; Hosken and Stockley 2004; Brennan and Prum 2015). During mammalian copulation, genital stimulation induces a cascade of physiological events that prime the female reproductive tract for embryo implantation and condition future female sexual behaviour (Diamond 1972; Pfau et al. 1999, 2001; Pavličev and Wagner 2016). Copulation has been shown to induce an increase in circulating Prolactin in both rats and humans (Erskine and Kornberg 1992; Blaicher et al. 1999; Exton et al. 1999), and this hormone is known to influence sexual behaviour and reproduction (Erskine and Kornberg 1992; Anderson-Hunt and Dennerstein 1995; Blaicher et al. 1999; Exton et al. 1999). In rats, there is a mating-induced surge of plasma Prolactin (Erskine 1995); vagino-cervical stimulated females show an acute increase in Prolactin within the first minutes of stimulation (Erskine and Kornberg 1992; Kornberg and Erskine 1994; Erskine 1995) and females that enter pseudopregnancy exhibit higher levels of circulating Prolactin than those that do not (Erskine and Kornberg 1992; Kornberg and Erskine 1994). Prolactin has also been linked with the rewarding system of sexual behaviour and the establishment of sexual satiety (Convey et al. 1971; Kamel
et al. 1975; Bronson and Desjardins 1982; Exton et al. 2001; Leeners et al. 2013). The mammalian baculum, therefore, could potentially play a role in delivering the stimulation to the female at copulation that is critical for successful reproduction.

(iv) Aims and Scope of this Thesis

In this thesis, my aim was to address gaps in our understanding of the function of the mammalian baculum, and the selective mechanisms responsible for its evolution. Given how much we know already about the house mouse, I have opted to use this model system in my research. Importantly, I took a holistic approach by considering the often-neglected female reproductive morphology and physiology, which is likely to be key in our understanding of the evolution of baculum morphology.

Previous research strongly suggests that the house mouse baculum plays a role in determining male fitness and is subject to sexual selection. In the first two chapters of this thesis, I explored the extent to which baculum shape is environmentally and genetically determined. Thus, in Chapter 1, I assessed phenotypic plasticity in baculum morphology in response to the risk of sperm competition perceived by male house mice during pre-adult development. Phenotypic plasticity allows an individual to adapt to changes in its environment and has been reported in a variety of life-history traits.

Consistent with theoretical predictions for models of post-copulatory sexual selection, previous studies have demonstrated that male house mice are capable of strategically allocating their ejaculate according to the perceived risk that they will engage in post-copulatory sexual competition (Ramm and Stockley 2009; Firman et al. 2013; Ramm et al. 2015). If the baculum is an important fitness trait in determining male competitive fertilisation success, I predicted that they will adjust their investment in the development of this trait depending on the perceived risk of sperm competition. Thus, I raised males in two different social environments, with or without cues of sperm


competition risk, and quantified variation in baculum morphology in response to the 

social environment using a landmark-based morphometrics approach.

To address the functionality of the house mouse baculum, I needed house mice with 
predictable extremes in genital morphology. Thus, in Chapter 2, I used a quantitative 

 genetic design to produce families that varied in genital morphology. This allowed me 
to quantify the amount of genetic variation and covariation underlying baculum 
morphology and vaginal tract size in wild house mice, something that has been done 
many times in invertebrates but never before in a mammal. Moreover, the quantitative 

 genetic design allowed me to obtain families of mice with known breeding values for 
 baculum shape. Thus, I applied a paternal half-sibling breeding design with 30 sires and 
78 dams and I assessed offspring for baculum morphology and female reproductive 
 tract morphology using Restricted Maximum Likelihood procedures.

Using eight families with breeding values for the widest and narrowest bacula, I 
generated families of mice with which to explore the adaptive value of baculum shape 
for male reproductive fitness. In Chapter 3, I asked whether baculum morphology has a 
causal effect on paternity success under non-competitive (monandrous) and competitive 
(polyandrous) mating scenarios and determine whether female genetic background 
mediates this effect. I evaluated how baculum morphology affects embryo implantation 
and viability in single mated females (monandrous) and used molecular markers to 
determine how baculum morphology affects paternity success when two males compete 
for fertilisation (polyandrous).

Finally, in Chapter 4, I specifically tested the hypothesis that the baculum is under 
sexual selection via its role in stimulation of the female reproductive tract during 
copulation. As in Chapter 3, I used males and females of known genetic background for 
baculum shape in staged mating interactions and examined the concentration of
circulating Prolactin in females following copulation, either 15 or 75 minutes after receiving an ejaculate. I quantified variation in baculum morphology and male copulatory behaviour in order to determine how these male traits influenced female hormonal responses to copulation.

Finally, I synthesised the findings presented in this thesis in an epilogue and outline some potential avenues for future research.
CHAPTER ONE: PHENOTYPIC PLASTICITY IN GENITALIA: BACULUM SHAPE Responds TO SPERM COMPETITION RISK IN HOUSE MICE

This chapter is presented as published in Proceedings of the Royal Society of London B: Biological Sciences, with minor changes to ensure consistency with the rest of my thesis.

1.1 Abstract

Males are known to adjust their expenditure on testes growth and sperm production in response to sperm competition risk. Genital morphology can also contribute to competitive fertilisation success but whether male genital morphology can respond plastically to the sperm competition environment has received little attention. Here, we exposed male house mice to two different sperm competition environments during their sexual development and quantified phenotypic plasticity in baculum morphology. The sperm competition environment generated plasticity in body growth. Males maturing under sperm competition risk were larger and heavier than males maturing under no sperm competition risk. We used a landmark-based geometric morphometric approach to measure baculum size and shape. Independent of variation in body size, males maintained under risk of sperm competition had a relatively thicker and more distally extended baculum bulb compared with males maintained under no sperm competition risk. Plasticity in baculum shape paralleled evolutionary responses to selection from sperm competition reported in previous studies of house mice. Our findings provide experimental evidence of socially mediated phenotypic plasticity in male genitalia.
1.2 Introduction

Phenotypic plasticity is defined as the capacity for a single genotype to produce multiple phenotypes in response to variation in the environment (Fordyce 2006). Phenotypic plasticity allows individuals to adapt to new conditions, or anticipate future conditions, by accessing environmental or social cues that predict environmental change (Whitman and Agrawal 2009). Many different types of traits can exhibit plasticity, which may lead to transient or permanent changes in morphological, physiological and/or behavioural phenotypes (Fordyce 2006). Phenotypic plasticity is a developmental process occurring within the lifetime of an individual. Nevertheless, it can be a major avenue of evolutionary divergence among populations (Pfennig et al. 2010). Adaptive phenotypic plasticity has been reported in a number of important life-history traits including predator defences, immune responses, and sexual traits important in reproductive competition (West-Eberhard 2003; Lyytinen et al. 2004; Schmid-Hempel 2005; Bretman et al. 2011).

Phenotypic plasticity in male allocation to reproductive competition has been described in a range of taxa (Bretman et al. 2011). Theoretical models predict that males should strategically allocate resources to increase ejaculate expenditure when faced with an increased risk of sperm competition (Parker and Pizzari 2010). Consistent with this prediction, empirical studies have reported phenotypic plasticity in ejaculate expenditure by males, cued by their social environment (Kelly and Jennions 2011). For example, males make adaptive changes in ejaculatory frequency (Candolin and Reynolds 2002; DelBarco-Trillo and Ferkin 2006), the size of the ejaculate (Garcia-Gonzalez and Gomendio 2004), the number of sperm within the ejaculate (Evans et al. 2003; delBarco-Trillo and Ferkin 2004; Smith et al. 2009), and the composition of seminal fluid (Simmons and Lovegrove 2017; Sloan et al. 2018) depending on their perceived risk of sperm competition. In house mice, exposure of males to potential
rivals induces increased levels of sperm production (Ramm and Stockley 2009; Firman et al. 2013), an increase in the production of key seminal fluid proteins (Ramm et al. 2015), and changes in mating behaviour, including a decrease in the number of thrusts, intromissions and duration of copulation, and an increase in the probability of repeated ejaculation (Preston and Stockley 2006). These studies of house mice illustrate how the sperm competition environment can induce phenotypic plasticity in a variety of male reproductive traits and that phenotypic plasticity need not be limited to sperm production.

There is now considerable evidence that male genital morphology is subject to sexual selection, and variation in genital shape can impact mating, insemination, fertilisation and overall male reproductive success (Eberhard 1985; Hosken and Stockley 2004; Simmons 2014). Preliminary evidence from ducks suggests that males may have the capacity to adjust the morphology of their penis in response to reproductive competition (Brennan et al. 2017). The ossified penis or baculum is a feature of several mammalian orders (Ramm 2007; Brindle and Opie 2016). In house mice, the shape of the baculum affects competitive male reproductive success (Stockley et al. 2013) and co-varies with the strength of selection from sperm competition among natural and experimentally evolving populations (Simmons and Firman 2014). The house mouse baculum is comprised of two structures, a proximal ossified and a distal cartilaginous structure (Glucksmann et al. 1976; Rodriguez et al. 2011). The growth of these structures occurs primarily during the first 60 days post-birth (Glucksmann et al. 1976) and is known to depend on androgens and endogenous oestrogen expression during pre-and post-natal development (Glucksmann et al. 1976; Yonezawa et al. 2011; Rodriguez et al. 2012).

In this study, we assessed whether house mouse baculum morphology exhibits phenotypic plasticity in response to sperm competition risk during pre-adult development. Given the wide-ranging effects of sperm competition risk on male
reproductive physiology and behaviour (Preston and Stockley 2006; Ramm and Stockley 2007, 2009; Firman et al. 2013; Ramm et al. 2015), and the importance of baculum shape for male reproductive success (Stockley et al. 2013), we might expect baculum morphology to show similar plasticity in response to cues in the social environment (Brennan et al. 2017). We thus raised males in two different social environments, either with or without cues to sperm competition risk and quantified variation in the shape of the baculum as well as a naturally selected bone, the hind femur. We expected males to show phenotypic plasticity in baculum morphology in response to the sperm competition environment, but not in the morphology of the hind femur.

1.3 Materials and Methods

(a) Experimental Animals

Wild house mice (*Mus musculus domesticus*) were sourced from an isolated population on Rat Island (28° 42’S, 113° 47’E) off the coast of Western Australia (n=100) and maintained in the laboratory at the University of Western Australia. The animals were held in constant temperature rooms (CTR; 24°C) on a reverse dark-light cycle (10:14) and provided with water and food *ad libitum*. Mice were outbred under common-garden conditions for two generations. Male and female pairs were housed together for a maximum of 14 days. When noted to be pregnant, females were housed alone. At weaning age (21 days) female and male offspring were separated; female offspring were housed in groups and male offspring housed individually. A total of 48 males and eight females were used in this study.

(b) Social Manipulation

We raised males in one of two social environments throughout their sexual development by manipulating their exposure to rival males and their odours. Two sibling males from
24 families were weighed and then randomly assigned to either a "Risk" or "No Risk" environment. Details of the experimental manipulation are described elsewhere (Firman et al. 2013). Briefly, males in both environments were housed individually in cages (16×33×12 cm). The individual cages in the “No Risk” environment were placed alone in a large plastic tub (49×74×41 cm), while those in the “Risk” environment were co-housed in a large plastic tub with those of two unrelated males. Males were housed under these conditions from weaning (21 days of age) until sexual maturity (90 days of age). The tubs were spread across two large (9x4 m) constant temperature rooms (CTR), such that each room contained four “Risk” tubs (total N males=12) and twelve “No risk” tubs (total N males=12). Tubs were spaced 60cm apart. Sexually mature females (N=4) were housed in their own cages within each CTR. Thus, females were placed in the centre of the room in large cages (28×46×13 cm) approximately equidistant to tubs containing males.

Each week, males in the “Risk” treatment were exposed to soiled chaff (15g) of the two rival males within their tub. For this, chaff was taken from the cage of each male and placed at the front of their rival male's cages. In the “No risk” treatment, males were ‘exposed’ to their own soiled chaff, i.e. it was moved from the back to the front of their cage. Once a fortnight “encounters” were conducted whereby males were released into the tub and allowed to roam freely for 30 minutes. In the “Risk” treatment, males were released one at a time and allowed to interact with their neighbouring males through the bars of their cages. “No risk” treatment males were released inside their tub but did not experience any interaction with other individuals. The same general procedures were performed using females; thus, once a fortnight, males of both treatments were exposed to female soiled chaff (15g) and interacted with a female through the bars of their cages.
(c) Baculum and Femur Morphometric Analyses

At 90 days of age, sexually mature males were sacrificed, and the baculum and right femur were dissected for analysis. At the time of dissection, the majority of the tissue surrounding the baculum and the femur was removed. However, to ensure complete tissue removal, both structures were stored overnight in 1ml of 5% KOH. Following this, the specimens were stored in 1ml of Dietrich’s Fixative solution.

Digital images of the baculum (ventral view) and femur (external lateral view) were taken using a binocular microscope at ×20 and ×10 magnification, respectively. Geometric morphometric analysis of bone shape was conducted using the software developed by Rohlf (2006), blind to the treatment group from which images were obtained. Landmarks were placed around the periphery of the baculum (36 sliding, 4 fixed) and the femur (71 sliding, 8 fixed) using the software tpsDig2 version 2.29 (for details see Fig.S1.1 in the online supplementary material). The software Tpsrelw version 1.65 was then used to extract relative warps (RWs) and centroid size. RWs represent the variation in shape relative to the consensus configuration across all samples (Zelditch et al. 2012c). In this study, we focused on those RWs that individually explained >10% of the variance for both the baculum and femur. Centroid size (square root of the summed distances of each landmark to the centroid in x and y distances) provided a multivariate measure of the size of each bone (Zelditch et al. 2012d). The repeatability of landmark placement was assessed by calculating the Euclidean distances between fixed landmarks for twelve individuals (six from each of the risk and non-risk treatment) on two separate occasions. We also assessed the repeatability of centroid size and shape scores derived from our geometric morphometric analyses of these landmarks (for details see Fig.S1.2 in online supplementary material).
(d) Statistical Analysis

Examination of the residuals identified outliers from the femur relative warps analysis (for details see Table S1 in the online supplementary material). Shapiro-Wilk tests confirmed that data residuals were normally distributed, and parametric tests were applied in all cases. We used linear mixed models fitted by maximum likelihood estimation using the lmer procedure in the lme4 R package (Bates et al. 2017). Significance values were extrapolated from Type II Wald chi-square tests using the ANOVA function in the car package of R (Fox et al. 2016). All morphological traits, except the RW scores, were log transformed prior to analysis. Male family identity and replicate tub identity were included in the model as random factors; log body length was entered as a covariate. Non-significant interaction terms were removed from statistical models.

We conducted bivariate line-fitting methods for estimating the relationships between baculum and femur centroid size and body length. We used SMATR version 2.0 freeware which allowed us to fit bivariate lines to the data and make inferences about such relationships (Warton et al. 2006). Following previous studies (Lüpold et al. 2004; Ramm et al. 2009; Tasikas et al. 2009), we opted to conduct standard major axis (SMA) regressions.

1.4 Results

Descriptive statistics for body size, genital and non-genital traits can be found in Table S1 of the online supplementary material. Individuals did not differ in body weight when first assigned to their social environment (weight at 21 days of age: “Risk” males: 8.14±0.28g; “No Risk” males: 8.17±0.28g; t_{46}=-0.079, P=0.937). However, at sexual maturity (ninety days of age) males reared in the risk environment were on average larger and heavier than males reared under no risk of sperm competition (length: “Risk”
males: $8.59\pm0.062\text{cm}$; “No Risk” males: $8.16\pm0.073\text{cm}$, $t_{45}=-4.47$, $P<0.001$. weight: “Risk” males: $18.19\pm0.383\text{g}$; “No Risk” males: $16.14\pm0.323\text{g}$; $t_{45}=-4.47$, $P<0.001$.

Baculum centroid size increased with body length (Figure 1.1a), but there was no significant interaction between body length and social environment ($\chi^2=0.945$, $P=0.331$), indicating that baculum allometry did not vary between the social environments. The common allometric slope (SMA slope= $0.9125\pm0.131$) did not differ statistically from 1.0 ($\chi^2=0.608$, $P=0.436$). Baculum centroid size did not differ significantly between social environments (Table 1.1). The same patterns of allometry were evident in a trait expected to be under natural selection (Figure. 1.1b). Thus, femur centroid size increased with body length (Figure. 1.1b), but there was no evidence for a significant interaction between social environment and body length ($\chi^2=0.037$, $P=0.859$), yielding a common slope (SMA slope= $1.122\pm0.144$) that did not differ from 1.0 ($\chi^2=0.851$, $P=0.356$). Femur centroid size did not differ between social environments (Table 1.2).
Table 1.1. Linear mixed model (LMM) of the effect of social environment (Social Env.) on the morphology of the baculum of male house mice.

<table>
<thead>
<tr>
<th>fixed effects</th>
<th>estimate</th>
<th>± se</th>
<th>type II, Wald χ²</th>
<th>p</th>
<th>random effects</th>
<th>variance</th>
<th>± sd</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Centroid</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>0.010</td>
<td>2.178</td>
<td>0.140</td>
<td></td>
<td>0.0001</td>
<td>0.0122</td>
</tr>
<tr>
<td>Body Length</td>
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<td>0.109</td>
<td>21.358</td>
<td>&lt;0.001</td>
<td></td>
<td>0.0002</td>
<td>0.0134</td>
</tr>
<tr>
<td><strong>RW1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>-0.053</td>
<td>0.266</td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.0001</td>
<td>0.0047</td>
</tr>
<tr>
<td>Social Env.</td>
<td>-0.030</td>
<td>0.006</td>
<td>26.163</td>
<td>&lt;0.001</td>
<td></td>
<td>&lt;0.0001</td>
<td>0.0019</td>
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<tr>
<td>Body Length</td>
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<td>0.061</td>
<td>0.003</td>
<td>0.713</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>RW2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>-0.172</td>
<td>0.175</td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Social Env.</td>
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<td>0.003</td>
<td>0.629</td>
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<td></td>
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<td>0.0078</td>
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<td>0.040</td>
<td>0.878</td>
<td>0.348</td>
<td></td>
<td></td>
<td></td>
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<td><strong>RW3</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>-0.394</td>
<td>0.131</td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.0001</td>
<td>0.0046</td>
</tr>
<tr>
<td>Social Env.</td>
<td>0.005</td>
<td>0.003</td>
<td>2.446</td>
<td>0.117</td>
<td></td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Body Length</td>
<td>0.087</td>
<td>0.030</td>
<td>8.402</td>
<td>0.004</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Figure 1.1** Allometric scaling of baculum centroid size (a) and femur centroid size (b) in male house mice. Blue symbols (greyscale – black) represent the “No Risk” social environment and red symbols (greyscale – grey) the “Risk” social environment. One regression line is fitted per treatment based on standardised major axis regression.
Table 1.2. Linear mixed model (LMM) of the effect of social environment (Social Env.) on the morphology of the hind femur of male house mice.

