A proteomic study of sperm competition in mammals

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

by

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Declaration of work conducted

All of the work presented within this thesis is my own, except for the following exceptions:

Within Chapter 5 histological processing of testicular tissue was performed by Miss Valerie Tilston (Veterinary Pathology, School of Veterinary Sciences, University of Liverpool). Also within Chapter 5, measurements of testicular architecture from histological sections were collected, under my supervision, by Miss Johanna Kabbert (MSc student, University of Bielefeld). Miss Emily Logie (MSc student, University of Liverpool) collected testes from EM&T Jackson abattoir, Knutsford, and performed some of the measurements of sperm morphology found within Chapter 5, under my supervision.

Abstract

Sperm competition, when sperm from more than one male compete to fertilise the same ova, has driven a diversity of adaptations. Increasingly, molecular techniques have been used to study the effect of post-copulatory sexual selection, including sperm competition, on proteins that are essential to reproduction. Genomic studies have revealed the rapid evolution of ejaculate proteins in polyandrous species. Additionally, there is evidence that gene expression can be altered plastically, in response to cues of sperm competition. Such studies are limited however, as the transcriptome does not always equal the proteome. Here, quantitative proteomics techniques are utilised to explore plasticity in reproduction, at a molecular level, in the house mouse (*Mus musculus domesticus*) (Chapters 2 – 4). In addition, adaptations to sperm competition are considered within mammalian testes and sperm proteins (Chapters 5 and 6).

Contrary to predictions of sperm competition theory, within rodents, sperm production is suppressed in subordinate males. In addition, dominant males develop significantly larger seminal vesicles. Here, quantitative proteomic analysis reveals that the composition of the secretion within the major accessory sex gland in house mice differs according to social status. Subordinate males produce a more concentrated protein secretion, which contains a greater proportion of the protein SVS2. This protein is essential in copulatory plug formation, and increasing the proportion of SVS2 within their seminal fluid may allow subordinate males to produce copulatory plugs of an equivalent size to those produced by dominant.

Within mammals, the oviduct is the site of fertilisation and can exert control over sperm storage and movement. As male house mice can plastically alter their ejaculate production and allocation according to local sperm competition risk, it is

feasible that females may alter the environment within the oviduct in response to similar cues of multiple mating, to maintain control of paternity and reduce the risk of polyspermy. I found no evidence for plasticity in the oviduct proteins of female house mice following either a high or low level of interactions with male house mice. Indeed, the oviduct proteome was more similar within siblings, balanced across treatments, than within treatment groups. I did find evidence for plasticity in female behaviour, when interacting with a novel male, and in female ano-genital distance.

Mothers in populations with high levels of sperm competition may benefit from producing more competitive male offspring. I analysed male offspring produced by female house mice that had encountered either a high or low level of male odour and interactions prior to pregnancy. Quantitative proteomic investigation revealed that ejaculate composition differed according to the level of male interaction their mother had encountered before pregnancy. In contrast, I found no evidence for variation in male mating behaviour, reproductive morphology or ejaculate size. This study reveals the potential for maternally driven, subtle alterations in ejaculate composition.

Sperm competition has driven the evolution of testicular architecture. Comparative analyses reveal that relatively larger testes also produce sperm more efficiently. Improved spermatogenesis efficiency is primarily due to a faster rate of each seminiferous epithelial cycle, thereby increasing sperm production rate. This trait is linked to sperm competition, and so sperm competition is selecting for a greater sperm output, primarily by increasing the rate of production.

Sexual conflict and post-copulatory sexual selection drive the rapid evolution of genes involved in reproduction. The reproductive proteomes of relatively closely related species may therefore be very different from each other. Comparative proteomic analysis of cauda epididymal samples from two groups of mammals, rodents and ungulates, reveals broad similarities in the sperm proteome. Closer analysis of proteins known to be involved in sperm – egg interactions suggests these proteins are very divergent, and exhibit a low level of sequence homology.

Chapter 1

General introduction

1.1 Chapter overview

Within this chapter I discuss the theoretical framework underpinning the studies presented in ***Chapters 2 – 6****.*

This thesis broadly investigates adaptations to sperm competition in mammals using modern proteomics methods. Within this chapter a review of relevant literature is presented to contextualise the theoretical background to the experiments I have undertaken. Sperm competition, sexual conflict and cryptic female choice are considered, highlighting evidence for adaptations and phenotypic plasticity in response to these selection pressures. Mammalian ejaculate production and composition is then discussed in some detail, as this is the focus of several chapters.

Finally, the use of proteomics to understand the reproductive proteome is discussed. Proteomics methods are a core technique within this thesis, used for Chapters 2, 3, 4 and 6, and so a thorough introduction is provided.

1.2 Sexual selection

Darwin's (1859) theory of natural selection describes how traits that confer an individual an advantage are more likely to be passed on to future generations: "This preservation of favourable variations and the rejection of injurious variations, I call Natural Selection" (Darwin 1859 pp 81). Sexual selection (Darwin 1871) differs from natural selection as it considers only those traits that act "in exclusive relation to reproduction" (Darwin 1871 pp 256). In general, sexual selection is expected to act more strongly on males than females (Darwin 1871; Bateman 1948; Trivers 1972). This is in part due to males experiencing a greater variance in fertilisation success than females, and the number of offspring a male produces correlating strongly with the number of mates he obtains (Bateman 1948). Furthermore, as females typically invest more than males in offspring production, they are expected to be more selective over their mates (Trivers 1972). These later ideas support Darwin's original paradigm of ardent males and coy, selective females (Darwin 1871). More recently, flaws in the Darwin-Bateman paradigm have been under discussion (Dewsbury 2005). The traditional interpretation of sexual selection regarding indiscriminate males and choosy females is considered too simplistic; however it holds that sexual selection is generally directional, and the sex encountering the higher cost through mating and offspring production will be the most choosy (Kokko and Monaghan 2001; Clutton-Brock 2007). Indeed, there are many examples within which there is stronger intrasexual competition between females than males (Andersson 1994; Dongen, Matthysen *et al.* 1998; Wong and Jennions 2003; Bro-Jørgensen 2007b; Kraaijeveld, Kraaijeveld-Smit *et al.* 2007; Clutton-Brock 2009).

Mechanisms of sexual selection broadly fall into two categories. Either intrasexual, competition between one sex for access to the other sex, or intersexual, attraction

by one sex to the other (Andersson 1994). As discussed above, this selection may act in either direction (Clutton-Brock 2007). Here, for simplicity, I will give examples whereby males are under greater selection. Male weaponry is a classic result of intrasexual competition, in addition to improving a males fighting ability these often serve as a reliable indicator of male quality and fitness (Clutton-Brock 1982; Kodric-Brown and Brown 1984; Malo, Roldan *et al.* 2005). There is a vast array of male traits that a female may use to select a high quality mate, including status, size, odour cues, vocal signals and visual displays (Searcy 1979; Andersson 1986; Andersson 1994; Clutton-Brock and McAuliffe 2009).

Darwin's discussion of sexual selection stopped firmly at the point of copulation. However, females are commonly polyandrous, mating with more than one male (Smith 1984; Birkhead and Møller 1998). This creates post-copulatory sexual selection, as the sperm from rival males compete to fertilise the same ova and females exert selection over which sperm are successful (Parker 1970; Eberhard and Cordero 1995; Birkhead and Pizzari 2002). This thesis primarily considers adaptations resulting from post-copulatory selection mechanisms (sperm competition, cryptic female choice, and sexual conflict) and so these will be discussed in greater detail.

1.2.1 Post-copulatory sexual selection

Parker's (1970) investigation of sperm competition within insects marked the beginning of a revolution in the field of sexual selection (Parker and Birkhead 2013). Polyandry is now known to be widespread across all taxa. Females benefit from mating multiply; gaining access to territory, nuptial gifts, reducing the chance of genetic incompatibility and increasing offspring production (Zeh and Zeh 1996; 1997; Arnqvist and Nilsson 2000; Jennions and Petrie 2000; Hosken and Stockley 2003). Following multiple mating, the sperm of more than one male compete to fertilise an ovum; this is known as sperm competition (Parker 1970). Post-copulatory selection by the female, the counterpart to sperm competition, is termed cryptic female choice (Thornhill 1983). Females are expected to adapt in order to maintain control over the paternity of their offspring when mating multiply (Eberhard 1996). Sperm

competition and cryptic female choice select for traits that confer an advantage to the male or the female. Often, the two sexes will have divergent evolutionary interests, creating sexual conflict (Parker 1979; Chapman, Arnqvist *et al.* 2003; Arnqvist and Rowe 2005). Adaptations to post-copulatory sexual selection are shaped by a combination of sperm competition, cryptic female choice, and sexual conflict (see Figure 1.1). These are discussed in more detail, within the context of this thesis, below.