<table>
<thead>
<tr>
<th>fixed effects</th>
<th>estimate</th>
<th>± se</th>
<th>type II, wald $\chi^2$</th>
<th>p</th>
<th>random effects</th>
<th>variance</th>
<th>±sd</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Centroid Size</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>5.937</td>
<td>0.564</td>
<td></td>
<td></td>
<td>Tub</td>
<td>0.0002</td>
<td>0.0126</td>
</tr>
<tr>
<td>Social Env.</td>
<td>0.003</td>
<td>0.012</td>
<td>0.113</td>
<td>0.772</td>
<td>Family</td>
<td>0.0001</td>
<td>0.0102</td>
</tr>
<tr>
<td>Body Length</td>
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<td>0.129</td>
<td>23.471</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>RW1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>0.169</td>
<td>0.223</td>
<td></td>
<td></td>
<td>Tub</td>
<td>0.0001</td>
<td>0.0085</td>
</tr>
<tr>
<td>Social Env.</td>
<td>-0.004</td>
<td>0.005</td>
<td>0.551</td>
<td>0.457</td>
<td>Family</td>
<td>&lt;0.0001</td>
<td>0.0047</td>
</tr>
<tr>
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<td>0.051</td>
<td>0.527</td>
<td>0.467</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>RW2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>0.016</td>
<td>0.145</td>
<td></td>
<td></td>
<td>Tub</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Social Env.</td>
<td>0.003</td>
<td>0.002</td>
<td>1.187</td>
<td>0.275</td>
<td>Family</td>
<td>&lt;0.0001</td>
<td>0.0038</td>
</tr>
<tr>
<td>Body Length</td>
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<td>0.033</td>
<td>0.023</td>
<td>0.877</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>RW3</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>0.145</td>
<td>0.108</td>
<td></td>
<td></td>
<td>Tub</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Social Env.</td>
<td>-0.002</td>
<td>0.002</td>
<td>1.162</td>
<td>0.281</td>
<td>Family</td>
<td>&lt;0.0001</td>
<td>0.0012</td>
</tr>
<tr>
<td>Body Length</td>
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<td>0.024</td>
<td>1.586</td>
<td>0.207</td>
<td></td>
<td></td>
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</tbody>
</table>

Geometric morphometric analysis of the baculum returned three relative warps (BRW) that each explained more than 10% of the variation in baculum shape (BRW1: 54.19%, BRW2: 15.92%, BRW3: 12.23%). BRW1 described variation in the relative thickness and extension of the baculum bulb along the shaft, and the thickness of the shaft itself (Figure 1.2). BRW2 described similar variation in the relative thickness of the baculum bulb (Figure 1.2), while BRW3 described variation in the rate of transition between bulb and shaft along the length of the baculum (Figure 1.2). Our analyses revealed a significant effect of social environment on BRW1 (Table 1.1; Figure 1.3a) but no effect on BRW2 and BRW3. Males from the “Risk” environment had a relatively thicker and more distally extended baculum bulb, and a relatively thicker baculum shaft compared to males from the “No risk” environment (Figure 1.3b). The interaction terms between social environment and body length were not significant (BRW1: $\chi^2=2.718$, $P=0.09$;
BRW2: $\chi^2=1.346, P=0.246$; BRW3: $\chi^2=0.210, P=0.647$) and were therefore removed from all analyses. Male body length had no effect on BRW1 or BRW2 but did explain some of the variation in BRW3 (table 1).

**Figure 1.2.** (a) Variation in baculum shape described by the extremes of relative warp 1 ($1^+, 1^-$), relative warp 2 ($2^+, 2^-$) and relative warp 3 ($3^+, 3^-$). The positions of the 40 landmarks around the periphery of the baculum for both positive and negative extremes are shown. The consensus shape (0) is displayed in the centre, with the four fixed landmarks identified as red symbols (greyscale – grey); (b) Morphospace plot of relative warp 1 against relative warp 2 with polygons encompassing individuals in each treatment. Blue symbols (greyscale – black) represent the “No Risk” social environment and red symbols (greyscale – grey) represent the “Risk” social environment.
Figure 1.3. Variation in baculum shape of male house mice from different sperm competition environments. (a) Mean (± 95% CL) score on the first relative warp; (b) Superimposition of Relative warp 1 thin-plate splines; Blue (Greyscale – black) represent the “No Risk” social environment, red shape (Greyscale – grey) represents the “Risk” social environment and white represent the consensus shape across all samples.

Geometric morphometric analysis of the hind femur returned three relative warps (FRW) that each explained more than 10% of the variation in femur shape (FRW1: 24.50%, FRW2: 17.34%, FRW3: 11.09%). FRW1 explained variation in the shape of the femur head, variation in the shape of the lesser trochanter, the position of the third trochanter and thickness of the femur shaft (Fig. S1.3). FRW2 explained variation in the position of the third trochanter, and the thickness of the femur shaft (Fig. S1.3). FRW3 explained variation in the relative length and thickness of the femur (Fig.S1.3). Femur shape was not influenced by social environment or male body length (Table 1.2). The
interaction terms between social environment and body length were not significant (FRW1: $\chi^2 = 0.514, P=0.474$; FRW2: $\chi^2 = 0.970, P=0.325$; FRW3: $\chi^2 = 0.665, P=0.415$) and were removed from the statistical models.

1.5 Discussion

Our study has revealed that male house mice exposed to rivals during sexual development grew a relative thicker baculum with a distally extended bulb, compared with males that did not experience rivals during their development. While phenotypic plasticity in male genitalia has been reported previously in response to temperature and diet in *Drosophila* (Andrade et al. 2005), and population density and wave-exposure in barnacles (Neufeld and Palmer 2008; Hoch 2009), there has been little evidence of socially mediated plasticity in genital morphology. A recent study of waterfowl reported how the penis of Lesser Scaup (*Aythya affinis*) grew longer in males housed together compared with those housed with a single female (Brennan et al. 2017). The authors considered this evidence preliminary because groups were not replicated and other environmental differences between groups could have explained their results. We provide replicated experimental evidence of phenotypic plasticity in male genital morphology. Socially induced plasticity was not apparent in a naturally selected bone, the hind femur, providing further support that the mammalian baculum is subject to sexual selection.

Remarkably, the variation in baculum shape that we observed between our sperm competition risk treatments reflects the genetic variation in baculum shape reported previously among populations of house mice (Simmons and Firman 2014). Among natural populations, males under high risk of sperm competition have evolved thicker bacula than males from populations with a relatively lower risk of sperm competition (Simmons and Firman 2014). Further, males from experimental populations subjected
to multiple generations of post-copulatory sexual selection were found to evolve thicker bacula compared to males from populations evolving under enforced monogamy (Simmons and Firman 2014). Consistent with these previous studies, we found no effect of sperm competition risk on baculum size (Ramm et al. 2009; Simmons and Firman 2014). Collectively, these studies support the hypothesis that it is the shape of male genitalia that is under sexual selection, and not genital size (Simmons 2014).

Considering that baculum thickness predicts male reproductive success under competitive conditions (Stockley et al. 2013), our data provide strong evidence that the mammalian baculum is a trait subject to selection via sperm competition, and shows that males can prepare for future sperm competition via adjustments in their genital morphology.

Variation in bone morphogenesis offers a potential mechanism for phenotypic plasticity in baculum shape. Sex steroids play a fundamental role in osteogenesis (Vidal et al. 2000; Bouillon et al. 2004; Vanderschueren et al. 2004, 2006), being actively responsible for longitudinal and radial bone growth during sexual maturation (Vanderschueren et al. 2004; Venken et al. 2006). Artificial manipulation of the expression or activation of androgen, oestrogen, oestrogen receptor alpha (ERα) and oestrogen aromatase enzyme, all lead to abnormal osteogenesis (Glucksmann et al. 1976; Vidal et al. 2000; Bouillon et al. 2004; Yonezawa et al. 2011; Rodriguez et al. 2012). The proximal segment of the mouse baculum is an ossified structure, and previous studies have shown that anti-androgen treatments arrest baculum development (Glucksmann et al. 1976). Studies conducted on rats and house mice have shown that during sexual maturation, androgen involvement in baculum growth is partly dependent on the aromatisation of oestrogen (Yonezawa et al. 2011; Rodriguez et al. 2012). ERα and oestrogen aromatase activation-expression has also been shown to be responsible for radial bone growth (Bouillon et al. 2004; Vanderschueren et al. 2006). Taken
together, these findings suggest the differential expression of ERα and/or differential local oestrogen aromatase enzyme expression as candidate mechanisms for the observed phenotypic plasticity in baculum thickness (Bouillon et al. 2004; Vanderschueren et al. 2006).

Our results also revealed socially mediated differences in adult size at sexual maturity. Androgen expression during sexual development is known to have a direct impact on spermatogenesis (O’Shaughnessy 2014) and indirect impact on body growth via growth hormone secretion (Lupu et al. 2001; Venken et al. 2006). A previous study of this same population of mice found that sperm competition risk induced higher sperm production (Firman et al. 2013) and differential body growth may be associated with higher expression of testosterone during the pre-adult developmental stages. Overall, the perception of rival males may influence the regulation and expression of sex steroids that affect a range of sexual traits preparing males for sperm competition.

Increased male body size in response to sexual competition may be important in establishing dominance over rivals, increasing a male's ability to gain access to receptive females, or be the first male to copulate. In house mice, dominance and fighting ability are both positively correlated with body mass (DeFries and McClearn 1970; Cunningham et al. 2013), and mating position is a strong predictor of paternity success, with the first male to mate siring the majority of offspring (Firman and Simmons 2008a). Larger male house mice deliver greater copulatory stimulation (Preston and Stockley 2006), and those with an evolutionary history of sperm competition have longer copulations and paternity success compared to males evolving under enforced monogamy (Klemme and Firman 2013), so it is conceivable that phenotypic plasticity in body size might be driven by pre-copulatory male-male competition over access to females. Indeed, socially-mediated plasticity in traits that contribute to pre-mating male contest competition has been demonstrated in studies of
invertebrates and vertebrates (Allen et al. 2011; Kasumovic et al. 2011; Simmons and Buzatto 2014; Huchard et al. 2016). When exposed to same-sex rivals, Kalahari meerkat individuals have been shown to adjust their growth according to that of their rivals, so-called competitive growth that prepares them for future sexual competition (Huchard et al. 2016). Phenotypic plasticity in pre- and post-copulatory competitive traits is expected when male investment in these traits is costly. For house mice, one cost of competitive traits may relate to sex steroid expression. In general, testosterone is known to suppress vertebrate immune function (Foo et al. 2017), which has the potential to impact lifespan, a well-documented cost of male investment in pre- and post-copulatory traits (Hunt et al. 2004; Robinson et al. 2006; Bretman et al. 2013).

The mechanism(s) by which post-copulatory sexual selection acts on baculum shape remains unknown. Of those proposed (Greenwald 1956; Ewer 1973; Dixson 1987), vaginal stimulation represents a plausible candidate. The baculum is an integral part of the glans penis (Rodriguez et al. 2011) and is likely to impart mechanical stimulation during copulation. House mouse sexual behaviour is characterised by multiple intromissions per mount and multiple mounts before the male reaches ejaculation (Bronson 1979). The baculum within the glans penis will impart rigidity to facilitate penetration as well as promote friction between the penis and the female reproductive tract (Greenwald 1956). Differences in baculum thickness may lead to variation in the degree of stimulation that a female receives during copulation. In particular, a more distal thickening of the basal bulb might promote greater levels of stimulation throughout a longer section of the vaginal tract, increasing neuroendocrine responses that affect sperm migration, embryo implantation rate, and embryo viability (Yang et al. 2009; Firman and Simmons 2012; Miki and Clapham 2013). Greater stimulation delivered by a thicker baculum may also influence female sexual behaviour by dampening their propensity to re-mate with a rival male (Eberhard 1996). Genital
stimulation is important in directing sexual behaviour, activating the brain reward system and reinforcing and facilitating sexual behaviour (Pfaus et al. 2012). Indeed, manipulation of neurochemical states of sexual reward has been shown to affect female preference (Pfaus et al. 2012).

In conclusion, we provide evidence of socially mediated phenotypic plasticity in male genital morphology. Our study adds to growing evidence that supports a role of sexual selection in the evolution of the mammalian baculum and re-enforces its role in male reproductive fitness (Stockley et al. 2013; Simmons and Firman 2014; Brindle and Opie 2016). Consistent with earlier studies of house mice, variation in baculum shape, but not size, was found to respond to socially mediated cues of sperm competition risk. Further investigation of the mechanism(s) underlying plasticity in baculum development, as well as those responsible for selection acting on the baculum, are required. In particular, we suggest that research on the role of sex steroids and their receptor distribution in the baculum will offer insight into the mechanisms that mediate plasticity in baculum shape.
Appendix 1.1 Supplementary Material

This appendix includes:

Supplementary Tables S1.1

Supplementary Figure S1.1 to S1.3
Table S1.1. Descriptive statistics of the morphological measurements taken from males reared in risk and no risk sperm competition environments.

<table>
<thead>
<tr>
<th>Trait</th>
<th>Treatment</th>
<th>N</th>
<th>Mean±SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Weight at 21 days of age (g)</td>
<td>No Risk</td>
<td>24</td>
<td>8.143±0.274</td>
</tr>
<tr>
<td></td>
<td>Risk</td>
<td>24</td>
<td>8.174±0.271</td>
</tr>
<tr>
<td>Body Weight at 90 days of age (g)</td>
<td>No Risk</td>
<td>24</td>
<td>16.138±0.316</td>
</tr>
<tr>
<td></td>
<td>Risk</td>
<td>24</td>
<td>18.187±0.375</td>
</tr>
<tr>
<td>Body Length (mm)</td>
<td>No Risk</td>
<td>24</td>
<td>81.628±0.719</td>
</tr>
<tr>
<td></td>
<td>Risk</td>
<td>24</td>
<td>85.940±0.612</td>
</tr>
<tr>
<td>Baculum Centroid size</td>
<td>No Risk</td>
<td>24</td>
<td>2967±21911</td>
</tr>
<tr>
<td></td>
<td>Risk</td>
<td>24</td>
<td>3053±27.048</td>
</tr>
<tr>
<td>Femur Centroid size</td>
<td>No Risk</td>
<td>23</td>
<td>6425±62.848</td>
</tr>
<tr>
<td></td>
<td>Risk</td>
<td>24</td>
<td>6664±53.472</td>
</tr>
<tr>
<td>Baculum RW1</td>
<td>No Risk</td>
<td>24</td>
<td>0.015±0.003</td>
</tr>
<tr>
<td></td>
<td>Risk</td>
<td>24</td>
<td>-0.012±0.004</td>
</tr>
<tr>
<td>Baculum RW2</td>
<td>No Risk</td>
<td>24</td>
<td>-0.002±0.002</td>
</tr>
<tr>
<td></td>
<td>Risk</td>
<td>24</td>
<td>-0.001±0.002</td>
</tr>
<tr>
<td>Baculum RW3</td>
<td>No Risk</td>
<td>24</td>
<td>-0.002±0.002</td>
</tr>
<tr>
<td></td>
<td>Risk</td>
<td>24</td>
<td>0.001±0.002</td>
</tr>
<tr>
<td>Femur RW1</td>
<td>No Risk</td>
<td>24</td>
<td>0.001±0.003</td>
</tr>
<tr>
<td></td>
<td>Risk</td>
<td>24</td>
<td>-0.005±0.003</td>
</tr>
<tr>
<td>Femur RW2</td>
<td>No Risk</td>
<td>23</td>
<td>-0.003±0.002</td>
</tr>
<tr>
<td></td>
<td>Risk</td>
<td>24</td>
<td>0.0003±0.002</td>
</tr>
<tr>
<td>Femur RW3</td>
<td>No Risk</td>
<td>23</td>
<td>0.005±0.002</td>
</tr>
<tr>
<td></td>
<td>Risk</td>
<td>23</td>
<td>0.001±0.001</td>
</tr>
</tbody>
</table>
**Figure S1.1.** Landmark placement on images of the baculum and femur of the house mouse, *Mus musculus domesticus*; *(a)* baculum showing the placement of 4 fixed landmarks (white, numbers: 1, 7, 21 and 35) and 36 semi-sliding landmarks (red) placed evenly between fixed landmarks around its periphery; *(b)* femur showing the placement of 8 fixed (white, numbers: 1, 5, 15, 30, 40, 53, 61 and 76) and 71 semi-sliding landmarks placed evenly between fixed landmarks around its periphery.

**Landmark placement details:** For the baculum, fixed landmarks demarked the most lateral, anterior and posterior positions of the baculum, homologous structures across all specimens. Sliding semi-landmarks were spaced evenly between the fixed landmarks (Zelditch et al. 2012b). For the femur, fixed landmarks were placed on homologous structures across specimens, and sliding semi-landmarks were spaced evenly between fixed landmarks.
Fig S1.2. Superimposition of repeated landmark placements on two replicate images of 12 baculum (a) and 12 femur (b) sample images. Samples have been scaled to unit centroid size and optimally superimposed. Blue symbols (greyscale – black) represent six bones from the “No Risk” social environment and red symbols (greyscale – grey) represent the six bones from the “Risk” social environment. Variations between forms, indicated by the scatter at each landmark, are due to variations in shape between samples rather than repeated measures of the same sample.

Repeatability analysis details: for the same sample, we obtained two images and placed landmarks on the independent images. We used intra-class correlation coefficient analysis (ICC) with the one-way random effects model to estimate the reliability of measurement, for both Euclidean distances between fixed landmarks and centroid size and trait shape measures from subsequent geometric morphometric analyses. Landmark
placement and resultant measures of centroid size and trait shape were significantly repeatable for both baculum and femur (Baculum: Euclidean distances, $R=0.980$, $P < 0.001$; baculum centroid size, $R=0.9873$, $P < 0.001$; baculum RW1, $R=0.983$, $P < 0.001$; baculum RW2, $R=0.975$, $P < 0.001$; baculum RW3, $R=0.972$, $P < 0.001$. Femur: Euclidean distances, $R=0.990$, $P < 0.001$; femur centroid size, $R=0.990$, $P < 0.001$; femur RW1, $R=0.990$, $P < 0.001$; femur RW2, $R=0.983$, $P < 0.001$; femur RW3, $R=0.976$, $P < 0.001$).
Figure S1.3. (a) Variation in femur shape described by relative warp 1 ($1^+$, $1^-$), relative warp 2 ($2^+$, $2^-$) and relative warp 3 ($3^+$, $3^-$). The positions of the 79 landmarks around the periphery of the femur for both positive and negative extremes are shown. The consensus shape (0) is displayed in the centre, with the eight fixed landmarks identified as red symbols (Greyscale – grey). (b) Morphospace plot of relative warp 1 against relative warp 2 with polygons encompassing individuals for each treatment. Blue symbols (Greyscale – black) represent the “No Risk” social environment and red symbols (Greyscale – grey) represent the “Risk” social environment. Femur structures: greater trochanter (a), head of femur (b), lesser trochanter (c), third trochanter (d), shaft (e).
CHAPTER TWO: QUANTITATIVE GENETIC INSIGHTS INTO THE COEVOLUTION OF MALE AND FEMALE GENITALIA IN A MAMMAL

This chapter is presented as accepted for publication (pending revisions) in Evolution, with minor changes to ensure consistency with the rest of my thesis.
2.1 Abstract

Male genitalia are among the most phenotypically diverse morphological traits, and sexual selection is widely accepted as being responsible for their evolutionary divergence. Studies of house mice suggest that the shape of the baculum (penis bone) affects male reproductive fitness and experimentally imposed postmating sexual selection has been shown to drive divergence in baculum shape across generations. Much less is known of the morphology of female genitalia and its coevolution with male genitalia. In light of this, we used a paternal half-sibling design to explore patterns of additive genetic variation and covariation underlying baculum shape and female vaginal tract size in house mice (Mus musculus domesticus). We applied a landmark-based morphometrics approach to measure baculum size and shape in males and the length of the vaginal tract and width of the vaginal cervix in females. Our results reveal significant additive genetic variation in house mouse baculum morphology and vaginal cervix width, as well as evidence for genetic covariation between male and female genital measures. Our data thereby provide novel insight into the potential for the coevolutionary divergence of male and female genital traits in a mammal.
2.2 Introduction

Male genitalia are known to be among the most phenotypically diverse structures in the animal kingdom (Eberhard 1985), and a growing number of studies have provided evidence that male genitalia are subject to sexual selection during copulation (Eberhard 1985; Arnqvist 1998; Simmons et al. 2009). Accordingly, male genital divergence has been found to correlate strongly with mating system, whereby male genitalia are typically more diverse in polygamous than in monogamous mating systems (Arnqvist 1998; Dixson 2012). There is now a wealth of evidence from polygamous species that sexual selection plays an essential role in the evolution of male genital morphology (Eberhard 1985, 2010; Hosken and Stockley 2004; Simmons 2014). Thus, variation in male genital morphology has been shown to influence mating, insemination, fertilisation and overall male reproductive success (Arnqvist and Danielsson 1999; Danielsson and Askenmo 1999; House and Simmons 2003; Hotzy and Arnqvist 2009; Wojcieszek and Simmons 2011; Stockley et al. 2013) in taxa ranging from insects to mammals.