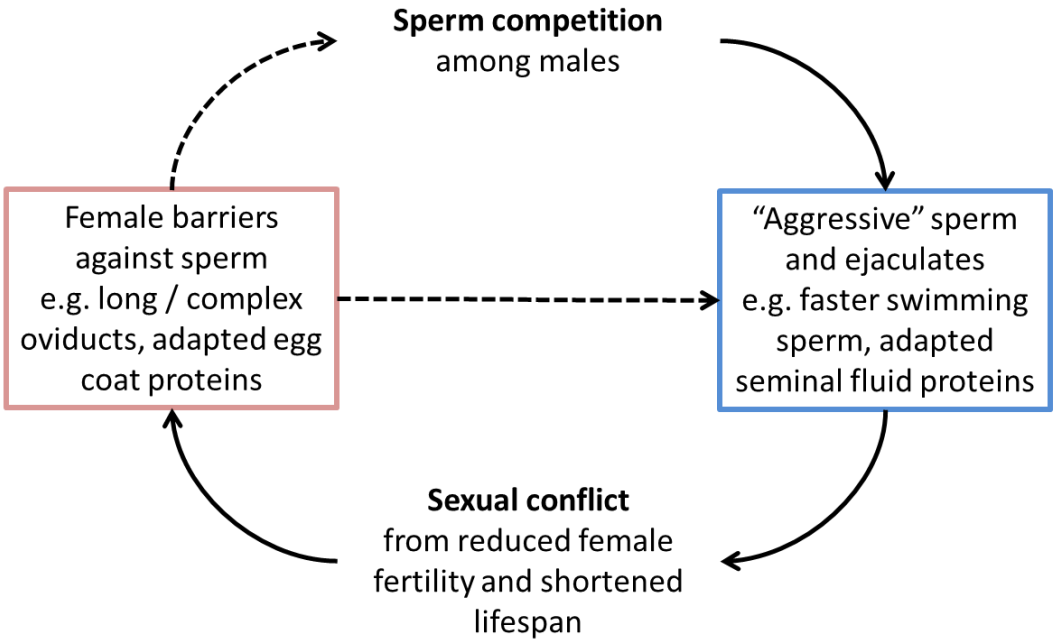
1.3 Ejaculates and sperm competition

Sperm competition is by far the most well studied mechanism of post-copulatory sexual selection. Diverse adaptations to male reproductive anatomy, physiology, and behaviour have evolved following selection via sperm competition (Smith 1984; Birkhead and Møller 1998; Dixson and Anderson 2004). These adaptations are generally either defensive, reducing the chance a female will mate multiply, or offensive, ensuring their ejaculate is more competitive (Parker 1970). A detailed discussion of all documented adaptations to sperm competition is beyond the scope of this thesis. Here, I focus on the ejaculate, particularly citing mammalian examples where possible.

1.3.1 Sperm

Since Leeuwenhoek's 1677 discovery of "animalcules" within ejaculates (Gilbert 2014), our understanding of the structure and function of sperm has changed drastically. Sperm can be roughly divided into two segments; a head and tail. Each sperm head contains a haploid nucleus and is capped by an acrosome. Prior to fertilisation, sperm shed the acrosome, revealing proteins that can bind to the zona pellucida (egg outer coat) and the oolemma (oocyte membrane) (Gilbert 2003). The sperm tail includes the mitochondria containing mid-piece and the flagellum, which propels the sperm. The sperm flagellum is a highly organised structure, consisting of tubulin, outer dense fibre proteins and A-kinase anchor proteins

Figure 1.1: The complex interplay between male adaptations to sperm competition and female counter-adaptations due to sexual conflict. Sperm competition selects for aggressive ejaculate traits. In response, females evolve barriers to avoid polyspermy and infertility (solid lines). Female barriers, in turn, alter the fitness effects of the male adaptations to sperm competition (dashed lines). Adapted from Arnqvist and Rowe 2005 (p123).



(Gilbert 2003; Gupta 2005). A comparative investigation of mammalian sperm proteins is presented within Chapter 6.

Although the major components of sperm are broadly conserved, the specific morphology of sperm is known to vary adaptively according to sperm competition levels (see Birkhead, Hosken *et al.* 2009 for a review of sperm adaptations). Muroid rodents in particular are known for the apical hooks present on their sperm heads (see Figure 5.2)(Breed 2005). These are more defined in species that have greater levels of polyandry (Immler, Moore *et al.* 2007; Šandera, Andrlíková *et al.* 2011). The hooks are predicted to increase swimming speed as the sperm can bind together to form sperm trains (Moore, Dvorakova *et al.* 2002). In the highly promiscuous species of deer mouse, *Peromyscus maniculatus*, sperm preferentially bind with other sperm from that male (Fisher and Hoekstra 2010). This even occurs when tested with sperm from a sibling male, highlighting how sophisticated preferential aggregation of sperm can be in highly polyandrous species (Fisher and Hoekstra 2010).

As well as adaptations to sperm morphology, sperm competition has led to differences in sperm length. A classic example of extreme diversity within reproductive biology is the 6cm long sperm found within *Drosophila bifurca* (Pitnick, Spicer *et al.* 1995). Within this species females are unwilling to re-mate unless they have previously mated with a sperm depleted male (Luck and Joly 2005). *D. bifurca* is an extreme example but highlights a trade-off between sperm size and sperm number. In species with multiple mating and sperm competition, males can gain an advantage by ejaculating a greater number of sperm (the raffle principle described in Section 1.3.1). It is therefore predicted that males could minimise sperm size in order to maximise sperm numbers, and this may be important in the evolution and maintenance of anisogamy (Parker 1982).

Empirical evidence for the influence of sperm competition on sperm size has been the subject of much dispute in recent years. Many studies have found no evidence for a link between sperm competition and sperm size (Briskie and Montgomerie 1992; Gage and Freckleton 2003; Ramm and Stockley 2010; Vrech, Olivero *et al.*

2014), although some find that sperm competition promotes the production of larger sperm (Briskie, Montgomerie *et al.* 1997; LaMunyon and Ward 1999; Tourmente, Gomendio *et al.* 2011a; Tourmente, Gomendio *et al.* 2011b). Within mammals, it has been suggested that metabolic constraints limit the capacity for sperm to increase greatly. When taking the metabolic rate into account within the analysis, sperm size is found to be larger in polyandrous species (Gomendio, Tourmente *et al.* 2011; Tourmente, Gomendio *et al.* 2011b; delBarco-Trillo, Tourmente *et al.* 2013). Larger sperm have a greater swimming velocity (Tourmente, Gomendio *et al.* 2011a) and advantages gained by producing larger sperm may explain the disparity between this result and initial predictions which do not take this into account (Parker 1982; 1993). An analysis of mammalian sperm morphology is presented within Chapter 5.

1.3.2 Sperm production

As sperm are under such strong selection, it is unsurprising that the sperm producing organs, the testes, have also adapted due to polyandry. Under the theory of a fair raffle, whereby all sperm have equal chances of fertilising the ova (Parker 1990), males may be selected to increase the quantity of sperm within an ejaculate in order to have a greater chance of fertilisation success. Increased relative testis mass, presumably to produce greater quantities of sperm, is a well-documented adaptation to sperm competition (e.g. mammals; Kenagy & Trombulak 1986, and fish; Stockley, Gage *et al.* 1997) and is often used as an indicator of mating system (for example in comparative studies of rodents; Ramm, Parker *et al.* 2005). Intraspecific studies have provided further evidence for this as the frequency of multiple paternity correlates with testes mass in isolated wild populations of house mice (Firman and Simmons 2008).

Further to sperm competition increasing testes mass, more recently there has been interest into the evolution of testis architecture (Ramm and Schärer 2014). Sperm production is supported by the Sertoli cells within the basement membrane of the seminiferous tubules. These specialised cells envelope the developing gametes,

providing essential nutrients, physical support and paracrine signals (Jégou 1992). The quantity of Sertoli cells is reasonably static after puberty (Kluin, Kramer *et al.* 1984; Herrera-Alarcón, Villagómez-Amezcua *et al.* 2007). This can therefore limit sperm production capacity as they can support the development of a limited number of germ cells (Johnson, Carter *et al.* 1994). Studies in New World Blackbirds (*Icteridae*) have found the testes of males with more competitive mating systems contain less interstitial tissue (Lüpold, Linz et a. 2009; also in *Mus* species, Montono, Arregui *et al.* 2012) and more efficient Sertoli cells (Lüpold, Wistuba *et al.* 2011), allowing for more efficient sperm production. Interestingly this has also been shown as an adaptive variation within a species, the marine flatworm *Macrostomum lignano*, with larger testes producing more sperm per unit area (Scharer and Vizoso 2007).

In addition to changes in testicular architecture, males of species with more competitive mating systems produce sperm at a faster rate, due to having a shorter spermatogenic cycle (Ramm and Stockley 2010; delBarco-Trillo, Tourmente *et al.* 2013) (additional details on mammalian sperm production found within Chapter 5). Therefore, mammalian testes evolving under sperm competition are relatively larger and produce sperm at a greater rate (Ramm and Stockley 2010). Furthermore, they produce a greater proportion of normal, acrosome intact sperm with increased motility (Gómez Montoto, Magaña *et al.* 2011). Further investigation of testicular architecture and sperm production can be found within Chapter 5.

1.3.3 Sperm competition games models

Sperm competition games models offer extensive predictions for a male's optimal sperm investment within differing roles and contexts (Parker 1990; Parker, Ball *et al.* 1997; Parker and Pizzari 2010; Parker, Lessells *et al.* 2013). These models seek to predict the evolutionarily stable strategy (ESS) of reproductive investment using an evolutionary games theory approach (Maynard Smith 1982), hence sperm competition games (Parker 1998). Predictions have been provided for various scenarios, for example in relation to the risk or intensity of sperm competition

(Parker, Ball *et al.* 1996; 1997), female mating status (Ball and Parker 2007), and sperm selection by females (Ball and Parker 2003). A full consideration of the complexities of these models is beyond the scope of this thesis, however, the experimental work presented within Chapter 2 is based on the "raffles and roles" models, and so these are discussed here in more detail.

The simplest model considers a fair raffle, in which the sperm of two competing males each have an equal chance of winning fertilisations. In this instance the male that ejaculates the most sperm has the greatest chance of siring offspring, similar to buying more tickets in a raffle (Parker 1990). However it is also possible that one male may have an advantage under sperm competition, in which case the situation is more similar to a loaded raffle. This may occur for example when the order of mating influences the outcome of sperm competition, or if one male is preferred by the female. Theory predicts that the number of sperm ejaculated by males mating in different roles will depend upon knowledge of their role within the loaded raffle, either favoured or disfavoured, and whether these roles can vary or are fixed. In a non-random raffle, whereby the roles are fixed, theory predicts that the male in the disfavoured role could increase sperm output to compensate for the lower value of his sperm (Parker 1990; Ball and Parker 2003).