Mechanisms of sexual selection proposed to account for the evolution of male genital morphology include cryptic female choice (CFC) and sexually antagonistic coevolution (SAC) (Eberhard 1996; Hosken and Stockley 2004; Firman et al. 2017). According to CFC theory, females increase their reproductive fitness by biasing paternity toward males whose genital morphology deliver more effective stimulation to the female (Eberhard 1996, 2010; Brennan and Prum 2015). Contrarily, SAC theory proposes that the reproductive interests of males and females are in conflict leading to an arms race for the control of reproduction (Chapman et al. 2003; Parker 2006; Brennan and Prum 2015). Male genitalia develop structures that promote their fitness, often through success in sperm competition, at a cost to female fitness. In turn, females counteract male costs via adaptations in their genitalia that alleviate the costs of mating (Arnqvist...
There is evidence that both mechanisms can account for the evolutionary divergence of genitalia among taxa (Hosken and Stockley 2004; Eberhard 2010; Simmons 2014; Brennan and Prum 2015). Among onthophagine dung beetles, male and female genitalia have been shown to coevolve both within and among species, where sexual selection has led to an increase in the internalisation of anchorage points within the female reproductive tract (genital pits) and the relative length of the male parameres that are inserted into these pits (Simmons and Garcia-Gonzalez 2011; Simmons and Fitzpatrick 2019). Previous studies indicate that both repeated mating and male quality can increase offspring viability without cost to the female (Simmons and Holley 2011) suggesting CFC as the likely mechanisms for the coevolutionary divergence of male and female genital structures (Simmons and Garcia-Gonzalez 2011; Simmons and Fitzpatrick 2019). In contrast, male seed beetle (Callosobruchus) genitalia are endowed with spines on the distal tip of the aedeagus that reduce female fitness by damaging the internal reproductive tract. Among species (Rönn et al. 2007), as well as populations within species (Dougherty et al. 2017), selection appears to counteract the fitness costs of spiny male genitalia through a thickening of the female reproductive tract walls. Similarly, genital coevolution via SAC is suggested in waterfowl where female genital tracts can exclude male genitalia, thus enabling females to resist forced copulations (Brennan et al. 2010). To our knowledge only a single study has looked at genital interactions in mammals: in four marine species, interactions between male and female genitalia suggest that both CFC and SAC may contribute to genital coevolution (Orbach et al. 2017).

For most mammalian species, CFC is believed to drive male genital evolution via its stimulatory role during copulation (Eberhard 1996; Lariviere and Ferguson 2002; Dixson 2012; Brennan and Prum 2015). Mammal copulation includes behaviours designed to stimulate the female reproductive tract and that are typically required for a
successful pregnancy (Eberhard 1996; Pavličev and Wagner 2016). Genital stimulation leads to a cascade of physiological events that prime the female reproductive tract for embryo implantation (Argiolas and Melis 2013). It is reasonable to argue, therefore, that it is the female ability to discern differences in genital stimulation that generates sexual selection on male genitalia among mammal species (Eberhard 1996; Hosken and Stockley 2004; Brennan and Prum 2015). The baculum or ossified penis is a common feature of mammalian genitalia (Ramm 2007; Stockley 2012; Brindle and Opie 2016; Schultz et al. 2016b). In house mice, baculum shape has been shown to respond to experimental manipulation of postmating sexual selection (Simmons and Firman 2014) and is a significant predictor of male reproductive success among free-ranging populations (Stockley et al. 2013). Moreover, it has been demonstrated that baculum shape responds plasticly to variation in the risk of postmating reproductive competition (André et al. 2018). Although the precise mechanism remains unknown, current research provides strong evidence that the house mouse baculum is subject to sexual selection (Stockley et al. 2013; Simmons and Firman 2014; André et al. 2018).

The baculum is a structural part of the penis (Rodriguez et al. 2011) that imparts mechanical stimulation during copulation by promoting friction between the penis and the female reproductive tract (Bronson 1979). Consequently, one potential mechanism for the evolution of the baculum via sexual selection is its role in vaginal stimulation (Greenwald 1956; Lariviere and Ferguson 2002).

Compared to the evolution of male genitalia, female genital diversity is significantly less well studied (Ah-King et al. 2014; Simmons 2014; Sloan and Simmons 2019). The few available studies report that female genitalia are highly complex and vary widely among closely related taxa (Pitnick et al. 1999; Polihronakis 2006; Puniamoorthy et al. 2010; Simmons and Fitzpatrick 2019). Female genitalia has also been demonstrated to interact with male genitalia and influence fertilisation success (Arnqvist and Danielsson...
Both theories of CFC and SAC predict the coevolution of male and female genital morphology (Mead and Arnold 2004) either in response to the sexual selection acting on interacting genital traits (CFC) or to selection in response to the costs that male genital traits impose (SAC). Comparative studies have provided evidence of correlative patterns of evolution between male and female genitalia (Arnqvist and Rowe 2002; Brennan et al. 2007; Rönn et al. 2007; Ilango and Lane 2009; Kuntner et al. 2009; Tatarnic and Cassis 2010; Orbach et al. 2017), but very few have investigated quantitative genetic variation of female genitalia or genetic covariation between female genitalia and male genitalia (Simmons and Garcia-Gonzalez 2011; Evans et al. 2013). To our knowledge, no such study exists for any mammalian species.

Previous studies of inbred strains of laboratory mice suggest that baculum morphology harbours underlying genetic variation (Schultz et al. 2016a). For example, the inbred strain D2 was found to possess a relatively thinner and shorter baculum when compared with males from the inbred strain B6, and the baculum shape of the recombinant offspring fall within the parental variance (Schultz et al. 2016a). However, no studies have examined variation in female reproductive tract morphology or its genetic basis. Here, we use a paternal half-sibling design to explore patterns of additive genetic variation and covariation underlying baculum shape and female vaginal tract size in wild house mice. Given the evidence of sexual selection acting on baculum shape (Stockley et al. 2013; Simmons and Firman 2014), theoretical models of CFC and SAC predict the evolution of significant additive genetic covariation between baculum morphology and female vaginal morphology.
2.3 Materials and Methods

(a) Experimental Animals and breeding design

Wild house mice (*Mus musculus domesticus*) were sourced from an isolated population on Rat Island (28° 42'S, 113° 47'E) off the coast of Western Australia and maintained in the laboratory at the University of Western Australia (*n* males = 36, *n* females = 104). The animals were held in constant temperature rooms (CTR; 24°C) on a reverse dark-light cycle (10:14) and provided with water and food *ad libitum*. We established a paternal half-sibling breeding design (Lynch and Walsh 1998) by pairing 30 sires to three dams (90 dams in total). Pairs were housed together for six days. Males were rested for four days between pairings with their three dams. After separating the mating pairs, females were monitored daily for the birth of pups. At weaning age (21 days) female and male offspring were separated; female offspring were housed with their full-siblings, and male offspring were housed individually. All procedures followed the guidelines for the ethical treatment of animals in research under UWA Animal Ethics Committee approval (03/100/1456).

(b) Morphometric analysis

(i) Males

At 90 days of age, males were euthanised, body length and body weight was recorded, and the baculum dissected for analysis. The majority of tissue surrounding the baculum was removed and the specimens stored overnight in 500 μl of 5% KOH to dissolve any remaining tissue. Specimens were then stored in 500 μl of Dietrich’s Fixative solution. Digital images of the baculum (ventral view) were captured using a binocular microscope at x20 magnification. Geometric morphometric analysis was conducted using the software developed by Rohlf (2006). Landmarks were placed around the periphery of the baculum (36 sliding, 4 fixed) using the software tpsDig2 v.2.29 (for
more details see André et al. 2018). The software Tpsrelw v.1.65 was then used to extract relative warps (RWs) and centroid size. We focused on those RWs that explained more than 10% of the variance in shape. Centroid size provided a multivariate measure of the size of the baculum (Zelditch et al. 2012a). We assessed the reliability of landmark placement and repeatability of our measurements by placing landmarks on two separate occasions for 20% of the analysed specimens. Our measurements of genital shape and size were highly repeatable (RW1: R= 0.996, F(58, 59) = 544.8, p < 0.001; RW2: R= 0.996, F(58,59) = 469.1, p < 0.001; RW3: R= 0.976, F(58, 59) = 81.2, p < 0.001; centroid size: R= 0.999, F(58, 59) = 2820.2, p < 0.001).

(ii) Females

At 90 days of age, females were euthanised, body length and body weight were recorded, and the reproductive tract was dissected for analysis. The female oestrous stage was determined by visual observation of the appearance of the vaginal opening (Byers et al. 2012). Digital images of the vaginal tract (ventral view) were taken using a binocular microscope at x10 magnification. Vaginal length and vaginal cervix width were taken using the software ImageJ v 1.46r. We assessed reliability of our vaginal measurements by re-measuring 20% of the specimens on three separate occasions. Our measurements of the vaginal length and vaginal cervix width were highly repeatable (vaginal length: R= 0.959, F(47,96) = 70.4, p < 0.001; vaginal cervix width: R= 0.918, F(47,96) = 34.7, p < 0.001).

(c) Quantitative genetic analyses

We used a paternal half-sibling breeding design (Lynch and Walsh 1998) to estimate patterns of additive genetic variation and covariation in male and female body size and genital traits. Quantitative variation was estimated using restricted maximum-likelihood procedures in the Im4 package of R v. 3.6.1. We fitted nested models for half-sib
designs that included dam nested within sire as random effects. As a possible source of phenotypic variation, we included logarithm of body weight as a fixed effect for the analyses of both male and female genitalia and oestrous stage for the analysis of female genitalia (Wilson 2008). Significance of sire additive genetic variance components were tested using likelihood ratio tests, where twice the difference in log-likelihood between hierarchically structured models was tested against a mixture $\chi^2$ distribution with zero and one degree of freedom respectively (Self and Liang 1987). Narrow-sense heritability was estimated from the ratio of additive genetic to total phenotypic variance (Lynch and Walsh 1998). For all estimates, standard errors were calculated by jackknifing across sire families (Roff 2014). For the analysis of male genitalia, we had a total of 293 male offspring from 30 sires (mean±s.e. 9.76±0.68 offspring per sire, range 4-18) and 77 dams (3.80±0.18 offspring per dam, range 1-9). For the analysis of female genitalia we had a total of 242 female offspring from 29 sires (8.34±0.35 offspring per sire, range 4-11) and 77 dams (3.14±0.13 offspring per dam, range 1-6). We calculated estimates of the coefficient of additive variance (CV_a) and evolvability (I_a) using the square root of the mean-standardised additive genetic variances, respectively (Garcia-Gonzalez et al. 2012). These metrics could not be calculated for baculum shape, where the mean values for relative warps are zero.

To estimate genetic covariance between male and female genital traits, we had a total of 28 sires with male and female offspring (10±0.71 offspring per sire range 4-18). Intersexual genetic correlations ($r_G$) were only calculated for traits that exhibited significant additive sire effects (Lynch and Walsh 1998). To estimate additive genetic covariances and between-sex genetic correlations by Restricted Maximum Likelihood procedures (Falconer and Mackay 1996), we followed the protocol proposed and described elsewhere (Via 1984; Evans et al. 2013). Briefly, males were randomly assigned to a full-sibling female within the family. For each randomly assembled
dataset, variances and covariances were estimated (procedure repeated over 500 times). Significance of intersexual genetic covariances was calculated by comparing z-scores for these estimates to the corresponding two-tailed significance levels from a standard normal probability distribution (Åkesson et al. 2008). Standard errors were estimated by jack-knifing across (half-sibling) sire families (Roff and Preziosi 1994; Roff 2014).

As we report below, our analysis revealed significant correlations between genital traits and body size, and oestrous stage for females. Our analysis also revealed a correlation between baculum size and RW3. Even though for some of the genital traits these correlations were non-significant, they have the potential to obscure intersexual genetic covariances between genital traits. Thus, to control for these phenotypic relationships we used residual values for male genital traits obtained from a linear model that included the logarithm of body weight, and in the case of RW3, also baculum centroid size. Likewise, for female genital traits we calculated residuals from a linear model that included the logarithm of female body weight and oestrous stage. We carried a sire-weighted least square analyses by regressing the sire family mean of both male and female traits, with families being weighted according to the number of offspring. Bootstrap resampling (10⁶) was applied to obtain the 95% confidence intervals, standard errors and p-values. We also applied half-sibling Pearson correlation analyses by the sire family mean trait of both male and female traits. While our half-sibling breeding design allows controlling for maternal effects and dominance variance, our relatively small and heterogeneous sample sizes lead to high error on variance and covariance estimations (Astles et al. 2006). We therefore also calculated sire family mean correlations. Although sire family mean correlations usually underestimate the magnitude of genetic correlations (Via 1984), they have the advantage of reducing error in family mean estimates inherent in small and heterogeneous sampling procedures (Astles et al. 2006). All statistical procedures were conducted in R v. 3.6.1.
2.4 Results

(a) Body Size

Our genetic analysis for trait size measurements for males and females had low levels of additive genetic variation and heritability (Table 2.1).

(b) Baculum Morphology

The geometric morphometric analysis of baculum shape produced three relative warps that each explained more than 10% of the variation: RW1 = 48.10%; RW2 = 21.87%; RW3 = 10.66%. RW1 described variation in the shape of the baculum bulb and its relative thickness, RW2 described variation in the relative thickness of the baculum bulb, while RW3 described variation in the shape of the proximal region of the baculum bulb as well as the transitional region between baculum bulb and shaft (Figure 2.1).

A preliminary analysis revealed that, baculum centroid size increased significantly with both body length ($\chi^2 = 31.55, p < 0.001$) and body weight ($\chi^2 = 116.82, p < 0.001$). Baculum centroid size exhibited a significant correlation with RW3 (Pearson $r = 0.260, p < 0.001$), but not with RW1 (Pearson $r = 0.039, p = 0.503$) or RW2 (Pearson $r = -0.001, p = 0.981$). Thus, because variation described by RW3 is in part determined by baculum size, baculum centroid size was included as a fixed factor in our quantitative genetic analysis as a known source of variation in RW3 (Wilson 2008).

Our genetic analysis revealed that all measurements of baculum shape and size had moderate to high levels of additive genetic variation and heritability (Table 2.1a).
Table 2.1. Quantitative genetic variation in male and female measurements of genital morphology. Restricted Maximum Likelihood procedures were used to estimate additive (V_a), residual (V_r) and phenotypic (V_p) components of variance, the estimate of heritability (h^2) and the coefficient of additive variance (C_V_a). Inference for significant additive genetic (V_A) effects were derived from log-likelihood ratio tests assuming a chi-square distribution with a mixture of zero and one degree of freedom. Significant p-values are shown in italics. Standard error (s.e.) estimates were calculated via jackknifing over sires.

<table>
<thead>
<tr>
<th>traits</th>
<th>mean (s.e.)</th>
<th>V_a (s.e.)</th>
<th>V_p (s.e.)</th>
<th>( \chi^2 )</th>
<th>p</th>
<th>h^2 (s.e.)</th>
<th>C_V_a (s.e.)</th>
<th>I_a (s.e.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Male traits</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body Length</td>
<td>78.383 (0.191)</td>
<td>4.067 (3.841)</td>
<td>10.903 (1.356)</td>
<td>1.794</td>
<td>0.092</td>
<td>0.38 (0.34)</td>
<td>0.026 (0.014)</td>
<td>0.052 (0.049)</td>
</tr>
<tr>
<td>Body Weight</td>
<td>18.977 (0.129)</td>
<td>1.259 (1.674)</td>
<td>5.009 (0.517)</td>
<td>0.512</td>
<td>0.231</td>
<td>0.24 (0.34)</td>
<td>0.059 (0.054)</td>
<td>0.066 (0.088)</td>
</tr>
<tr>
<td>RW1 †</td>
<td>0.007 (0.124)</td>
<td>0.032 (0.018)</td>
<td>0.047 (0.005)</td>
<td>5.637</td>
<td>0.009</td>
<td>0.68 (0.36)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>RW2 †</td>
<td>-0.060 (0.084)</td>
<td>0.013 (0.006)</td>
<td>0.021 (0.002)</td>
<td>4.818</td>
<td>0.014</td>
<td>0.59 (0.26)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>RW3 †</td>
<td>-0.083 (0.058)</td>
<td>0.008 (0.002)</td>
<td>0.009 (0.001)</td>
<td>9.534</td>
<td>0.001</td>
<td>0.81 (0.21)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Baculum Size ‡</td>
<td>3.827 (0.008)</td>
<td>15.025 (6.778)</td>
<td>16.616 (1.733)</td>
<td>6.704</td>
<td>0.005</td>
<td>0.87 (0.37)</td>
<td>0.032 (0.007)</td>
<td>0.004 (0.002)</td>
</tr>
<tr>
<td>(b) Female traits</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body Length</td>
<td>80.042 (0.254)</td>
<td>5.062 (7.017)</td>
<td>16.212 (2.062)</td>
<td>0.584</td>
<td>0.222</td>
<td>0.29 (0.45)</td>
<td>0.028 (0.031)</td>
<td>0.063 (0.087)</td>
</tr>
<tr>
<td>Body Weight</td>
<td>15.376 (0.116)</td>
<td>0.854 (1.039)</td>
<td>3.314 (0.311)</td>
<td>0.678</td>
<td>0.205</td>
<td>0.26 (0.31)</td>
<td>0.060 (0.038)</td>
<td>0.055 (0.067)</td>
</tr>
<tr>
<td>Vaginal Length</td>
<td>7.069 (0.059)</td>
<td>0.086 (0.172)</td>
<td>0.683 (0.094)</td>
<td>0.472</td>
<td>0.246</td>
<td>0.14 (0.26)</td>
<td>0.041 (0.064)</td>
<td>0.012 (0.024)</td>
</tr>
<tr>
<td>Vaginal Cervix Width</td>
<td>3.600 (0.030)</td>
<td>0.083 (0.041)</td>
<td>0.151 (0.020)</td>
<td>3.259</td>
<td>0.036</td>
<td>0.47 (0.25)</td>
<td>0.080 (0.022)</td>
<td>0.023 (0.011)</td>
</tr>
</tbody>
</table>

† All variance estimates for relative warp (RW) scores are \( \times 10^{-2} \)

‡ All variance estimates for centroid size are \( \times 10^{3} \)
Figure 2.1. Variation in baculum shape across sire families. The position of the 40 landmarks around the periphery of the baculum for both negative and positive extremes are shown. (a) baculum shape distribution across sire families for relative warp 1; (b) baculum shape distribution across sire families for relative warp 2; (c) baculum shape distribution across sire families for relative warp 3.
(c) Vaginal morphology

Our initial analysis revealed that, vaginal length was positively correlated with body weight \( (\chi^2 = 50.525, p < 0.001) \), but it did not exhibit a significant correlation with body length \( (\chi^2 = 0.193, p = 0.660) \). Vaginal cervix width was positively correlated with body length \( (\chi^2 = 7.999, p = 0.004) \) and body weight \( (\chi^2 = 17.383, p < 0.001) \). Both vaginal length and vaginal cervix width were significantly affected by the stage of the oestrous cycle \( (\text{vaginal length}: \chi^2 = 16.017, p = 0.024; \text{vaginal cervix width}: \chi^2 = 96.817, p < 0.001) \). Our genetic analysis revealed that only vaginal cervix width exhibited statistically significant additive genetic variation and heritability (Table 2.1b).