The non-random roles model of optimising investment in sperm production is particularly relevant for species with social dominance relationships. In this system males should 'know' their role within a competitive mating: favoured (dominant) or disfavoured (subordinate). Empirical studies comparing dominant and subordinate males of non-mammalian species support these predictions, as subordinate males produce and ejaculate more sperm than dominant males (Liljedal and Folstad 2003; Pizzari, Cornwallis *et al.* 2003); however evidence from rodents suggests the contrary (Koyama and Kamimura 2000; Lemaitre, Ramm *et al.* 2012; Schradin, Eder *et al.* 2012). This could be due to physiological constraints of the subordinate male being in a state of chronic stress (Blanchard, Sakai *et al.* 1993), or they may suppress sperm production to appear less competitive to the dominant male. As rodents have

prominent accessory sex glands, which are larger with increased sperm competition (Ramm, Parker *et al.* 2005; Lemaitre, Ramm *et al.* 2010), the importance of non-sperm ejaculate components may be particularly relevant within these species.

1.3.4 Seminal fluid

As sperm cells are essential for fertilisation, it is understandable that they have garnered a lot of attention. However, ejaculates are a complex mixture and there is growing interest in ejaculate composition in relation to the outcomes of post-copulatory sexual selection (Chapman 2001; Perry, Sirot *et al.* 2013). Components of the seminal fluid have diverse functions such as increasing sperm motility (Kawano and Yoshida 2007), altering female behaviour to reduce the chance she will remate (Mane, Tompkins *et al.* 1983) and forming a copulatory plug (Williams-Ashman 1984; Dewsbury 1988; Dixson and Anderson 2002). In mammals, components of the seminal fluid can initiate ovulation (Waberski, Claassen *et al.* 1997) and assist the movement of sperm through the reproductive tract by increasing uterine contractions (Suarez and Pacey 2006).

Copulatory plugs are a commonly found adaptation to sperm competition (Devine 1975; Shine, Mason *et al.* 2000; Dixson and Anderson 2002; Timmermeyer, Gerlach *et al.* 2010). They increase the movement of sperm into the reproductive tract, act as a slow releasing sperm store, and provide a physical barrier reducing the chance of re-mating (Smith 1984; Carballada and Esponda 1992; Shine, Mason *et al.* 2000; Dean 2013). The plug can be formed through the coagulation of seminal fluid components (Voss 1979; Smith 1984; Simmons 2001), emasculation of male genitals (often found in spiders e.g. Fromhage and Schneider 2006) or coagulation of kidney products (common in snakes e.g. Devine 1975). Interestingly, there is evidence for a female derived copulatory plug, which can prevent unwanted copulations (Kuntner, Gregoric *et al.* 2012). More commonly, copulatory plugs are male derived and predicted to limit the rate of female re-mating (Parker 1970). There is some empirical evidence for this (*Cavia porcellus*: Martan and Shepherd 1976, *Agelena limbata*: Masumoto 1993, *Drosophila hibisci*: Polak, Wolf *et al.* 2001).

Theory predicts the evolution of plug displacement will be common (Fromhage 2012) and there are many examples whereby the copulatory plug is ineffective at reducing female re-mating (Mosig and Dewsbury 1970; Dewsbury and Baumgardner 1981; Jia, Duan *et al.* 2002; Moreira and Birkhead 2003; Parga 2003; Timmermeyer, Gerlach *et al.* 2010). Plugs may be removed or displaced by males (Mosig and Dewsbury 1970; Hartung and Dewsbury 1978; Timmermeyer, Gerlach *et al.* 2010) or females (e.g. *Sciurus carolinensis:* Koprowski 1992) and are often consumed by either sex (Mosig and Dewsbury 1970; Koprowski 1992). Therefore, chastity enforcement is unlikely to be a widespread function of the copulatory plug. Instead, there is increasing evidence that copulatory plugs commonly improve fertility through aiding sperm retention and transit within the female tract (Dewsbury 1988; Carballada and Esponda 1992; Jia, Duan *et al.* 2002; Ramm, Parker *et al.* 2005; Dean 2013).