(d) Genetic covariance between male and female genital morphology

Intersexual genetic correlations between genital traits were estimated only for traits that exhibited significant additive genetic variation. REML procedures did not return statistically significant genetic correlation between baculum shape or size with vaginal cervix width (Table 2.2). However, when controlling for the effects on genital traits of body size in males and females, and oestrous stage in females, we observed a statistically significant intersexual correlation between RW1 and vaginal cervix width (Table 2.2). The sign of the genetic correlation between RW1 for baculum shape and female vaginal cervix width indicated that increases in cervix width of females was genetically correlated with a relative thickening and more distal positioned baculum bulb in males (Figure 2.1, Figure 2.2a). We also found a marginally significant positive genetic correlation between baculum size and cervix width (Figure 2.2d) when controlling for body weight and oestrous stage (Table 2.2).
**Figure 2.2.** Genetic covariance between female and male genital morphology. The relationships are illustrated by plots of sire family means (± s.e) for both male and female genital traits. (a) vaginal cervix width and male relative warp 1; (b) vaginal cervix width and male relative warp 2; (c) vaginal cervix width and male relative warp 3; (d) vaginal cervix width and baculum size
Table 2.2. Intersexual genetic correlations for male and female reproductive traits. Estimation of intersexual genetic correlations by three statistical procedures. (a) estimates ($r_G$) and standard errors (s.e.) were calculated from the mean values of 500 randomly resampled datasets (see main text) using Restricted Maximum likelihood procedures. The 95% confidence interval (CI) for these estimates were calculated based on randomly resampled datasets. Standard errors (s.e.) estimates ($r_G$) were calculated via jackknifing over (half-sibling) sire families, (b) estimates from weighted regressions are based on the correlation of sire family means for each trait, with families being weighted according the number of offspring when controlling for covariates (see main text). Bootstrap resampling ($10^6$) was applied to obtain the 95% confidence intervals, standard errors and $p$-values; (c) estimates ($r$) and confidence intervals when controlling for covariates (see main text) by Pearson correlation analysis (see main text). Pearson correlations are based on the correlation of sire family means for each trait. Significant $p$-values are shown in italics.

<table>
<thead>
<tr>
<th>Traits</th>
<th>(a) REML</th>
<th></th>
<th>(b) Weighted Regression</th>
<th></th>
<th>(c) Pearson Correlation</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rg (s.e.)</td>
<td>CI.</td>
<td>$p$</td>
<td>Rg (s.e.)</td>
<td>CI.</td>
<td>$p$</td>
</tr>
<tr>
<td>RW1</td>
<td>-0.311 (0.561)</td>
<td>-0.443; -0.168</td>
<td>0.58</td>
<td>-0.389 (0.127)</td>
<td>-0.746; -0.248</td>
<td>0.005</td>
</tr>
<tr>
<td>RW2</td>
<td>0.089 (0.495)</td>
<td>-0.022; 0.194</td>
<td>0.86</td>
<td>0.026 (0.222)</td>
<td>-0.660; 0.212</td>
<td>0.858</td>
</tr>
<tr>
<td>RW3</td>
<td>0.049 (0.405)</td>
<td>-0.045; 0.144</td>
<td>0.92</td>
<td>-0.027 (0.172)</td>
<td>-0.217; 0.456</td>
<td>0.832</td>
</tr>
<tr>
<td>Baculum Size</td>
<td>0.584 (0.617)</td>
<td>0.489; 0.685</td>
<td>0.34</td>
<td>0.386 (0.225)</td>
<td>0.024; 0.904</td>
<td>0.091</td>
</tr>
</tbody>
</table>
2.5 Discussion

We found significant levels of additive genetic variance underlying house mouse baculum morphology and vaginal cervix width, and evidence for an intersexual genetic correlation between these male and female genital traits. To our knowledge, this is the first study to employ a quantitative genetic approach to examine the coevolution of male and female genital traits in a mammalian species.

Here we show that significant levels of additive genetic variation in baculum size and shape are present in a natural population of wild house mice. Previous research on the house mouse baculum has shown that baculum width is a predictor of male reproductive success in free ranging populations (Stockley et al. 2013) and the robustness of the shaft and shape of the baculum bulb covaries with the strength of selection from sperm competition among natural populations (Simmons and Firman 2014). Importantly, the variation in baculum shape that we found to harbour significant additive genetic variation (RW1, RW2 and RW3) is qualitatively similar to the shape variation that was found to evolve in response to experimentally imposed postmating sexual selection (Simmons and Firman 2014). Collectively, these studies of house mice show that baculum shape has the genetic variance required for its evolutionary divergence in response to sexual selection.

Baculum shape harboured moderate to high levels of additive genetic variance and heritability. The magnitude of the heritable variation is consistent with previous estimates for morphological traits generally (Mousseau and Roff 1987; Roff 2002). Classic models of female choice predict that additive genetic variance in traits under selection should be depleted, thus precluding female choice (Kirkpatrick and Ryan 1991; Pomiankowski and Moller 1995). Nevertheless, a variety of resolutions to the lek paradox have been proposed, including the idea that traits subject to female choice
capture variation in mutations arising across the genome (Pomiankowski and Moller 1995; Rowe and Houle 1996; Tomkins et al. 2004; Kotiaho et al. 2008). Baculum size and shape are expected to be under the control of many genes, and so the maintenance of additive genetic variance might be expected (Falconer and Mackay 1996; Lynch and Walsh 1998). Indeed, a previous study found that the size and shape of the mouse baculum are determined by three quantitative trait loci (QTL); two QTL account for variation in baculum size and one QTL accounts for variation in baculum shape (Schultz et al. 2016a). At least sixteen genes are responsible for the observed variation in baculum morphology (Schultz et al. 2016a), allowing scope for mutation-selection balance to maintain quantitative genetic variation despite sexual selection acting on this trait.

Furthermore, although baculum shape may be subject to sexual selection (Ramm et al. 2009; Simmons and Firman 2014), it is one of multiple traits that contribute to male reproductive fitness (Ramm and Stockley 2009; Firman et al. 2013; Ramm et al. 2015) so that the intensity of selection may be insufficient to deplete genetic variance in the face of mutational variance. Finally, the house mouse baculum has also been reported to respond plastically to the sperm competition environment (André et al. 2018). Theoretical models predict that phenotypic plasticity can weaken the strength of selection acting on a trait and so contribute to the maintenance of genetic variance (Price et al. 2003; Price 2006).

We found significant additive genetic variation in vaginal cervix width but not vaginal length. The mammalian female reproductive tract undergoes a multitude of physiological and morphological changes throughout the oestrous cycle (Allen 1922; Champlin et al. 1973; Byers et al. 2012). Accordingly, the vaginal tract is susceptible to a wide range of variation due to oestrous stage. Changes in female reproductive tract morphology and physiology are highly regulated by the neuroendocrine system.
(Brenner and West 1975), and physiological changes are reflected in changes of contractility of the smooth muscle that surrounds the vaginal tract, vaginal blood flow and vasocongestion (Giraldi et al. 2002; van Helden et al. 2017). Here, even though we controlled for females’ oestrous stage in our statistical model, our inability to detect significant additive genetic variation in vaginal tract length could be due to different oestrous cycle conditions at the time of specimen preparation. In contrast, our ability to detect significant additive genetic variation in vaginal cervix width may be a consequence of the fact that the vaginal cervix is composed mainly of connective tissue, which means that cervix width is likely to be a less phenotypically plastic trait compared to vaginal tract length, and therefore less susceptible to major changes in morphology during the oestrus cycle (García-Villar et al. 1982).

Our data provide evidence of intersexual genetic covariation between baculum shape and size and vaginal cervix width. Genetic correlations between male and female genital traits are expected from the coevolutionary dynamics of sexual selection and sexual conflict acting on male and female genitalia (Brennan and Prum 2015). Although our data provide support for male and female genital coevolution, understanding the underlying mechanism(s) requires further study. Female choice via the reproductive tract may exert selection on baculum shape better equipped to deliver stimulation (Brennan and Prum 2015). A study of tsetse fly (Glossina pallida) found that modifications to the cercal teeth of male genitalia that affect sperm storage, ovulation and the probability of female remating, was dependent on female perception of stimulation during copulation (Briceño and Eberhard 2009). Likewise, in domestic pigs (Sus domesticus), males possess a corkscrew filament at the tip of the penis that, during copulation, stimulates the sow’s cervix ridges (Bonet et al. 2013). A lack of stimulation of the cervix ridges is known to reduce pregnancy rates (Bonet et al. 2013). The mammal female vaginal tract facilitates the sensory capacity to discern differences in
male genital morphology (Hilliges et al. 1995) by being highly innervated (Hilliges et al. 1995; Barry et al. 2017; van Helden et al. 2017) and regulated by the neuroendocrine system (Brenner and West 1975). House mouse vaginal tract innervation includes sympathetic, parasympathetic and sensory fibres (Barry et al. 2017) and possesses regional, temporal and mating status differences in innervation (Georgas et al. 2015; Barry et al. 2017). Differential distribution of nerve endings on the cervix and vaginal walls may lead to different ability to discern genital stimulation. If female house mice are able to bias reproductive outcomes based on genital stimulation, we expect the coevolution of the female ability to discern genital stimulation with male genital morphology. As our study was restricted to quantifying variation in gross morphology of the vagina, future research on vaginal innervation is required to identify whether mechanical stimulation is the selective mechanism responsible for the coevolution of male and female genital traits in house mice.

In conclusion, we found significant additive genetic variation in baculum size and shape and the width of the vaginal cervix. We also found evidence of genetic covariation between baculum size and shape and vaginal cervix width. Future studies of the coevolution of mammalian female genitalia should focus on the sensory physiology of the female tract. Patterns of vaginal innervation and variation in hormonal profiles of females following copulatory stimulation are likely to offer further insight into how female genitalia as a preference trait impose selection for the evolution of male genital morphology.
CHAPTER THREE: BACULUM SHAPE AND PATERNITY SUCCESS IN HOUSE MICE: EVIDENCE FOR GENITAL COEVOLUTION DRIVEN BY CRYPTIC FEMALE CHOICE?
3.1 Abstract

Sexual selection is believed to be responsible for the rapid divergence of male genitalia, which is a widely observed phenomenon across different taxa. Among mammals, cryptic female choice is thought to explain these evolutionary patterns, for example through the stimulatory role of male genitalia and female ‘choice’ favouring males with a given genital morphology. Recent research on house mice has suggested that baculum (penis bone) shape can respond to experimentally imposed sexual selection. Here, we explore the adaptive value of baculum shape by performing two experiments that examine the effects of male and female genitalia (both independent and interacting) on male reproductive success. Thus, we selected house mice (*Mus musculus domesticus*) from families characterised by extremes in baculum shape (relative width) and examined paternity success in both non-competitive (monogamous) and competitive (polyandrous) contexts. Our analyses revealed that the baculum shape of competing males influenced competitive paternity success, but that this effect was dependent on the breeding value for baculum shape of the family from which females were derived. Our data provide novel insight into the potential role of cryptic female choice on the coevolution of the house mouse baculum and lend support to the stimulatory hypothesis for mammalian genital evolution.
3.2 Introduction

In sexually reproducing species with internal insemination, male external genitalia is essential for deposition of the ejaculate into the female reproductive tract. Animal species in which females mate with multiple males are known to exhibit rapid divergent evolution in male genitalia (Eberhard 1985; Arnqvist 1998), and there is now substantial evidence for the role of sexual selection in male genital evolution (Eberhard 1985, 2010; Hosken and Stockley 2004; Simmons 2014). Three mechanisms of sexual selection have been proposed for the evolution and coevolution of male and female genital morphology; sperm competition, cryptic female choice and sexually antagonistic coevolution (Eberhard 1996, 2010; Simmons 2014; Brennan and Prum 2015; Firman et al. 2017).

Sexually antagonistic coevolution occurs when the reproductive interest of males and females are not aligned, instigating an arms race between male and female genitalia over the control of mating and fertilisation (Chapman et al. 2003; Parker 2006; Eberhard 2010; Brennan and Prum 2015). Sexually antagonistic coevolution may arise in the context of sperm competition over fertilisations (Chapman et al. 2003; Hosken and Stockley 2004; Sloan and Simmons 2019) and/or female resistance to mating (Eberhard 2010). For example, in seed beetles (Callosobruchus) males possess spines on the distal tip of the aedeagus that puncture the reproductive tract to deliver seminal fluid proteins into the haemolymph that affect sperm competition success (Hotzy and Arnqvist 2009; Hotzy et al. 2012). Damage to the female reproductive tract reduced female lifespan, and sexually antagonistic selection has favoured the coevolution of thicker female reproductive tracts, seen both among populations and species (Rönn et al. 2007; Dougherty et al. 2017). In contrast, sexual selection by cryptic female choice proposes that females increase their reproductive fitness by biasing paternity outcome toward males whose genital morphology deliver more effective stimulation (Eberhard
There are two cryptic female choice hypotheses which differ in the nature of benefits that females receive from their choice (Hosken and Stockley 2004; Greenfield et al. 2014), the Fisherian and “good genes” hypotheses (Hosken and Stockley 2004; Brennan and Prum 2015; Sloan and Simmons 2019). According to Fisherian selection, females choose males that possess genital morphology that is better suited to stimulate their reproductive tract and produce male offspring that will possess the same genital morphology, as well as female offspring that exhibit the same preference for stimulation (Hosken and Stockley 2004; Brennan et al. 2010; Greenfield et al. 2014). The good genes hypothesis predicts that, in addition to attractive sons, females obtain increased offspring viability due to covariance between the stimulatory genital trait and general male genetic quality (Firman et al. 2017; Sloan and Simmons 2019). Like the antagonistic sexual selection, models of cryptic female choice predict the coevolution of male genital trait and female preference. Regardless of the precise mechanism, there is now a wealth of evidence that sexual selection can act on male genital morphology through the effect of male genital traits on mating, insemination, fertilisation and overall male reproductive success (Arnqvist and Danielsson 1999; Danielsson and Askenmo 1999; House and Simmons 2003; Rodríguez et al. 2004; Briceño and Eberhard 2009; Hotzy and Arnqvist 2009; Wojcieszek and Simmons 2011; Stockley et al. 2013).

While the coevolution of male and female genitalia has been documented in a growing number of taxa (Sloan and Simmons 2019) studies of the coevolution of male and female genitalia in mammals are limited to a single study (Orbach et al. 2017). The interaction between male and female genitalia of four different marine species suggests that both cryptic female choice and sexually antagonistic coevolution may both contribute to genital coevolution (Orbach et al. 2017). The mammalian ossified penis or baculum is a common feature of male genitalia across most mammalian orders (Ramm
In house mice (*Mus musculus domesticus*), it is now evident that the shape of the baculum plays an essential role in postmating sexual selection and has the potential to influence male fitness. For example, we recently discovered that the shape of the house mouse baculum harbours significant additive genetic variance (André et al. 2020 Chapter 2), and has been shown to respond to experimentally imposed sexual selection (Simmons and Firman 2014). Interestingly, baculum shape also exhibits plasticity in response to the social environment, with an elevated risk of sperm competition leading to qualitatively similar changes in baculum shape to the evolutionary response seen under elevated sexual selection (André et al. 2018). Moreover, mouse baculum morphology has been shown to influence male reproductive success in free-ranging experimental populations; males with a relatively wider baculum are more successful at securing paternity under competitive scenarios (Stockley et al. 2013). Although the precise mechanism of sexual selection acting on the mouse baculum remains to be demonstrated, for most mammalian species cryptic female choice is thought to be an important driver of male genital evolution via its stimulatory role during copulation (Greenwald 1956; Eberhard 1996; Dixson 2012; Brennan and Prum 2015). As an ossified structure, the baculum may have evolved to provide rigidity to the penis and so provide stimulation to the female reproductive tract (Greenwald 1956). Female genital stimulation is important in mammals and is known to play a key role in priming the reproductive tract for embryo implantation by inducing neuroendocrine and physiological changes during and after copulation (Argiolas and Melis 2013). For mammal species, it is, therefore, reasonable to predict that female ability to discern variation in genital stimulation generates sexual selection on male genitalia (Eberhard 1996, 2010; Hosken and Stockley 2004; Brennan and Prum 2015). Copulation in various mammals includes behaviours with the specific function of stimulating the female reproductive tract, many of which are required for a successful
pregnancy (Eberhard 1996; Pavličev and Wagner 2016). Mammalian vaginal tracts are typically highly innervated (Hilliges et al. 1995; Barry et al. 2017; van Helden et al. 2017) making them putative female preference traits that discern sensory stimulation from male genital traits (Hilliges et al. 1995).

In this study, we aimed to establish whether baculum size and shape have a causal effect on paternity success under both non-competitive and competitive mating scenarios and to determine whether this effect was mediated by variation among females. We thus evaluated the effect of baculum morphology on embryo implantation rate and embryo viability in single mated females (monandrous matings), and the contribution of baculum size and shape to paternity success when two males compete for fertilisation (polyandrous matings). We selected individuals from families known to lie at the extremes of genetic variation in baculum shape (herein referred to as “wide” and “narrow”), and therefore had prior knowledge of the breeding values for genital morphology in our experimental pairings. Following the stimulation hypothesis (Greenwald 1956; Lariviere and Ferguson 2002; Brennan and Prum 2015), we expected that males with a relatively thick baculum would induce higher implantation rates and increased embryo viability compared to those males with a relatively narrow baculum. In polyandrous matings, we predict that paternity success should depend on the shape of a male’s baculum shape, the shape of his rival’s baculum and on the genetic background of females. Further, if sexual selection is responsible for the coevolution of male genital traits and female preference traits (van Helden et al. 2017), we predict that the fitness effects of variation in baculum shape would depend on the breeding value for baculum morphology of the family from which females were derived.
3.3 Materials and Methods

(a) Source population and experimental animals

Male and female laboratory-reared house mice (*Mus musculus domesticus*) were second and third-generation outbred descendants of wild individuals sourced from an isolated population on Rat Island located off the coast of Western Australia (28° 42´S, 113°47´E). The source population was used to establish a paternal half-sibling breeding design to estimate patterns of additive genetic variation in baculum morphology, as described elsewhere (André et al. 2020 Chapter 2). Briefly, we selected a subset of the offspring to conduct a morphometric analysis of baculum morphology from 30 paternal half-sibling families (90 maternal full-sibling families). The analysis revealed that all baculum shape measurements had moderate to high levels of additive genetic variation and heritability (André et al. 2020 Chapter 2). Baculum shape was described mainly by variation in the relative width of the baculum bulb.

For the experiments described here, we selected one male and one female from the eight sire families at each extreme of baculum shape variation, for simplicity referred to as relatively narrow and relatively wide. These mice were outbred under common garden conditions to generate families with relatively narrow or wide bacula. Male and female pairs were housed together for a maximum of 14 days. When noted to be pregnant, females were separated housed alone. At weaning age (21 days of age) female and male offspring were separated; female offspring were housed with their female siblings, and male offspring were housed individually. We used a sample of these offspring (F2) for our first experiment that examined the effect of baculum shape on reproductive success in a non-competitive context (monogamous matings). The remaining offspring were used to generate grand-offspring (F3) for our second experiment in which we examined the effect of baculum shape on competitive fertilization success (polyandrous matings).
Baculum shape aligned with the expected baculum shape extremes in both the F2 and F3 generations (for details, see electronic supplementary material, Fig.S3.1, Fig.S3.2 and Table S3.1). All animals were held in constant temperature rooms (CTR; 24°C) on a reverse dark-light cycle (10:14) and provided with water and food *ad libitum*. During pre-adult maturation, females were exposed once a week to unfamiliar and unrelated male odours, alternating between fresh male soiled chaff and fresh male urine. Females were virgin at the time of experiments, and males were sexually experienced by allowing them to mate with one unrelated female eight days prior to experimentation.