Within primates and rodents, the seminal proteins forming the plug have been reasonably well studied. These proteins are known as the semenoclotins in primates. In rodents the major components of the seminal fluid are the seminal vesicle secretory (SVS) proteins I to VII, named in order of descending size. The semenoclotins I & II, and SVS proteins I, II & III, are known to be a rapidly evolving substrate of transglutaminase (REST) (Lin, Luo *et al.* 2002). Catalysis of these proteins to coagulate and form the copulatory plug is initiated by the ejaculate specific transglutaminase, protein-glutamine gamma-glutamyltransferase 4 (Tgm4), which is released by the anterior prostate or coagulating gland (Williams-Ashman, Beil *et al.* 1980; Williams-Ashman 1984; Lin, Luo *et al.* 2002; Dean 2013). There is evidence for selection, driven by female promiscuity resulting in sperm competition, on the size of the seminal vesicle glands that produce these proteins, (Dixson 1998; Ramm, Parker *et al.* 2005), the proteins that form the copulatory plug (Jensen-Seaman and Li 2003; Dorus, Evans *et al.* 2004; Clark and Swanson 2005; Carnahan and Jensen-Seaman 2008; Ramm, Oliver *et al.* 2008; Ramm, McDonald *et al.* 2009; Carnahan-Craig and Jensen-Seaman 2014) and the size of the plug (Dixson and Anderson 2002;

Ramm, Parker *et al.* 2005). Thus, the copulatory plug is a widespread adaptation to sperm competition.

1.3.5 Predictions for seminal fluid production

A long-standing theoretical focus on male investment in sperm numbers in the sperm competition literature has shifted recently to include the importance of seminal fluid in predicting fertilisation outcomes (Cameron, Day *et al.* 2007; Perry, Sirot *et al.* 2013; Dhole and Servedio 2014). The model produced by Cameron *et al.* (2007) considers the energy a male will expend on sperm and seminal products respectively and finds this should be different for males in favoured and disfavoured roles, changing with the level at which seminal fluid can alter fertilisation success. This model agrees with previous predictions, suggesting that in all instances the disfavoured male should invest more in sperm than the favoured male (Parker 1990; Cameron, Day *et al.* 2007). As the level of bias created by seminal fluid products increases, by proffering a greater advantage in a competitive mating, it is predicted that the favoured male should invest more in seminal products compared to sperm (Cameron, Day *et al.* 2007). These predictions are tested within Chapter 2.

1.4 Cryptic female choice

As females typically invest more than males in offspring production, they are expected to exert greater control over the quality of their mates (Trivers 1972). When mating multiply, post-copulatory selection of which sperm gain successful fertilisations, or cryptic female choice, can increase a female's control over the paternity of their offspring (Thornhill 1983; Birkhead and Møller 1993; Eberhard 1996). There are diverse post-copulatory mechanisms by which females can exert preference for one male's sperm over another. These include, but are not limited to, sperm ejection, controlling the timing of ovulation or oviposition, soliciting further matings and digesting sperm from previous ejaculations (reviewed in Eberhard 1996).

Observing convincing evidence for cryptic female choice, particularly in relation to specific mechanisms of sperm selection, can be challenging due to the difficulties in disentangling male versus female mediated effects (Birkhead 1998; 2000; Eberhard 2000; Kempenaers, Foerster *et al.* 2000; Pitnick and Brown 2000). Nonetheless, there are some striking examples of sperm selection by females. I will only highlight two here due to limited space. Firstly, although female feral fowl are coerced into copulations with subdominant males, they are able to selectively eject the sperm of subdominant males to increase the proportion of offspring sired by the dominant male (Pizzari and Birkhead 2000). Secondly, Comb jellys (*Beroe ovata*) have an exceptional level of choice over fertilisation within the egg (Carré and Sardet 1984). Polyspermy, when more than one sperm fuse with the egg, is common in this species. At fertilisation, the pronuclei of multiple sperm remain fixed within the egg surface. The pronuclei of the egg then travels around the egg to each of the sperm pronuclei, before choosing which to fuse with (Carré and Sardet 1984). Within rodents, sperm are able to discriminate between sperm from relatives (Fisher and Hoekstra 2010), suggesting there is a molecular mechanism by which sperm can be recognised as belonging to individuals. It is certainly feasible that mechanisms of sperm selection may occur within female reproductive tract of mammals.

1.4.1 Cryptic female choice within mammals

The initial, and most obvious, opportunity for sperm selection occurs promptly after mating as females can eject recently inseminated sperm (Ginsberg and Huck 1989; Manier, Belote *et al.* 2010). Within mammals, sperm that are not immediately ejected travel to the oviduct. This is the final stage of the female reproductive tract that sperm have to negotiate, and the site of fertilisation. Prior to ovulation, sperm can be stored in the sperm storage organs at the bottom of the oviduct before being slowly released as required (Suarez 2008b). The oviduct may also mediate sperm selection (Holt and Fazeli 2010). Molecules within the oviduct cause differential motility in sperm sub-populations (Satake, Elliott *et al.* 2006). Selective proteolysis of zonadhesin, a sperm membrane bound protein that binds to the zona pellucida coating on the egg, creates fragments with differing binding activity levels within pigs

(Gupta 2005). Molecular mechanisms of control over sperm activity within the oviduct are likely to be widespread. At present, knowledge of this is only limited. More broadly, oviduct morphology has also adapted in response to sperm competition. In mammals, oviduct length correlates with sperm numbers (Gomendio and Roldan 1993) and relative testis size (Anderson, Dixson *et al.* 2006), both indicators of sperm competition. Similarly in birds, increased complexity of the female tract co-occurs with increased sperm length (Briskie and Montgomerie 1992) and penis length (Brennan, Prum *et al.* 2007).

The final level of female control is at the gamete level. IVF assays have shown that selection is as strong on ovum defensiveness as it is on sperm competitiveness within closely related *Mus* species (Martín-Coello, Benavent-Corai *et al.* 2009). Within the house mouse, increased ova defensiveness has been shown as both a plastic response to social interactions indicating high or low levels of multiple mating, and a product of coevolution, following experimental evolution of monogamous and polyandrous selection lines (Firman and Simmons 2013; Firman, Gomendio *et al.* 2014).

1.5 Sexual conflict

Sexual conflict occurs when adaptations that confer an advantage in one sex lead to fitness costs or constraints in the other (Parker 1979; Chapman, Arnqvist *et al.* 2003; Arnqvist and Rowe 2005; Parker 2006). Male ejaculates are continually selected by fertilisation success. These adaptations, although beneficial in males, may be detrimental to females (Chapman, Liddle *et al.* 1995; Rice and Holland 1997). As well as the need to counter-act harmful, indeed even toxic (Rice 1996), male adaptations, females will also aim to reduce the chances of embryolethal polyspermy and to ensure the best quality male fertilises her ova (Frank 2000; Gilbert 2000; Arnqvist and Rowe 2005). When coupled with sperm competition, accelerating the evolution of male reproductive traits, and cryptic female choice, driving adaptations within females, this accentuates the rate of antagonistic co-evolution between the sexes

(Rice and Holland 1997; Stockley 1997; Martín-Coello, Benavent-Corai *et al.* 2009) (see Figure 1.1).

1.5.1 Sexual conflict and antagonistic co-evolution

Sexually antagonistic coevolution is driven by divergent genomic optima of the sexes (Rice and Holland 1997). Studies of experimental evolution in insects are increasingly providing evidence for sexually antagonistic coevolution acting on genital morphology, mating behaviour, weaponry, and ejaculate mediated behaviour in females (Rice 1996; Holland and Rice 1999; Arnqvist and Rowe 2002; Hosken and Stockley 2004; Wigby, Chapman *et al.* 2004; Edward, Poissant *et al.* 2014). Long term studies of wild populations of mammals have also been a useful resource for testing sexually antagonistic coevolution. Horn size of soay sheep (*Ovis aries)* is associated with greater longevity in females, but a reduced lifespan for males (Robinson, Pilkington *et al.* 2006). Similarly within the red deer, males with relatively high fitness sire daughters with relatively low fitness (Foerster, Coulson *et al.* 2007). These examples highlight the maintenance of genetic variation in sexually selected traits within wild populations, as often genetic benefits accrued within one sex will be detrimental in the other.

Substances within the seminal fluid are commonly implicit in sexual conflict (Chapman 2001). Within *Drosophila*, seminal fluid proteins (sfps) are known to influence the female after copulation (reviewed in Sirot, LaFlamme *et al.* 2009). Components of the seminal fluid increase egg laying rate, reduce remating rate and shorten the female's lifespan (Chen, Stumm-Zollinger *et al.* 1988; Fowler and Partridge 1989; Chapman, Liddle *et al.* 1995). Evidence for the toxicity of seminal fluid is further provided using experimental evolution; females under monogamous selection lines become less resistant to the toxicity of seminal fluid (Rice 1996; Holland and Rice 1999).

1.5.2 Rapid evolution of reproductive proteins

Molecular evolution is accelerated in situations of co-evolution, due to adaptations being continually counter adapted, for example in host infection selection lines (Paterson, Vogwill *et al.* 2010). Within the reproductive system this relationship is further accelerated as traits are not constrained by survivability as they may be under natural selection (Swanson, Yang *et al.* 2001; Swanson and Vacquier 2002; Turner and Hoekstra 2008). There is evidence for this effect in the proteins of sperm (Dorus, Wasbrough *et al.* 2010; Vicens, Lüke *et al.* 2014; Vicens, Tourmente *et al.* 2014), seminal vesicle products (Dean, Clark *et al.* 2009), female reproductive tracts (Swanson, Wong *et al.* 2004) and oocytes (Swanson, Yang *et al.* 2001). The evolution of reproductive proteins in response to sperm competition has been of growing interest, with comparative studies investigating rodents (SVS II and Protamine 2 Ramm, McDonald *et al.* 2009; Martin-Coello, Dopazo *et al.* 2009) and primates (SEMG2 Dorus, Evans *et al.* 2004; Jenson-Seaman and Li 2003).

The female reproductive proteins are less well studied with reference to molecular evolution; however it is an area that is receiving more interest. Positive selection has been identified as acting on ZP2 and ZP3, proteins directly involved in sperm – egg binding (Swanson, Yang *et al.* 2001; Turner and Hoekstra 2006), and oviductal glycoprotein (OGP) (Swanson, Yang *et al.* 2001). These studies utilise genomic techniques to determine the rate of evolution and have found that glycosylation sites around the functional sperm binding regions of proteins are under the fastest selection. Proteomics techniques could also be used to clarify protein alterations, which may lead to functional differences, within the female reproductive tract. This could give us insights into molecules essential to sperm selection.