(b) Monandrous matings

Experimental matings were conducted when mice were approximately three months of age. At the onset of the dark phase, each female was inspected for oestrous condition by examining the appearance of the vaginal opening (Byers et al. 2012). If in oestrous, females were paired with a male. Mating sessions started approximately 5 hours into the dark cycle when female sexual receptivity is at its peak (Gomendio et al. 1998). Pairs were inspected for the presence of a mating plug every hour for evidence of a complete ejaculatory series, and therefore successful mating (Dietrich et al. 1992). Following mating, females were kept until mid-pregnancy (14 days of gestation) before being euthanised and dissected. The number and viability of the embryos (i.e., whether they were undergoing active development or being resorbed) were recorded (Firman and Simmons 2012).

(c) Polyandrous matings

Mating procedures were the same as those described for monandrous matings except that females were mated consecutively with two different males. Mating pairs were observed continuously for a maximum of three hours or until ejaculation was observed. Because we were investigating the stimulatory role of the house mouse baculum and not
its role on dislodging the mating plug, we removed the mating plug after each ejaculation. In this way, we ensured that each female received two ejaculations and that neither male was allowed to have its mating plug remain longer than 15 minutes. Thus, after a 15-minute rest period, the first male’s mating plug was removed from the female by gently pressing her against the side of the handling bin and dislodging it with a blunt probe. The female was then paired with the second male and again allowed to interact for a maximum of three hours or until fifteen minutes after receiving the second ejaculation. The second male’s plug was removed as described above. Once matings were completed, females were placed in a clean box with nesting material and kept until mid-pregnancy (14 days of gestation) before being euthanised and dissected. Embryos were stored in 100% ethanol prior to paternity analysis as described below.

(d) Paternity assignment

For individuals used in the competitive matings, DNA was extracted for both parents and both viable and non-viable embryos (parents $n=222$, embryos $n=566$). DNA was extracted from the parental liver and embryonic head using the EDNA HISPEX extraction kit (Fisher Biotec, Subiaco, Western Australia). Paternity was assigned to 519 embryos by screening ten microsatellite loci (D1Mit17, D2Mit1, D4Mit22, D6Mit138, D10Mit14, D11Mit4, D13Mit1, D14Mit132, D15Mit13, D18Mit17) (Dietrich et al. 1992; Sutter 2015). Unlabelled primers were obtained from Integrated DNA Technologies (Singapore, Singapore). Labelled primers were obtained from Applied Biosystems (Foster City, California) (VIC) and Alpha DNA (Montreal, Canada) (FAM, PET &NED). Primers were divided into two multiplexes (Multiplex 1: D1Mit17, D10Mit14, D11Mit4, D14Mit132, D18Mit17; Multiplex 2: D2Mit1, D4Mit22, D6Mit138, D13Mit1, D15Mit13) in 10 μl reactions. Reactions contained eight μl of a multiplex kit (Qiagen, Doncaster, Victoria), 0.1 μM of labelled forward primer mixed with 0.1 μM of unlabelled forward primer, 0.2 μM of unlabelled reverse
primer, and ~ 200 ng of template DNA. The thermocycling annealing temperature varied according to the multiplex; multiplex 1 annealing temperature was 60°C and multiplex 2 was 55°C. The rest of the thermocycling profile was identical: 5 min denature at 94°C, 30 cycles of 94°C for 30 s, 60°C/55°C for 30 s and 72°C for 1 min, followed by 72°C for 3 min. PCR products (2.5 μl) were run on an ABI3730 Sequencer, sized using Genescan-500 LIZ size standard and genotyped using Genemapper software (v3.0; Life Technologies). Paternity was assigned by manual exclusion.

(e) Morphometric analysis

Seven days after mating, males were euthanised, body weight was recorded, and the baculum dissected for geometric morphometric analysis (GMA). Details of baculum dissection and morphometric analysis are described elsewhere (André et al. 2018). Briefly, after baculum dissection, digital images were captured, and geometric morphometric analysis conducted using the software Tpsrelw 1.65 developed by Rohlf (2006). Landmarks were placed around the periphery of the baculum (36 sliding, 4 fixed) and relative warps (RWs) and centroid size were extracted. We focused on those RWs that each explained more than 10% of the variation in shape and on centroid size. We assessed the reliability of landmark placement and repeatability of our measurements by placing landmarks on two separate occasions for 20% of the analysed specimens for both generations. Our measurements of genital shape and size were highly repeatable (for details, see electronic supplementary material, table S3.2.).

(f) Statistical analysis

All statistical analyses were performed in R, version 3.6.1 (R Core Team 2015). For the non-competitive experiment, we explored the effect of baculum morphology and its interaction with female genetic background (wide/narrow) on the number of implanted embryos and embryo viability. To analyse embryo viability, we used generalised linear
mixed models (GLMMs) with Laplace approximation and binomial error distributions. The number of viable embryos was included as the dependent variable and the total number of embryos as the binomial denominator. In our GLMM of embryo number, we used a Poisson error distribution. For all analyses, baculum size was rescaled by centring and dividing by two standard deviations. In some cases, more than one male and/or female had to be used from the same full-sibling family, so male and female family were modelled as random effects in the analyses.

In the polyandrous matings, we investigated whether the interaction between 1st and 2nd male baculum morphology and female genetic background affected the paternity share of the 2nd male (P2). We reduced the number of predictor variables by subtracting the 1st male RWs and centroid size from those of the 2nd male, providing a measure of relative baculum shape for competing males. Since RW scores are centred to zero, we transformed them by adding a positive constant (10) to facilitate interpretation. Second male paternity share was analysed by GLMM with Laplace approximation and binomial error distribution, and with the number of embryos sired by the second male included as the dependent variable and the total number of embryos as the binomial denominator. Both 1st and 2nd male and female family were included as random effects.
3.4 Results

(a) Monandrous matings

The geometric morphometric analysis returned two relative warps (RW) that each explained more than 10% of the variation: RW1 = 44.58%; RW2 = 26.56%. Both RWS described variation in the relative thickness of the baculum bulb and shaft (Figure 3.1a). Baculum morphology and its interaction with female family background had no significant effect on the number of embryos (interaction terms - RW1: estimate = -0.275 ± 4.338, p = 0.95; RW2: estimate = -5.975 ± 5.750, p = 0.29; baculum size = -0.024 ± 0.171, p = 0.88) or on their viability (interaction terms - RW1: estimate = -10.119 ± 17.112, p = 0.54; RW2: estimate = 6.531 ± 21.766, p = 0.62; Baculum Size = -0.272 ± 0.707, p = 0.44). For full analyses, see Table S3.3 and S3.4 in the electronic supplementary material.

(b) Polyandrous matings

The geometric morphometric analysis again returned two relative warps each explaining more than 10% of the variation in baculum shape: RW1 = 37.59%; RW2 = 29.80%. The analysis was qualitatively similar to that for males in the monandrous experiment. Thus, both RWs described variation in the relative thickness of the baculum bulb and shaft (Figure 3.1b).

Mating position (first or second male) had no effect on the proportion of embryos sired (Wilcoxon signed-rank test with continuity correction, V = 1004.5, p = 0.11; mean P2 = 0.45 ± 0.15). However, there was a significant interaction between RW2 and female genetic background on the proportion of offspring sired by the second male, P2 (Table 3.1). Thus, among females from families with a breeding value for narrow bacula, P2 decreased as second male’s baculum, relative to the first male, became wider (Figure 3.2). Yet, among females from families with a breeding value for wide bacula, P2
increased as the second male’s baculum, relative to the first male, became wider (Figure 3.2). In other words, among ‘narrow’ females, maximum P2 (47.12% ± 4.85%) was achieved with a relatively narrow baculum, and among ‘wide’ females, maximum P2 (41.79% ± 5.46%) was achieved with a relatively wider baculum. RW1, baculum size and their interaction with female genetic background had no significant effects on second male parentage (Table 3.1).

Table 3.1. General Linear Mixed Model (GLMM) of the effect of baculum shape of the first male relative to the second male and its interaction with the female’s genetic background on second male parentage. Random effects variance: 1st ♂ Family (0.614 ± 0.783), 2nd ♂ Family (0.476 ± 0.689) and ♀ Family (0.0001 ± 0.0001)

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<th>type III, wald $\chi^2$</th>
<th>$p$</th>
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<td></td>
</tr>
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<td>RW1</td>
<td>0.793 (7.619)</td>
<td>0.010</td>
<td>0.92</td>
</tr>
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<td>RW2</td>
<td>6.921 (7.543)</td>
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<td>Baculum Size</td>
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<td>0.98</td>
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<tr>
<td>♀ Extreme</td>
<td>0.493 (0.267)</td>
<td>3.404</td>
<td>0.06</td>
</tr>
<tr>
<td>RW1 × ♀ Extreme</td>
<td>-7.252 (9.509)</td>
<td>0.582</td>
<td>0.44</td>
</tr>
<tr>
<td>RW2 × ♀ Extreme</td>
<td>-23.26 (11.444)</td>
<td>4.133</td>
<td>0.04</td>
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<tr>
<td>Baculum Size × ♀ Extreme</td>
<td>1.796 (2.818)</td>
<td>0.406</td>
<td>0.52</td>
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</table>
Figure 3.1. Variation in baculum shape. (a) Among males in the monandrous experiment and (b) Among males in the polyandrous experiment. Variation in baculum shape described by the extreme of relative warp 1 ($1^-$, $1^+$), relative warp 2 ($2^-$, $2^+$) and consensus shape (0). The positions of the 40 landmarks around the periphery of the baculum for both positive and negative extremes are shown.
Figure 3.2. Effects of relative baculum width (RW2 first male – second male) and female genetic background on second male parentage (P2). The plot shows the predict values (marginal effects) of the difference in baculum shape between 1st and 2nd male (RW2s) for males mating to females from a family with a breeding value for relatively wide bacula (red) and relatively narrow bacula (blue), with 95% confidence intervals. Negative values on the x-axis represent 2nd males with a narrower baculum than the 1st male, and positive values represent 2nd males with a wider baculum than the 1st male. A score of zero represents competing males with the same shaped baculum. Baculum shapes at the extremes of shape variation of shown for qualitative interpretation.
3.5 Discussion

Polyandry is a common mating strategy across many species (Birkhead 1998; Simmons 2001; Bretman and Tregenza 2005), including the house mouse (Dean et al. 2006; Firman and Simmons 2008c). In natural mouse populations, the rate of multiple paternity can reach levels of up to 70%, indicating that postcopulatory male competition is intense (Dean et al. 2006; Firman and Simmons 2008c). In a previous study on the source population used in these experiments, we reported that 38% of females captured in the field had litters sired by two or more males (Firman and Simmons 2008c). Our previous research has also established that (i) baculum shape harbours significant additive genetic variation (André et al. 2020 Chapter 2), (ii) can respond to the experimental manipulation of postcopulatory sexual selection (Simmons and Firman 2014), and (iii) males from the population that seeded this experiment had relatively wider bacula than males from populations with lower levels of multiple paternity (Simmons and Firman 2014). Here, we found that baculum shape influenced male fitness when female house mice mated multiply, and thereby provide evidence that baculum shape contributes to male reproductive success. Moreover, our results show that the effect of baculum shape on male competitive fitness is dependent on the breeding value for baculum shape for the family from which females were derived. Collectively, these studies provide strong support for the role of postcopulatory sexual selection in the evolution of the house mouse baculum.

Our analyses revealed that baculum shape did not affect male fitness when females mated monandrously. This finding provides evidence that it is the effect of the baculum on a male’s ability to induce sperm transport and/or utilisation, rather than his ability to affect embryo implantation and/or embryo survival, since all males, regardless of the shape of their baculum attained equal reproductive success when mating in a non-competitive context. The cryptic female choice hypothesis posits that females discern
stimulation and bias paternity outcome to those males whose genital morphology delivers better stimulation (Eberhard 1996, 2010; Brennan and Prum 2015). Female mammals are therefore required to receive stimulation from at least two males in the same oestrous cycle to exercise their choice. Evidence that supports the role of female sensory perception in imposing selection on the baculum comes from a significant interaction effect on paternity success, between the female’s genetic background and the relative baculum shapes of competing males. A Fisherian mechanism of cryptic female choice predicts the establishment of genetic covariation between male genital trait and female preference (Eberhard 1996; Mead and Arnold 2004). The supposition is that females with the preference should bias paternity toward males with the preferred trait(s) so that genes for the preference and trait become linked (Prum 2010; Greenfield et al. 2014). For example, in Stalk-eyed flies (Cyrtodiopsis dalmanni) artificial selection for increased and decreased male eye stalk length across thirteen generations resulted in correlated evolution between the strength of female preferences for long and short eye stalks, respectively (Wilkinson and Reillo 1994; Wilkinson et al. 1998). Likewise, among populations of guppies (Poecilia reticulata), the strength of female preference for orange is associated with the amount of orange on males (Houde and Endler 1990; Houde 1994). We found that when mated to two males, females from families characterised by males with a relatively wide baculum showed a second male paternity advantage toward the male with the relatively wider baculum, while females from families characterised by males with a relatively narrow baculum showed a second male paternity advantage toward the male with the relatively narrower baculum. Intriguingly, this interaction effect is entirely compatible with a mechanism of cryptic female choice based on the Fisher process.

Recently, we showed that baculum shape covaries with the width of the genital cervix, consistent with the genetic coevolution of male and female genitalia predicted by
models of sexual selection (André et al. 2020 Chapter 2). While certainly possible, it seems unlikely that cervix width alone can be responsible for the biasing of paternity in response to baculum shape that we have observed. Similarly to other mammalian species, the neuronal arrangement of the house mouse vaginal tract is comprised of sympathetic, parasympathetic and sensory fibres (van Helden et al. 2017), and is susceptible to regional, temporal and mating experience difference in innervation (Barry et al. 2017; van Helden et al. 2017). Variation in baculum width may lead to variation in the stimulation of individual nerve fibres and/or the overall stimulation that females receive and perceive during copulation. Females from different genetic backgrounds (narrow/wide) may favour males with different baculum widths due to the type of neuronal fibres being stimulated as well as the number of, and the degree to which the neuronal fibres are stimulated. Further, females from families with breeding values for relatively narrow and wide bacula may vary in the distribution and/or quantity of nerve fibres surrounding the reproductive tract that may consequently lead to a differential perception of stimulation according to baculum width. Future studies should examine more closely variation in female genital tract innervation and sensory responses to variation in baculum shape.

In conclusion, our data show that baculum shape affects male competitive fertilisation success when female house mice mate with more than one male. Our finding of an interaction between the difference in baculum shape between rival males and female genetic background supports a role for cryptic female choice in the coevolution of male and female genitalia in this species. Research on female tract innervation and variation in the neuroendocrine response following copulatory stimulation are likely to bring a more nuanced understanding of how female genitalia as a preference trait impose selection for the evolution of male genital morphology.
Appendix 3.1 Supplementary Material

This appendix includes:

Supplementary Tables S3.1 to S3.4

Supplementary Figure S3.1 to S3.2
**Figure S3.1.** Variation in baculum shape of second-generation laboratory reared house mice derived from the extremes of baculum shape (Wide/Narrow). *(a)* Mean (± 95% CI) score of the first relative warp (RW1). *(b)* Mean (± 95% CI) score of the second relative warp (RW2). Baculum shape thin-plate splines: left – wide background, right – narrow background.

(a)

(b)
**Figure S3.2.** Variation in baculum shape of third-generation laboratory reared house mice descended from families from the extremes of baculum shape (Wide/Narrow). *(a)* Mean (± 95% CI) score of the first relative warp (RW1). *(b)* Mean (± 95% CI) score of the second relative warp (RW2). Baculum shape thin-plate splines: left – wide background, right – narrow background.
Table S3.1. Linear mixed model (LMM) of baculum shape of F₂ and F₃ house mice descended from families from the extremes of baculum shape. (a) Second generation (n = 104); (b) Third generation (n=214).

<table>
<thead>
<tr>
<th>fixed effects</th>
<th>estimate ± (se)</th>
<th>type II, Wald χ²</th>
<th>p</th>
<th>random effects</th>
<th>variance ± sd</th>
</tr>
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<tbody>
<tr>
<td>(a) 2ⁿᵈ Generation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RW1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>intercept</td>
<td>-0.071 (0.042)</td>
<td></td>
<td></td>
<td>family</td>
<td>&lt;0.001 0.001</td>
</tr>
<tr>
<td>extreme</td>
<td>-0.013 (0.004)</td>
<td>8.37</td>
<td>0.003</td>
<td></td>
<td></td>
</tr>
<tr>
<td>body weight</td>
<td>0.027 (0.015)</td>
<td>3.43</td>
<td>0.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RW2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>intercept</td>
<td>0.031 (0.036)</td>
<td></td>
<td></td>
<td>family</td>
<td>&lt;0.001 0.003</td>
</tr>
<tr>
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<td>0.002 (0.003)</td>
<td>0.27</td>
<td>0.61</td>
<td></td>
<td></td>
</tr>
<tr>
<td>body weight</td>
<td>-0.012 (0.013)</td>
<td>0.83</td>
<td>0.36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Centroid Size</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>intercept</td>
<td>0.423 (1.209)</td>
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<td></td>
<td>family</td>
<td>0.050 0.224</td>
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<tr>
<td>extreme</td>
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<td>0.57</td>
<td></td>
<td></td>
</tr>
<tr>
<td>body weight</td>
<td>-0.135 (0.425)</td>
<td>0.11</td>
<td>0.75</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(b) 3ʳᵈ Generation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RW1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>intercept</td>
<td>-0.058 (0.032)</td>
<td></td>
<td></td>
<td>family</td>
<td>&lt;0.001 0.005</td>
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<tr>
<td>extreme</td>
<td>-0.004 (0.003)</td>
<td>1.92</td>
<td>0.16</td>
<td></td>
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</tr>
<tr>
<td>body weight</td>
<td>0.021 (0.011)</td>
<td>3.55</td>
<td>0.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RW2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>intercept</td>
<td>0.039 (0.025)</td>
<td></td>
<td></td>
<td>family</td>
<td>&lt;0.001 0.007</td>
</tr>
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<td>extreme</td>
<td>0.014 (0.003)</td>
<td>14.69</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
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<td>body weight</td>
<td>-0.016 (0.008)</td>
<td>3.48</td>
<td>0.06</td>
<td></td>
<td></td>
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<tr>
<td>Centroid Size</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>intercept</td>
<td>-3.382 (0.814)</td>
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<td>family</td>
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<td>extreme</td>
<td>0.053 (0.121)</td>
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<td>16.64</td>
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Table S3.2 Repeatability analysis. (a) Second generation, (b) Third generation. We used intra-class correlation coefficient analysis (ICC) with the one-way random effects model to estimate the reliability of measurement for landmark placement.

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<th>F</th>
<th>p</th>
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<td>RW1</td>
<td>21</td>
<td>0.992</td>
<td>0.982 - 0.997</td>
<td>266</td>
<td>&lt;0.0001</td>
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<td>21</td>
<td>0.982</td>
<td>0.958 - 0.993</td>
<td>113</td>
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<td>Centroid Size</td>
<td>21</td>
<td>0.996</td>
<td>0.991 - 0.998</td>
<td>537</td>
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<tr>
<td>(b) 3rd Generation</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RW1</td>
<td>42</td>
<td>0.974</td>
<td>0.953 - 0.986</td>
<td>76.2</td>
<td>&lt;0.0001</td>
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Table S3.3. General Linear Mixed Model (GLMM) of the effect of baculum morphology and its interaction with female genetic background on the number of embryos.

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<th>variance ±sd</th>
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</thead>
<tbody>
<tr>
<td>intercept</td>
<td>2.059 (0.059)</td>
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<td></td>
<td>♂ Family</td>
<td>&lt;0.0001 &lt;0.0001</td>
</tr>
<tr>
<td>RW1</td>
<td>0.408 (2.725)</td>
<td>0.022</td>
<td>0.88</td>
<td>♂ Family</td>
<td>&lt;0.0001 &lt;0.0001</td>
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<td>RW2</td>
<td>1.447 (3.493)</td>
<td>0.172</td>
<td>0.68</td>
<td>♂ Family</td>
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<td>Size</td>
<td>0.052 (0.109)</td>
<td>0.228</td>
<td>0.63</td>
<td>♂ Family</td>
<td>&lt;0.0001 &lt;0.0001</td>
</tr>
<tr>
<td>♀Extreme</td>
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<td>0.267</td>
<td>0.61</td>
<td>♂ Family</td>
<td>&lt;0.0001 &lt;0.0001</td>
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<tr>
<td>RW1 × ♀Extreme</td>
<td>-0.275 (4.338)</td>
<td>0.004</td>
<td>0.95</td>
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<td>&lt;0.0001 &lt;0.0001</td>
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<tr>
<td>RW2 × ♀Extreme</td>
<td>-5.975 (5.750)</td>
<td>1.079</td>
<td>0.29</td>
<td>♂ Family</td>
<td>&lt;0.0001 &lt;0.0001</td>
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<tr>
<td>Size × ♀Extreme</td>
<td>-0.024 (0.171)</td>
<td>0.019</td>
<td>0.88</td>
<td>♂ Family</td>
<td>&lt;0.0001 &lt;0.0001</td>
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Table S3.4. General Linear Mixed Model (GLMM) of the effect of baculum morphology and its interaction with female genetic background on embryo viability.

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<th>random effects</th>
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<td>2.874 (0.318)</td>
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<td>RW1</td>
<td>4.631 (11.741)</td>
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<td>0.923 (14.699)</td>
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<td>Size</td>
<td>0.885 (0.511)</td>
<td>3.006 0.08</td>
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</tr>
<tr>
<td>♂ Extreme</td>
<td>-0.354 (0.347)</td>
<td>1.044 0.18</td>
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<td>♂ Family</td>
<td></td>
</tr>
<tr>
<td>RW1 × ♂ Extreme</td>
<td>-10.11 (17.11)</td>
<td>0.349 0.54</td>
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<td>♂ Family</td>
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<tr>
<td>RW2 × ♂ Extreme</td>
<td>6.531 (21.766)</td>
<td>0.09 0.62</td>
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<td>♂ Family</td>
<td></td>
</tr>
<tr>
<td>Size × ♂ Extreme</td>
<td>-0.272 (0.707)</td>
<td>0.148 0.44</td>
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<td>♂ Family</td>
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CHAPTER FOUR: THE EFFECT OF BACULUM SHAPE ON MATING-INDUCED PROLACTIN RELEASE IN FEMALE HOUSE MICE
4.1 Abstract

Male genitalia have been shown to be subject to rapid divergent evolution and sexual selection is believed to be responsible for this pattern of evolutionary divergence. Genital stimulation during copulation is an essential feature of sexual reproduction. In mammals, the male intromittent genitalia induces a cascade of physiological and neurological changes in females that promote pregnancy. Previous studies of the house mouse have suggested that the shape of the baculum (penis bone) affects male reproductive success and can respond to experimentally imposed variation in sexual selection. Vagino-cervical stimulation has shown to induce a peak of Prolactin release that has been linked with pregnancy success and the establishment of sexual satiety. Here, we explore the hypothesis that the baculum is subject to sexual selection due to a stimulatory function during copulation. We selected male and female house mice (*Mus musculus domesticus*) from families with breeding values at the extremes of baculum shape to run two series of experimental matings in which we examined the concentration of Prolactin in the blood of females either 15 (“early”) or 75 (“late”) minutes after ejaculation. Our results provide evidence of a mating-induced release of Prolactin in the female house mouse early after ejaculation, the level of which is dependent both on the shape of the baculum and male sexual behaviour. Our data provide novel insight into the potential mechanism(s) of sexual selection acting on the mammalian baculum.
4.2 Introduction

Male genitalia are widely recognised as being among the most highly variable and evolutionarily divergent of all animal structures (Eberhard 1985). There is now considerable evidence across a variety of invertebrate and vertebrate taxa that male genital morphology is subject to sexual selection (Eberhard 1985; Arnqvist 1998; Langerhans et al. 2005; Simmons et al. 2009; Cayetano et al. 2011; Evans et al. 2011; Firman and Simmons 2011; Mautz et al. 2013; Simmons and Firman 2014), and that variation in genital morphology can impact mating, insemination, fertilisation and overall male reproductive success (Arnqvist and Danielsson 1999; Danielsson and Askenmo 1999; House and Simmons 2003; Hotzy and Arnqvist 2009; Wojcieszek and Simmons 2011; Stockley et al. 2013).

In species with internal fertilisation, male and female genitalia interact mechanically during copulation so that genital coevolution is expected via mechanisms of sexual selection including sexually antagonistic coevolution and cryptic female choice (Eberhard 1996, 2010; Hosken and Stockley 2004; Simmons 2014; Brennan and Prum 2015; Firman et al. 2017). Theories of cryptic female choice propose that females gain indirect benefits from mate choice by biasing paternity outcomes toward males whose genital morphology deliver more effective stimulation, producing sons better equipped to deliver stimulation to choosy females and, if stimulatory ability covaries with general health and vigour, offspring of higher general viability (Eberhard 1996, 2010; Brennan and Prum 2015; Firman et al. 2017; Eberhard and Lehmann 2019). In contrast, sexually antagonistic coevolution proposes that male and female reproductive interests differ, leading to an arms race over reproductive outcomes (Chapman et al. 2003; Parker 2006; Eberhard 2011; Simmons 2014; Brennan and Prum 2015). Under sexual conflict, male genital structures evolve to promote their own reproductive fitness at a cost to female fitness (Chapman et al. 2003; Arnqvist and Rowe 2005; Eberhard 2011). Male genital
adaptations subsequently lead to the evolution of female adaptations to reduce the cost imposed by males (Chapman et al. 2003; Parker 2006; Eberhard 2010). Empirical studies have offered support for both cryptic female choice and sexually antagonistic mechanisms of sexual selection. In dung beetles (Simmons and Garcia-Gonzalez 2011; Simmons and Holley 2011; Simmons and Fitzpatrick 2019), bushcrickets (Wulff et al. 2015, 2017; Wulff and Lehmann 2016), tsetse fly (Briceño and Eberhard 2009) and damselfly (Córdoba-Aguilar 1999, 2006) the coevolution of interacting male and female genital traits is consistent with a model of cryptic female choice. In contrast, the evolution of male and female genital traits in seed beetles (Rönn et al. 2007; Hotzy et al. 2012; Dougherty et al. 2017), water striders (Arnqvist and Danielsson 1999) and waterfowl (Brennan et al. 2007, 2010) appear consistent with a model of sexually antagonistic coevolution. Although it is now accepted that mammalian genitalia are subject to sexual selection (Ramm 2007; Dixson 2012; Mautz et al. 2013; Simmons and Firman 2014; André et al. 2018, 2019 Chapter 3), there is a general lack of evidence for the mechanism(s) by which sexual selection acts in this taxon.

The ossified penis or baculum is a feature of several mammalian orders (Ramm 2007; Brindle and Opie 2016), however its adaptive function is yet to be established. Three different hypotheses have been proposed to explain baculum evolution. The bone may impart rigidity to facilitate intromission (Long 1969), assist in the transportation of sperm (Dixson 2012) and/or stimulate the female during copulation (Greenwald 1956; Eberhard 1985). Here, we use house mice (Mus musculus domesticus) to test the stimulation hypothesis for the evolution of the mammalian baculum. In house mice, baculum shape has been shown to covary with the strength of selection from sperm competition among natural and experimentally evolving populations (Simmons and Firman 2014), to harbour significant additive genetic variance (André et al. 2020 Chapter 2), and to exhibit developmental plasticity in response to potential risk of sperm
competition (André et al. 2018). Baculum shape was also found to influence male reproductive success in free-ranging experimental populations; males with a wider baculum were found to sire a greater proportion of offspring per litter (Stockley et al. 2013). Our most recent work has revealed that the shape of the house mouse baculum influenced paternity success when males compete for fertilisations, but not when females mated monandrously (André et al. 2019 Chapter 3). Moreover, the effect of baculum shape on a male’s competitive fertilisation success depended on the genetic background of the female; specifically, males with wide bacula obtained a greater paternity share when mating with females having a genetic background for wide bacula (André et al. 2019 Chapter 3). Taken together, these findings suggest that sexual selection might act on male genital evolution via a stimulatory role of the baculum during copulation (Greenwald 1956; Eberhard 1996, 2010; Ramm 2007; Brennan and Prum 2015).

In a number of mammalian species, female genital stimulation is essential for successful pregnancy (Chester and Zucker 1970; Eberhard 1996; Pavličev and Wagner 2016). The mammalian vaginal tract is highly innervated (Hilliges et al. 1995; Barry et al. 2017; van Helden et al. 2017) and regulated by the neuroendocrine system (Brenner and West 1975) making it a candidate structure for the perception of stimulation that imposes sexual selection on male genitalia (Eberhard 1996; Hosken and Stockley 2004; Brennan and Prum 2015). During mammalian copulation, genital stimulation induces a cascade of physiological events that prime the female reproductive tract for embryo implantation and condition future female sexual behaviour (Diamond 1972; Pfau 1999; Pfau et al. 2001; Pavličev and Wagner 2016). Copulation has been shown to induce an increase in circulating Luteinizing Hormone, Prolactin and Oxytocin in both rats and humans (Erskine and Kornberg 1992; Blaicher et al. 1999; Exton et al. 1999), and these hormones are known to influence sexual behaviour and reproduction (Erskine and
Kornberg 1992; Anderson-Hunt and Dennerstein 1995; Blaicher et al. 1999; Exton et al. 1999). Prolactin, in particular, is involved in a multitude of physiological processes (Freeman et al. 2000; Ben-Jonathan et al. 2008). In rats, there is a mating-induced surge of plasma Prolactin (Erskine 1995); vagino-cervical stimulated females show an acute increase in Prolactin within the first minutes of stimulation (Erskine and Kornberg 1992; Kornberg and Erskine 1994; Erskine 1995) and females that enter pseudopregnancy exhibit higher levels of circulating Prolactin than those that do not (Erskine and Kornberg 1992; Kornberg and Erskine 1994; Erskine 1995). Prolactin has also been linked with the rewarding system of sexual behaviour and the establishment of sexual satiety (Convey et al. 1971; Kamel et al. 1975; Bronson and Desjardins 1982; Exton et al. 2001; Leeners et al. 2013). Studies of mice, rats, bulls and humans have all found that following ejaculation/orgasm both males and females experience a peak in serum Prolactin (Convey et al. 1971; Kamel et al. 1975; Bronson and Desjardins 1982; Exton et al. 2001; Leeners et al. 2013). Moreover, in rats, there is evidence that high levels of circulating Prolactin (hyperprolactinemia) leads to the inhibition of sexual function at the motivational and physiological levels (Dudley et al. 1982; Bailey et al. 1984; Bole-Feyset et al. 1998; Krüger et al. 2002).

In this study, we aimed to determine whether baculum shape affects genital stimulation, and female perception of stimulation, by examining circulating Prolactin levels in females following copulation. We thus quantified (i) variation in baculum shape, (ii) various components of male mating behaviour, and (iii) circulating plasma Prolactin levels in females, either 15 (“early”) or 75 (“late”) minutes after ejaculation. We sourced our experimental subjects from families known to lie at the extremes of genetic variation in baculum shape (herein referred to as “wide” and “narrow”). If sexual selection acts on the baculum via a stimulatory role during copulation, we predicted that males with a relatively wide baculum would induce greater female Prolactin levels after
less copulatory behaviour, while males with a relatively narrow baculum would be required to deliver greater levels of copulatory behaviour to achieve equivalent levels. Further, if the female vaginal tract acts as a preference trait in cryptic female choice, we expected that the effect of baculum morphology would depend on the genetic background (wide or narrow baculum morphology) from which females were derived.

4.3 Materials and Methods

(a) Source population and experimental animals

Male and female laboratory-reared house mice (Mus musculus domesticus) were fourth generation, outbred descendants of wild house mice sourced from an isolated population on Rat Island (28° 42’S, 113°47’E) off the coast of Western Australia. The source population was used to estimate patterns of additive genetic variation in baculum morphology, where a subset of the F₁ offspring was selected for geometric morphometric analysis of baculum size and shape, as described elsewhere (André et al. 2020 Chapter 2). The analysis demonstrated that variation in the relative width of the baculum bulb harboured high to moderate levels of additive genetic variation and heritability (André et al. 2020 Chapter 2). We selected eight of 30 F₁ sire families from each extreme of baculum shape and outbred them under a common garden environment to generate families with relatively wide and narrow bacula, as described elsewhere (André et al. 2020 Chapter 2). Mating pairs were housed for 14 days. After that period, females were housed alone and checked for pregnancy. At weaning age (21 days of age) the sexes were separated; female offspring were housed with female siblings, and male offspring were housed individually.

For the experiments described herein, we used the F₄ offspring of the selected families to examine the effect of baculum shape on the endocrine response of females to copulation. Baculum shape from the F₄ generation aligned with the F₁ “wide” and
“narrow” baculum shape extremes (for details, see electronic supplementary material, Fig. S4.1 and Table S4.1). All animals were held in constant temperature rooms (CTR; 24°C) on a reverse dark-light cycle (10:14) and provided with water and food *ad libitum*. During pre-adult maturation, females were exposed once a week to unfamiliar and unrelated male odours, alternating between fresh male soiled chaff and fresh male urine. Females were virgin at the time of experiments, and males were sexually experienced by mating them with two unrelated randomly assigned females, eight days prior to their use in experiments.

(b) Treatment (mated) and Control (unmated) Groups

For the early and late treatment groups, matings were conducted when mice were approximately 80 to 120 days of age. At the onset of the dark phase, females were inspected for oestrous condition by examining the appearance of the vaginal opening (Byers et al. 2012). When in oestrous, females were paired with a male approximately 5 hours into the dark phase (i.e., when female sexual receptivity is at its peak) (Gomendio et al. 1998). Mating pairs were observed and recorded for a maximum of three hours or until an ejaculation was observed. Mating sessions were aborted if, in the first 45 minutes, males did not show any mating interest and/or females did not exhibit signs of receptivity (lordosis behaviour and/or solicitation behaviour). Mating sessions were video-recorded for the assessment of male copulatory behaviour. Following ejaculation, the mating pair were separated. A subset of females were anaesthetised ~15 minutes after ejaculation (early; *n* = 43), while the remaining females were placed in a clean box with nesting material before being anaesthetised ~75 minutes after ejaculation (late; *n* = 45). To provide an estimate of the baseline levels of circulating Prolactin with which to contrast the treatment groups, blood samples were collected from a control group (*n* = 20). The control group consisted of receptive, but unmated, females drawn from both
genetic backgrounds (relatively wide vs. narrow baculum). A blood sample was collected prior to euthanasia (details below).

(c) Blood Collection and Prolactin Concentration Assessment

Blood samples were collected from females by terminal cardiac puncture 15 minutes after applying an anaesthetic of ketamine and medetomidine (75 mg/kg + 1 mg/kg) via peritoneum injection. Blood samples were allowed to coagulate for 30 minutes at room temperature (25°C) before being prepared by 4°C centrifugation at 3250 rpm for 20 minutes. Aliquots of 50 μl were stored at -80°C until the assays were performed. The concentration of Prolactin was determined using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Abcam, ab100736) in accordance with the manufacturer’s instructions. All samples (n = 108) and standards were assayed in duplicate and reproducibility was assessed by the analysis of the coefficient of variance between the duplicates of each sample. For all samples, the coefficient of variance did not exceed 15% (4.772%, 0.2% to 13.4%), and so all samples were included for subsequent analyses. Examination of plasma Prolactin data identified five outliers (mean ± 2×sd), one in the control group and two outliers for the early and late groups, that were removed from the analysis.

(d) Male Copulatory Behaviour

The copulatory behaviour of male house mice is typically characterised by a variable number of mount attempts and a variable number of mounts with intromissions, where a single intromission consists of the insertion of the penis into the vaginal tract (McGill 1962). A copulatory series includes all mount attempts and mounts with variable numbers of intromissions and ends with the deposition of the ejaculate and copulatory plug (McGill 1962). Thus, we recorded the (i) number of mounts with intromissions, (ii)
total number of intromissions, (iii) copulation duration (time of all mounts with intromission), and (iv) ejaculation latency (time from the first mount to ejaculation).

(e) Baculum Morphometric Analysis

Seven days after mating, males were euthanised, body weight was recorded, and the baculum dissected for geometric morphometric analysis. Details of baculum dissection and morphometric analysis are described elsewhere (André et al. 2018). Briefly, a total of 40 landmarks were placed around the periphery of the baculum, and relative warps (RWs) and centroid size were extracted using the software Tpsrelw 1.65 developed by Rohlf (2006). We focused on centroid size and the RWs that individually explained more than 10% of the variation in shape. We assessed the reliability of landmark placement and repeatability of our measurements by placing landmarks on two separate occasions for 20% of the bacula. Our measurements of baculum shape and size were highly repeatable (for details, see electronic supplementary material, Table S4.2).

(f) Statistical Analysis

All statistical analyses were performed in R, version 3.6.1 (R Core Team 2015). To explore whether mating per se had an effect on the levels of circulating Prolactin across treatment groups, we applied a permutational multivariate analysis of variance (PerMANOVA) with log body weight as a covariate, followed by a post-hoc Dunn’s test with Bonferroni correction. PerMANOVA was used for this analysis as transformations could not normalise the residuals from the model. To assess whether Prolactin levels were affected by the interaction between baculum shape, male copulatory behaviour and female genetic background (wide or narrow) in the early and late treatment groups, we used linear mixed models (LMMs) fitted by maximum-likelihood estimation with Laplace approximation. Because more than one individual was used from each full-sibling family, the male and female family were included as
random effects. Significance values were extrapolated from Type III Wald $\chi^2$ tests. We transformed variables to approach normality using $\log(x + 1)$ transformation, with the exception of “ejaculation latency”, which was transformed using $\sqrt{x + 1}$. For all analyses, baculum size was rescaled by centring and dividing by two standard deviations. After transformation, behavioural variables were similarly rescaled. Copulatory behaviour was reduced to a single variable using a principal component analysis (PCA). Prolactin concentration was reciprocal transformed to approach normality.

4.4 Results

(a) Copulation-induced Prolactin Concentration
Prolactin concentration differed significantly across the three groups ($F= 10.42, r^2= 0.176, p =0.001$). A greater Prolactin concentration was observed in the early group relative to the concentration of plasma Prolactin in either the unmated control ($Z= -4.173, p < 0.001$) and late group ($Z= 4.080, p < 0.001$) (Figure 4.1). Prolactin concentration in the late group did not differ from that observed in the unmated control females ($Z= -0.967, p =0.99$) (Figure 4.1).
(b) Baculum Morphology and Prolactin Concentration

(i) Early group

The geometric morphometric analysis returned two relative warps (RW) that each explained more than 10% of the variation in baculum shape (RW1: 43.48%, RW2: 32.44%). Both RWs described variation in the shape of the baculum bulb, specifically in the relative width and extent of the bulb along the baculum shaft (referred to for simplicity as relative width, Figure 4.2a). Baculum centroid size exhibited no significant correlation with either RWs (RW1: Pearson $r = 0.122$, $p = 0.45$; RW2: Pearson $r = -0.027$, $p = 0.86$). The PCA of copulatory behaviour returned one component with an eigenvalue greater than one. This principal component explained 81.5% of the variation.
in copulatory behaviour. PC1 was positively loaded by the number of mounts, the number of intromissions, copulation duration and ejaculation latency (Table 4.1a) and can be interpreted as the total amount of copulatory behaviour delivered prior to ejaculation. Baculum RW2 and its interaction with copulatory behaviour had a significant effect on Prolactin concentration (Table 4.2). To visualise the interaction, we plotted the effect of copulation behaviour on Prolactin concentration separately for females mated to males with baculum shapes above (relatively wide) and below (relatively narrow) the mean for RW2 (Figure 4.3). Post-hoc analysis revealed that Prolactin concentration was highest among females mated to males with a relatively wide baculum but decreased with increasing copulatory behaviour ($\chi^2 = 16.543, p < 0.0001$). In contrast, for females mated with males with a relative narrow baculum, there was no significant effect of copulatory behaviour on Prolactin concentration ($\chi^2 = 0.190, p = 0.66$) (Figure 4.3). RW1, baculum size and female genetic background had no significant effect on Prolactin concentration in the early group (for details, see electronic supplementary material, Table S4.3 and S4.4).
Figure 4.2. Variation in baculum shape. (a) Males used in the Early Group, (b) Males used in the Late group. Variation in baculum shape described by the extreme of relative warp 1 ($1^-, 1^+$), relative warp 2 ($2^-, 2^+$) and consensus shape (0). The positions of the 40 landmarks around the periphery of the baculum for both positive and negative extremes are shown.
Table 4.1. Observed copulatory behaviour traits, their variability indices, and results from a PCA from Early (a) and Late (b) groups. Eigenvectors in bold were interpreted as contributing significantly to the principal component (PC1). SD, standard deviation.

<table>
<thead>
<tr>
<th>Behavioural Trait</th>
<th>mean</th>
<th>sd</th>
<th>PC1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>(a) Early</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of mounts †</td>
<td>5.55</td>
<td>4.06</td>
<td>0.522</td>
</tr>
<tr>
<td>Number of Intromissions †</td>
<td>100.25</td>
<td>73.76</td>
<td>0.515</td>
</tr>
<tr>
<td>Copulation duration (s) †</td>
<td>61.93</td>
<td>40.02</td>
<td>0.534</td>
</tr>
<tr>
<td>Ejaculation latency (s) ‡</td>
<td>1458</td>
<td>1498</td>
<td>0.420</td>
</tr>
<tr>
<td>Eigenvalue</td>
<td>3.26</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% explained</td>
<td></td>
<td>81.50%</td>
<td></td>
</tr>
<tr>
<td><strong>(b) Late</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of mounts †</td>
<td>6.91</td>
<td>6.12</td>
<td>0.504</td>
</tr>
<tr>
<td>Number of Intromissions †</td>
<td>116.35</td>
<td>116.26</td>
<td>0.501</td>
</tr>
<tr>
<td>Copulation duration (s) †</td>
<td>77.46</td>
<td>71.85</td>
<td>0.525</td>
</tr>
<tr>
<td>Ejaculation latency (s) ‡</td>
<td>1848</td>
<td>1537</td>
<td>0.469</td>
</tr>
<tr>
<td>Eigenvalue</td>
<td>3.371</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% explained</td>
<td></td>
<td>84.28%</td>
<td></td>
</tr>
</tbody>
</table>

† Log(x+ 1) transformed for the PCA;
‡ Sqrt(x + 1) transformed for PCA
Table 4.2. Linear mixed model (LMM) of the effect of baculum shape (RW2), male copulatory behaviour (PC1), female genetic background and their interactions on levels of circulating prolactin for females sampled Early after ejaculation.

<table>
<thead>
<tr>
<th>fixed effects</th>
<th>estimate ± (se)</th>
<th>type III, wald $\chi^2$</th>
<th>$p$</th>
<th>random effects</th>
<th>variance ± sd</th>
</tr>
</thead>
<tbody>
<tr>
<td>intercept</td>
<td>0.325 (0.024)</td>
<td></td>
<td></td>
<td>♂ Family</td>
<td>0.0177 ± 0.0421</td>
</tr>
<tr>
<td>RW2</td>
<td>-1.853 (1.494)</td>
<td>1.54</td>
<td>0.21</td>
<td>♂ Family</td>
<td>&lt;0.0001 ± &lt;0.0001</td>
</tr>
<tr>
<td>PC1</td>
<td>0.039 (0.015)</td>
<td>7.11</td>
<td>0.007</td>
<td>Family</td>
<td></td>
</tr>
<tr>
<td>♂ Background</td>
<td>0.023 (0.032)</td>
<td>0.51</td>
<td>0.47</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RW2 × PC1</td>
<td>-1.833 (0.719)</td>
<td>6.49</td>
<td>0.01</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RW2 × ♂ Background</td>
<td>-0.967 (1.902)</td>
<td>0.26</td>
<td>0.61</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC1 × ♂ Background</td>
<td>-0.006 (0.021)</td>
<td>0.09</td>
<td>0.76</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RW2 × PC1 × ♂ Background</td>
<td>0.358 (1.053)</td>
<td>0.11</td>
<td>0.73</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 4.3. Effects of baculum shape (RW2) and male copulatory behaviour (PC1) on the levels of plasma prolactin for females Early group after ejaculation. The plot shows the interaction between male copulatory behaviour (PC1) and males with a RW2 above the mean (Red) and below the mean (Blue), with 95% confidence interval.
(ii) Late group

The geometric morphometric analysis again returned two relative warps (RW) that each explained more than 10% of the variation in baculum shape (RW1: 41.49%, RW2: 28.85%). As for males in the early group, RW1 and RW2 both described variation in the shape of the baculum bulb (Figure 4.2b). Baculum centroid size did not correlate significantly with either RW (RW1: Pearson r = -0.134, p= 0.39; RW2: Pearson r = -0.244, p= 0.11). The PCA of copulatory behaviour returned one principal component with an eigenvalue greater than one. The principal component explained 84.3% of the variation in copulatory behaviour and was positively loaded by the number of mounts, number of intromissions, copulation duration and ejaculation latency (Table 4.1b). PC1 can be interpreted as the total amount of copulatory behaviour delivered prior to ejaculation. Neither baculum morphology, copulatory behaviour or female genetic background affected Prolactin concentration (see electronic supplementary material, Table S4.5, S4.6 and S4.7).

4.5 Discussion

We found that female house mice had significantly higher concentrations of Prolactin 15 minutes after copulation, compared with unmated controls or mated females assayed 75 minutes after copulation. Moreover, the Prolactin concentration in recently mated females was dependent on both baculum shape and the amount of copulatory behaviour performed by the male prior to ejaculation. Our data are consistent with the hypothesis that vagino-cervical stimulation by the baculum generates a release of Prolactin in females following copulation, and thereby provide novel support for the stimulation hypothesis for the evolution of the mammalian baculum.

A mating-induced surge in Prolactin has been documented in a number of mammalian species (Convey et al. 1971; Kamel et al. 1975; Erskine and Kornberg 1992; Krüger et
In males, the mating-induced Prolactin surge is closely associated with ejaculation (Convey et al. 1971; Kamel et al. 1975; Krüger et al. 1998) and is thought to be responsible for the establishment of the post-ejaculatory refractory period (Krüger et al. 2002). In humans, the Prolactin surge is sustained for one hour after ejaculation (Krüger et al. 1998; Exton et al. 2001) and it is thought to play a key role in the control of sexual arousal following copulation (Krüger et al. 2002). Human studies have found that when females report having experienced an orgasm, there is an increase of circulating Prolactin and a decrease in sexual motivation (Exton et al. 2001; Leeners et al. 2013). A high level of circulating Prolactin (hyperprolactinemia) has been shown to induce a reduction in both sexual motivation and function (Krüger et al. 2005). Non-human animal studies have further shown that high levels of Prolactin lead to sexual impairment (Dudley et al. 1982; Doherty et al. 1985, 1986). In both male and female rats, high levels of plasma Prolactin lead to an increase in the latency to engage in copulation, decreasing male copulatory behaviour and reducing female receptivity through inhibition of lordosis, the female behaviour that facilitates intromission (Dudley et al. 1982). Polyandry is a common mating strategy for most rodent species, including the house mouse where the rate of multiple paternity in natural populations can be as high as 70% and with as many as three males siring pups within a single litter (Dean et al. 2006; Firman and Simmons 2008c). Male competitive fertilisation success in mammals depends largely on the interval between rival male copulations (Gomendio et al. 1998). In rats for example, the interval between the deposition of competing ejaculates influences the paternity success of the second male, whereby longer intervals reduce the probability of second male paternity (Coria-Avila et al. 2004). Thus, a stimulation-driven female Prolactin release may serve as an effective paternity assurance strategy by reducing the propensity of females to re-mate shortly following
ejaculation. Ultimately, this would increase the interval between competing ejaculates and provide an advantage to males that mate first.

We found a significant interaction effect between baculum shape and the amount of copulatory behaviour on Prolactin levels in recently mated females. Males with a relatively wide baculum needed to deliver less copulatory behaviour to generate higher levels of Prolactin in females, compared with males with a relatively narrow baculum. Under the risk of sperm competition, it has been demonstrated that males tend to invest less in copulatory behaviour and ejaculate sooner (Preston and Stockley 2006). The ability to induce a Prolactin surge suitable for the maintenance of the corpus luteum in females with less copulatory behaviour may allow males with a wide baculum to ensure the probability of siring a greater proportion of offspring and also to more quickly gain access to additional receptive females to increase their reproductive success. Even though we did not find an effect of the genetic background of females on post-copulation Prolactin levels, we cannot exclude the possibility of variation in female sensitivity to stimuli as seen in rats (Wilson et al. 1965). The overall higher circulating Prolactin levels in females shortly after copulation may have masked subtle among-individual variation in female sensitivity to stimulation.

In a previous study we found that baculum shape influences fertilisation success when males compete for fertilisations (André et al. 2019 Chapter 3). Sperm need to reach the oocyte to achieve fertilisation and three different mechanisms have been proposed to explain sperm transport in the oviduct: thermotaxis, rheotaxis and chemotaxis (Cerezales et al. 2015). In the house mouse, both sperm chemotaxis and rheotaxis have been shown to be important for sperm transport (Oliveira et al. 1999; Eisenbach and Giojalas 2006; Miki and Clapham 2013). Copulation in house mice induces secretion and fluid flow within the oviduct (Miki and Clapham 2013), and there is a swelling of the uterus and significant increase in uterine fluid in the first hours after mating that
lowers fluid viscosity and clears debris from the oviduct, facilitating sperm rheotaxis (Miki and Clapham 2013). Importantly, the increase of uterine fluid necessary for sperm transport is controlled by the release of Prolactin at copulation; the inhibition of Prolactin release was found to suppress the normal increase of uterine fluid after mating (Miki and Clapham 2013). It is plausible to suggest therefore, that variation in mating-induced Prolactin release due to stimulation delivered by males with different baculum shapes and different amounts of copulatory behaviour might lead to differential rates of increase in the uterine fluid after mating and affect a male’s subsequent fertilisation success.

Although our previous work has reported no effect of baculum shape on embryo implantation rate or early embryo viability per se (André et al. 2019 Chapter 3), our findings here highlight the potential that baculum shape, via influencing prolactin release, might contribute to a successful reproductive outcome. In rodents, Prolactin also plays a central and modulatory role in pregnancy (Freeman et al. 2000; Bachelot and Binart 2007; Ben-Jonathan et al. 2008). For example, Prolactin receptor-deficient female house mice are almost entirely sterile due to failed embryo implantation (Ormandy et al. 1997). Prolactin is responsible for corpus luteum maintenance (luteotropic action) and progesterone production (Freeman et al. 2000; Bachelot and Binart 2007; Ben-Jonathan et al. 2008) and as a result, the development of the uterine endometrium necessary for blastocyst implantation (Erskine 1995). Qualitative variation in vagino-cervical stimulation has been shown to generate variation in Prolactin release; when females control the pace of mating Prolactin levels are higher than when females are unable to do so (Erskine and Kornberg 1992). Considerable variation in female sensitivity to mating stimulation has also been documented, with some females requiring more or less stimulation to reach the threshold required for acute Prolactin release (Wilson et al. 1965). Although there is no evidence that Prolactin release affects
pseudopregnancy in house mice, a previous study has shown that vagino-cervical stimulation before artificial insemination increases the average number of implanted embryos per pregnant female (Leckie et al. 1973). Moreover, a wide range of stimuli inputs are capable of inducing pseudopregnancy in house mice, while extremes of stimulation do not, which demonstrates that an optimum level of vaginal stimulation is required to induce the maintenance of the corpus luteum and pregnancy (Diamond 1970).

In conclusion, our study suggests that, as in other species of mammal, female house mice likely experience mating-induced release of Prolactin. We provide evidence that the level of plasma Prolactin following copulation is influenced by baculum shape and the copulatory behaviour of males, lending novel support to the stimulation hypothesis for the evolution of the mammalian baculum. Our findings are compatible with both cryptic female choice and sexual conflict mechanisms of sexual selection. For example, cryptic female preferences based on stimulation may determine the probability that a copulating male will sire a significant proportion of the female’s litter. In this way, females could exert selection for the evolution of baculum shape and its stimulatory abilities (Eberhard 1996, 2010; Eberhard and Lehmann 2019). However, male ability to inhibit female remating via prolactin release may lead to a reduction in the benefits that females gain from polyandry. Polyandry in house mice has been found to result in greater postbirth pup survival and to facilitate postcopulatory inbreeding avoidance (Firman and Simmons 2008b,a). While delaying female remating may reduce the risk of competition with rival ejaculates, it may not be in the best interests of polyandrous females. Furthermore, female sensory preferences may make females vulnerable to overstimulation, which could result in excess sperm at the site of fertilisation and an increased the risk of polyspermy (Fraser and Maudlin 1978, 1979; Snook et al. 2011). Sexual conflict might then favour reduced sensitivity to stimulation in females and/or
reduced levels of Prolactin following overstimulation. Such a scenario is consistent with
the finding that female mice require an optimal level of stimulation to achieve
pregnancy (Diamond 1970; Leckie et al. 1973) and our finding that for males with a
relatively wide baculum, greater copulatory behaviour was associated with lower levels
of Prolactin after copulation. Future studies should focus on providing a more detailed
picture of the copulation-induced release of Prolactin, perhaps using with multiple
blood sampling per female, before and after receiving an ejaculation, and by applying
ultra-sensitive ELISAs. In so doing we will gain greater insight into the dynamics of
copulation-induced Prolactin release and the mechanism(s) by which sexual selection
drives the (co)evolution of genital morphology.
Appendix 4.1

This appendix includes:

Supplementary Tables S4.1 to S4.7

Supplementary Figure S4.1
Figure S4.1. Variation in baculum shape of fourth-generation laboratory-reared house mice derived from families with different baculum shape extremes (Wide/Narrow). (a) Median (± 1.5×IQR) score of the first relative warp (RW1). (b) Median (± 1.5×IQR) score of the second relative warp (RW2). Baculum shape thin-plate splines: left – wide background, right – narrow background.
Table S4.1. Linear mixed model (LMM) of baculum shape for fourth generation laboratory reared house mice (n= 177) that were derived from families selected from the extremes of the distribution of baculum shape (Wide/Narrow).

<table>
<thead>
<tr>
<th>fixed effects</th>
<th>estimate ± (se)</th>
<th>type II, wald $\chi^2$</th>
<th>p</th>
<th>random effects</th>
<th>variance ± sd</th>
</tr>
</thead>
<tbody>
<tr>
<td>RW1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>intercept</td>
<td>0.018 (0.034)</td>
<td>Family</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>extreme</td>
<td>0.017 (0.003)</td>
<td>26.91</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>body weight</td>
<td>-0.009 (0.012)</td>
<td>0.67</td>
<td>0.41</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RW2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>intercept</td>
<td>-0.017 (0.029)</td>
<td>Family</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>extreme</td>
<td>-0.011 (0.004)</td>
<td>7.56</td>
<td>0.005</td>
<td></td>
<td></td>
</tr>
<tr>
<td>body weight</td>
<td>0.008 (0.010)</td>
<td>0.63</td>
<td>0.43</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Centroid Size</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>intercept</td>
<td>-5.523 (0.762)</td>
<td>Family</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>extreme</td>
<td>0.103 (0.121)</td>
<td>0.72</td>
<td>0.39</td>
<td></td>
<td></td>
</tr>
<tr>
<td>body weight</td>
<td>1.902 (0.263)</td>
<td>52.19</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table S4.2. Repeatability analysis for baculum size and shape. We used intra-class correlation coefficient analysis (ICC) with the one-way random effects model to estimate the reliability of measurement for landmark placement.

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>ICC</th>
<th>Confidence Interval</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>RW1</td>
<td>36</td>
<td>0.989</td>
<td>0.982 - 0.995</td>
<td>189</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>RW2</td>
<td>36</td>
<td>0.985</td>
<td>0.97 - 0.992</td>
<td>130</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Centroid Size</td>
<td>36</td>
<td>0.996</td>
<td>0.992 - 0.998</td>
<td>499</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>
Table S4.3. Linear mixed model (LMM) of the effect of baculum shape (RW1), male sexual behaviour (PC1), female genetic background and their interaction on copulation induced circulating prolactin for the Early (fifteen-minute) group.

<table>
<thead>
<tr>
<th>fixed effects</th>
<th>estimate ± (se)</th>
<th>Wald $\chi^2$</th>
<th>$p$</th>
<th>random effects</th>
<th>variance ± sd</th>
</tr>
</thead>
<tbody>
<tr>
<td>intercept</td>
<td>0.317 (0.026)</td>
<td></td>
<td></td>
<td>$\varnothing$ Family</td>
<td>0.0018 ± 0.0432</td>
</tr>
<tr>
<td>RW1</td>
<td>1.401 (1.823)</td>
<td>0.59</td>
<td>0.44</td>
<td>$\varnothing$ Family</td>
<td>&lt;0.0001 ± &lt;0.0001</td>
</tr>
<tr>
<td>PC1</td>
<td>0.007 (0.014)</td>
<td>0.31</td>
<td>0.57</td>
<td>$\varnothing$ Family</td>
<td></td>
</tr>
<tr>
<td>$\varnothing$ Background</td>
<td>0.019 (0.035)</td>
<td>0.29</td>
<td>0.58</td>
<td>$\varnothing$ Family</td>
<td></td>
</tr>
<tr>
<td>RW1 × PC1</td>
<td>-0.227 (0.868)</td>
<td>0.06</td>
<td>0.79</td>
<td>$\varnothing$ Family</td>
<td></td>
</tr>
<tr>
<td>RW1 × $\varnothing$ Background</td>
<td>-3.025 (2.233)</td>
<td>1.83</td>
<td>0.17</td>
<td>$\varnothing$ Family</td>
<td></td>
</tr>
<tr>
<td>PC1 × $\varnothing$ Background</td>
<td>0.012 (0.020)</td>
<td>0.35</td>
<td>0.55</td>
<td>$\varnothing$ Family</td>
<td></td>
</tr>
<tr>
<td>RW1 × PC1 × $\varnothing$ Background</td>
<td>-1.071 (1.106)</td>
<td>0.93</td>
<td>0.33</td>
<td>$\varnothing$ Family</td>
<td></td>
</tr>
</tbody>
</table>
Table S4.4. Linear mixed model (LMM) of the effect of baculum size male sexual behaviour (PC1), female genetic background and their interaction on copulation induced circulating prolactin for the Early (fifteen-minute) group.

<table>
<thead>
<tr>
<th>fixed effects</th>
<th>estimate ± (se)</th>
<th>type III, Wald $\chi^2$</th>
<th>$p$</th>
<th>random effects</th>
<th>variance ± sd</th>
</tr>
</thead>
<tbody>
<tr>
<td>intercept</td>
<td>0.310 (0.027)</td>
<td></td>
<td></td>
<td></td>
<td>0.0361 ± 0.0601</td>
</tr>
<tr>
<td>Baculum Size</td>
<td>0.048 (0.046)</td>
<td>1.07</td>
<td>0.30</td>
<td>♂ Family</td>
<td>0.0601</td>
</tr>
<tr>
<td>PC1</td>
<td>0.015 (0.013)</td>
<td>1.43</td>
<td>0.23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>♀ Background</td>
<td>0.046 (0.036)</td>
<td>1.71</td>
<td>0.19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baculum Size × PC1</td>
<td>0.300 (0.024)</td>
<td>1.54</td>
<td>0.21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baculum Size × ♀ Background</td>
<td>0.096 (0.071)</td>
<td>1.85</td>
<td>0.17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC1 × ♀ Background</td>
<td>0.017 (0.019)</td>
<td>0.76</td>
<td>0.38</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baculum Size × PC1 × ♀ Background</td>
<td>0.019 (0.043)</td>
<td>0.18</td>
<td>0.67</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table S4.5. Linear mixed model (LMM) of the effect of baculum shape (RW1), male sexual behaviour (PC1), female genetic background and their interaction on copulation induced circulating prolactin for the Late (seventy-five-minute) group.

<table>
<thead>
<tr>
<th>fixed effects</th>
<th>estimate ± (se)</th>
<th>type III, Wald $\chi^2$</th>
<th>$p$</th>
<th>random effects</th>
<th>variance</th>
<th>± sd</th>
</tr>
</thead>
<tbody>
<tr>
<td>intercept</td>
<td>0.445 (0.035)</td>
<td></td>
<td></td>
<td>♂ Family</td>
<td>0.0008</td>
<td>0.0293</td>
</tr>
<tr>
<td>RW1</td>
<td>-2.603 (1.744)</td>
<td>2.22</td>
<td>0.13</td>
<td>♂ Family</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>PC1</td>
<td>0.015 (0.018)</td>
<td>0.72</td>
<td>0.39</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>♂ Background</td>
<td>0.011 (0.053)</td>
<td>0.04</td>
<td>0.83</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RW1 × PC1</td>
<td>1.893 (1.208)</td>
<td>2.45</td>
<td>0.11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>♂ Background × PC1</td>
<td>2.252 (2.969)</td>
<td>0.57</td>
<td>0.44</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC1 × ♂ Background</td>
<td>-0.029 (0.031)</td>
<td>0.87</td>
<td>0.34</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RW1 × PC1 × ♂ Background</td>
<td>-2.152 (1.629)</td>
<td>1.74</td>
<td>0.18</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table S4.6. Linear mixed model (LMM) of the effect of baculum shape (RW2), male sexual behaviour (PC1), female genetic background and their interaction on copulation induced circulating prolactin for the Late (seventy-five-minute) group.

<table>
<thead>
<tr>
<th>fixed effects</th>
<th>estimate ± (se)</th>
<th>type III, Wald $\chi^2$</th>
<th>p</th>
<th>random effects</th>
<th>variance ±sd</th>
</tr>
</thead>
<tbody>
<tr>
<td>intercept</td>
<td>0.455 (0.032)</td>
<td></td>
<td></td>
<td>♂ Family</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>RW2</td>
<td>-0.752 (2.049)</td>
<td>0.13</td>
<td>0.71</td>
<td>♀ Family</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>PC1</td>
<td>0.012 (0.017)</td>
<td>0.46</td>
<td>0.49</td>
<td></td>
<td></td>
</tr>
<tr>
<td>♀ Background</td>
<td>0.0001 (0.050)</td>
<td>0.0001</td>
<td>0.99</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RW2 × PC1</td>
<td>2.079 (1.069)</td>
<td>3.79</td>
<td>0.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RW2 × ♀ Background</td>
<td>4.377 (3.242)</td>
<td>1.79</td>
<td>0.18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC1 × ♀ Background</td>
<td>-0.037 (0.028)</td>
<td>1.81</td>
<td>0.18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RW2 × PC1 × ♀ Background</td>
<td>-3.242 (2.099)</td>
<td>2.38</td>
<td>0.12</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table S4.7. Linear mixed model (LMM) of the effect of baculum size, male sexual behaviour (PC1), female genetic background and their interaction on copulation induced circulating prolactin for the Late (seventy-five-minute) group.

<table>
<thead>
<tr>
<th>fixed effects</th>
<th>estimate ± (se)</th>
<th>type III, Wald $\chi^2$</th>
<th>$p$</th>
<th>random effects</th>
<th>variance</th>
<th>± sd</th>
</tr>
</thead>
<tbody>
<tr>
<td>intercept</td>
<td>0.448 (0.034)</td>
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<td></td>
<td>♀ Family</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Baculum Size</td>
<td>-0.004 (0.066)</td>
<td>0.004</td>
<td>0.95</td>
<td>♀ Family</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>PC1</td>
<td>0.008 (0.018)</td>
<td>0.19</td>
<td>0.66</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>♀ Background</td>
<td>-0.001 (0.051)</td>
<td>0.0002</td>
<td>0.99</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baculum Size × PC1</td>
<td>-0.052 (0.028)</td>
<td>3.47</td>
<td>0.06</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baculum Size × ♀ Background</td>
<td>0.019 (0.107)</td>
<td>0.03</td>
<td>0.85</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC1 × ♀ Background</td>
<td>-0.030 (0.029)</td>
<td>1.06</td>
<td>0.31</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baculum Size × PC1 × ♀ Background</td>
<td>0.041 (0.053)</td>
<td>0.59</td>
<td>0.44</td>
<td></td>
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</tr>
</tbody>
</table>
EPILOGUE
Animal genitalia are recognised as being among the most highly variable and evolutionary divergent of all animal structures. The mammalian baculum is a classic example, and its evolution remains one of the most puzzling enigmas of mammalian morphology (Eberhard 1985; Stockley 2012). There is a wealth of literature on potential mechanisms that may be responsible for genital evolution and more specifically, the evolution of male genitalia (Simmons 2014). However, there is a wide disparity in the number of studies on invertebrate and vertebrate genital evolution. For the few studies that have examined the evolution of the mammalian baculum, evidence for a role for sexual selection is mixed (Lüpold et al. 2004; Miller and Nagorsen 2008; Ramm et al. 2009; Schulte-Hostedde et al. 2011; Brindle and Opie 2016). Nevertheless, previous studies on house mice have provided strong support for the role of post-copulatory sexual selection in baculum evolution (Stockley et al. 2013; Simmons and Firman 2014; Schultz et al. 2016a).

Evidence for the role of sexual selection in the evolution of the house mouse baculum comes from studies conducted on natural populations of house mice (Simmons and Firman 2014). By sampling, geographically isolated populations of wild mice found on offshore islands along the west coast of Western Australia, Firman and Simmons (2008b) identified variation in baculum shape that persisted after three generations of common-garden rearing and was associated with population variation in the level of post-copulatory sexual selection. Male house mice from populations with high rates of mixed paternity within litters, a direct measure of the strength of post-copulatory sexual selection, had a relative wider baculum than mice from populations experiencing low rates of mixed paternity litters. To unequivocally confirm the role of sexual selection in the evolution of the house mouse baculum, the same investigators maintained experimental populations under two regimes of post-copulatory sexual selection (with or without selection) for 27 generations and found that sexual selection resulted in a
significant divergence in baculum morphology which was consistent with the variation seen among natural populations of mice; males from populations evolving under post-copulatory sexual selection (polyandry) evolved to have a relatively wider baculum than males from populations evolving without selection (monogamy) (Simmons and Firman 2014). Consistent with these findings, independent evidence of sexual selection came from Stockley et al. (2013) who demonstrated that variation in baculum morphology contributed to a male’s reproductive success in free-ranging populations. Specifically, the authors found evidence of selection for baculum width (Stockley et al. 2013).

More recently, Schultz et al. (2016) used inbred strains of laboratory house mice to identify candidate genes associated with baculum morphology (Schultz et al. 2016a). In this study, the authors showed that three quantitative trait loci determined the size and shape of the house mouse baculum, two accounting for the variation in baculum size and one accounting for variation in baculum shape (Schultz et al. 2016a). Taking into account the genome position of the quantitative trait loci, the authors proposed 16 candidate genes that might contribute to variation in baculum morphology. Furthermore, by crossing different inbred strains of laboratory mice, the authors provided evidence for significant additive genetic variance and heritability of the baculum among these highly inbred laboratory strains (Schultz et al. 2016a).

Collectively, the results from studies of house mice suggest that baculum morphology contributes to male fitness and can evolve in response to post-copulatory sexual selection. However, the precise mechanism by which baculum morphology affects male fitness remains unknown. Thus, in my thesis, I conducted a series of experiments to explore the selective mechanism behind the evolution of the house mouse baculum.

In Chapter 1, I have provided experimental evidence for socially mediated phenotypic plasticity in baculum morphology, re-enforcing support for its role in post-copulatory sexual selection. In Chapter 2, I found significant additive genetic variation in baculum
morphology in a natural population of house mice. Importantly, for the first time in any mammal, I provide evidence consistent with the coevolution of the baculum with female reproductive tract morphology: vaginal cervix width was found to harbour significant additive genetic variance that was genetically correlated with baculum width. In Chapter 3, I showed that baculum shape influences male reproductive success, but only in a competitive context. Interestingly, the effect of baculum morphology on male fitness was dependent on the breeding value for baculum shape of the family from which females were derived. This novel insight lends further support for the potential role of cryptic female choice in the coevolution of female and male genital traits and is consistent with the stimulation hypothesis for mammalian genital evolution. Finally, I test this idea in Chapter 4. I found that female house mice experience a mating-induced release of Prolactin that it is dependent both on the morphology of the mating male’s baculum and his behaviour during copulation, providing support for the stimulation hypothesis for the evolution of the mammalian baculum. Together, my results provide novel insight into the selective mechanism(s) underlying the evolution of the mammalian baculum and provide promising avenues for future research.

As is true of any research endeavour, my conclusions come with caveats and raise many new questions. I found that baculum shape covaries with the width of the genital cervix. While female cervix width may exert selective pressure on baculum morphology, it seems highly unlikely to be the only trait involved. Baculum shape and not size have shown to covary with the strength of selection from sperm competition (Simmons and Firman 2014) and to exhibit developmental plasticity in response to a potential risk of sperm competition (André et al. 2018). Moreover, it was baculum width and not length that was found to influence male reproductive success (Stockley et al. 2013). During copulation, variation in baculum width would mainly exert its effect on the vaginal walls rather than the cervix. The female vaginal tract measurements I made fail to
capture the possible extent of intersexual correlation between male and female genitalia. More comprehensive measurements of the female tract are necessary. Vaginal casts might have allowed me to extract greater detail in vaginal shape and how it relates to variation in baculum shape. However, although I attempted to obtainment three-dimensional casts of reproductive tracts, this unfortunately proved impossible. The vaginal tract is susceptible to a wide range of variation due to the oestrous stage, with changes of contractility of the smooth muscle that surrounds the vaginal tract, vaginal blood flow and vasocongestion (Giraldi et al. 2002; van Helden et al. 2017). The dissection of females at different stages of their oestrous cycle led to intrinsic variations in their reaction to the injected resin (Bateson #17 resin). Not only did variation in contractility of the smooth muscle lead to variation in muscle distention and so the insertion of resin, but also the resin being exothermic resulted in variation in further muscle distention. Thus, my attempt at casts generated high levels of measurement error and the castes proved unreliable for quantitative estimates of natural vaginal shape. In future studies, females should be dissected at the receptive stage of the cycle where we can infer the “environment” to which the male penis would be exposed and find a methodology, perhaps using silicone, to avoid exothermic reactions. Even if three-dimensional measures of vaginal shape had been possible, I suspect that these would have been unlikely to have provided much more insight into male and female genital coevolution than did my simple linear measurements used in Chapter 2 because they cannot fully address the issue of genital stimulation.

Mammalian copulation includes behaviours designed to stimulate the female reproductive tract (Eberhard 1996), and copulation is known to lead to a cascade of physiological events that prime the female reproductive tract for embryo implantation and successful pregnancy (Argiolas and Melis 2013; Pavličev and Wagner 2016). Therefore, the ability of females to discern genital stimulation given by potential sires is
a plausible mechanism by which selection on male genitalia operates in mammalian species (Eberhard 1996; Hosken and Stockley 2004; Brennan and Prum 2015). The vaginal tract is widely innervated and susceptible to regional, temporal and mating experience differences (Barry et al. 2017; van Helden et al. 2017). Variation in the distribution and/or quantity of nerve endings in the female reproductive tract may lead to differences in their ability to perceive stimulation. Future studies of the coevolution of mammalian genitalia should, therefore, investigate the sensory physiology of the female vaginal tract rather than its simple morphology. Female vaginal tract histology would allow us to establish patterns of vaginal innervation throughout the vaginal walls and cervix, and to check for correlations between baculum shape and regions of higher innervation. A comprehensive study would thus investigate both the morphology and sensory physiology of the vaginal tract. Micro-computed tomography might be a useful approach here as it would not only provide us with a three-dimensional perspective of the reproductive tract but would also, with the use of radiolabelled antibodies to neuronal cells, reveal the pattern of vaginal innervation across the entire vaginal tract. Three-dimensional microCT imaging of male and female genitalia during copulation would also provide greater insight into how the various regions within the vagina interact with the baculum.

My finding of a mating-induced elevation in Prolactin provides some support for the stimulatory capacity of the house mouse baculum. However, more detailed studies are now needed to examine the full extent of the effect of baculum morphology and copulatory behaviour on the female’s neuroendocrine system. Future studies should use multiple blood sampling per female. With multiple blood sampling by cannulation, we could infer whether the acute release of Prolactin occurs at different points after ejaculation for females that mated with males with different baculum morphologies and that delivered different amounts of copulatory behaviour. The ability to induce quicker
peaks of Prolactin after ejaculation may lead to variation in the onset of uterine fluid flow and consequently lead to variation in sperm transportation rates (Miki and Clapham 2013). Furthermore, the analysis of brain activation and the release of other hormones such as oxytocin, progesterone and luteinising hormone would provide a broader picture of the stimulatory role of the mammalian baculum. During mammalian copulation, various brain regions are activated (Georgiadis et al. 2012). Studies in rodents show that different brain regions are associated with different aspects of sexual behaviour (Veening and Coolen 1998; Argiolas 1999) and that the activation of these regions is related to the amount of stimulation received during copulation (Coolen et al. 1996). Thus, an analysis of how baculum morphology coupled with copulatory behaviour induces variation in brain activation would provide more robust evidence of the stimulatory role of the baculum.

My data do not allow me to comment on the precise mechanism of sexual selection most likely to lead to the evolution of the mammalian baculum; cryptic female choice versus sexual antagonistic coevolution. My results in Chapter 3 re-enforce the role of post-copulatory sexual selection acting on the baculum because baculum shape only affected male fitness when females mated with two males. In rodents, Prolactin is involved in a multitude of physiological processes (Freeman et al. 2000; Ben-Jonathan et al. 2008) with vagino-cervical stimulation inducing an acute release of Prolactin (Erskine 1995). Acute release of Prolactin is only observed when females enter pseudopregnancy and it has been linked with the rewarding system of sexual behaviour and the establishment of sexual satiety (Convey et al. 1971; Kamel et al. 1975; Bronson and Desjardins 1982; Exton et al. 2001; Leeners et al. 2013). Moreover, in rodents, Prolactin is involved in the maintenance of the corpus luteum and progesterone production, that is necessary for the embryo implantation (Erskine 1995). Thus, selection may act on the baculum via its role in pregnancy initiation or in inducing
sexual satiety in the female. However, given that baculum morphology did not affect the number of embryos implanted or early embryo viability following monogamous mating, I argue that selection on the baculum most likely acts via its stimulatory effects on the duration of sexual satiety and/or sperm transportation. Thus, I would argue that both cryptic female choice and sexual conflict are likely to be involved in the evolution of the mammalian baculum via male fertilisation success. Under a cryptic female choice scenario, females may bias paternity according to variation in the stimulation received by rival males. In this way, females could exert selection for the evolution of baculum shape and its stimulatory abilities. Male stimulatory abilities may induce differential rates of increase in the uterine fluid release after mating and affect sperm transport through the female reproductive tract and subsequent fertilisation success (Miki and Clapham 2013). Stimulation may also induce female sexual satiation and increase the time between successive matings for females, potentially reducing the benefits that females gain from polyandry (Firman and Simmons 2008c,b). The role of Prolactin in the establishment of sexual satiety is not unequivocal (Drago 1984). In the literature, we found conflicting reports of the effect of high levels of circulating Prolactin in female rats with both the facilitation as well as the inhibition of lordosis behaviour (Dudley et al. 1982; Drago 1984). Human studies report inhibition of sexual wanting in females with high levels of Prolactin (hyperprolactinemia) (Krüger et al. 2005; Leeners et al. 2013). Both human and non-human animal studies show effects of circulating levels of Prolactin that are not observed immediately after copulation (Drago 1984; Egli et al. 2010) and so, it is necessary to be cautious in claiming a connection between hyperprolactinemia and sexual satiety.

Distinguishing between cryptic female choice and sexual conflict mechanisms of sexual selection will require carefully designed experiments that will allow us to infer whether stimulation by the baculum generates fitness costs to female reproductive success.
Under sexually antagonistic coevolution, the reproductive interests of males and females are thought to be in conflict, leading to an arms race for the control of reproduction (Chapman et al. 2003; Parker 2006; Brennan and Prum 2015). Female house mice are receptive for approximately five hours (Bronson 1979) and have been shown to benefit from accepting multiple matings (Firman and Simmons 2008b,c). Male interests lie in siring the maximum number of offspring per litter, so if male stimulation leads to establishing sexual satiety in the female and/or inducing an increased rate of sperm transportation (Miki and Clapham 2013), it could reduce the risk of sperm competition for males but decrease the benefits that females can gain from polyandry, generating sexual conflict. A test of the sexual conflict hypothesis would, therefore, involve determining whether females that have copulated with males that have a wide baculum have a reduced mating frequency during their receptive period, and a subsequent reduction in their reproductive success, compared with females that have copulated with males that have a narrow baculum. An alternative approach might be to test whether females develop resistance to stimulation by reducing the neuro-endocrine response to stimulation or by evolving a neuro-endocrine negative feedback to stimulation. To counter-act a possible exploitation of female sensory responses, females may develop resistance to sexual stimulation by a reduction in the increase of prolactin when stimulated over their sensory threshold. Even by conducting these experiments, distinguishing the role of cryptic female choice versus sexual conflict would be difficult since, cryptic female choice can generate sexual conflict so that the two mechanisms are inextricably linked (Gavrilets et al. 2001). Indeed, mathematical models have demonstrated that female choice can arise as a mechanism by which females reduce costs of mating (Gavrilets et al. 2001; Arnqvist and Rowe 2005); even if there is selection for increased resistance, females may accrue indirect benefits from the production of offspring that are more equipped to exploit female sensory bias.
In conclusion, my studies support the role of post-copulatory sexual selection in the
evolution of the house mouse baculum and have provided novel insight into how genital
stimulation may contribute to the evolution of the mammalian baculum.
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