1.6 Phenotypic plasticity

Phenotypic plasticity occurs when a genotype is capable of producing variation in the phenotype due to changes in the environment (Via and Lande 1985). This occurs due

to a range of environmental cues, but here I will limit my discussion to those relating to varying levels of sperm competition. I also discuss differences in offspring phenotype due to the maternal environment. Plasticity in ejaculate production and maternal effects are investigated in Chapter 2 and Chapter 4.

1.6.1 Evidence for plasticity in mammalian ejaculate production

In wild populations it is common that sex ratio and population density may fluctuate. In the house mouse this leads to varying levels of multiple paternity within litters (Dean, Ardlie *et al.* 2006). This fluctuation automatically alters the extent of sexual selection due to variation in the numbers of mates, or competitors available. Experimentally changing the perceived number of competitors alters male investment in sperm production in the house mouse (Ramm and Stockley 2009a), and accessory gland size in the bank vole (Lemaitre, Ramm *et al.* 2010). Furthermore, protein turnover within the seminal vesicles of house mice is strikingly fast (Claydon, Ramm *et al.* 2012), showing the potential for plasticity in seminal vesicle protein production. Indeed, *Drosophila* are able to plastically alter the quantity of specific proteins within their ejaculates, according to the level of competition (Wigby, Sirot *et al.* 2009).

Plasticity in reproductive traits may also be caused by variation in social status. In mammals, dominant bank voles ejaculate more sperm and possess larger seminal vesicles than subordinates (Lemaitre, Ramm *et al.* 2012). In fish species that have distinct mating roles, males that sneak copulations increase the speed of ejaculated sperm when paired with a smaller male, possibly by a seminal fluid mediated effect (Smith and Ryan 2011). There is further evidence for this in the cichlid *Telmatochromis vittatus,* which has sneaks, pirates (larger males with sneak behaviour) and territorial males. In this species, ejaculate tactics vary dependant on a male's role at each fertilisation event and territorial males will ejaculate sperm with increased longevity in response to piracy risk (Ota, Heg *et al.* 2010). Similarly, sperm of male grass gobys (*Zosterisessor ophiocephalus*) of differing roles is known to be affected by the seminal fluid of other males (Locatello, Poli *et al.* 2013),

suggesting that seminal fluid proteins are altered according to their social status; however this has not been tested. Plasticity in the production of seminal fluid proteins is investigated in Chapter 2.

1.6.2 Maternal effects

Whilst discussing phenotypic plasticity, I should briefly introduce maternal effects. Maternal effects, in response to environmental cues of the likely level of multiple mating experienced prior to pregnancy, are investigated within Chapter 4.

Maternal effects occur when a mother alters the future characteristics of her offspring, in a manner that is unrelated to the offspring genotype (Bernardo 1996; Wolf and Wade 2009). Altering offspring phenotype to suit current environmental conditions, which are likely to fluctuate naturally, can ensure a greater chance of offspring success (Mousseau and Fox 1998b). There is widespread evidence for maternal effects within diverse taxa, including mammals (Mousseau and Fox 1998b; Ryan and Vandenbergh 2002; Simpson and Miller 2007; Henriksen, Rettenbacher *et al.* 2011; Dantzer, Newman *et al.* 2013).

Rodents are commonly used as a model for maternal effects, particularly in response to nutrition and stress. Maternal nutrition can affect offspring development, metabolism and hormone production (Trombulak 1991; Liang and Zhang 2006; Gluckman, Lillycrop *et al.* 2007; Dantzer, Newman *et al.* 2013). Social and predator stressors during pregnancy causes variation in offspring growth, behaviour, anogenital distance and endocrine function (Pratt and Lisk 1989; Zielinski, Vandenbergh *et al.* 1991; Kaiser, Kruijver *et al.* 2003; Bian, Wu *et al.* 2005; Siegeler, Sachser *et al.* 2011). In addition to maternal effects during pregnancy, maternal grooming behaviour during lactation influences offspring phenotype through epigenetic alterations in the pups response to stress (Weaver, Cervoni *et al.* 2004).

As well as influencing individual offspring, it is hypothesised that females will alter the sex ratio of their litters according to their current quality and nutritional state;

with high quality females gaining more from producing high quality sons (Trivers and Willard 1973). When the measure of female quality is taken at the time of conception, there is evidence for this phenomenon in mammals (Clutton-Brock and Iason 1986; Cameron 2004; Sheldon 2004).

An ability to match offspring phenotype to local conditions may be particularly useful for females within populations that have fluctuations in their density, and so varying levels of competition for resources, territory and mates (Dantzer, Newman *et al.* 2013). In species with multiple mating, variation in populations concurrently alters the incidence of sperm competition (Firman and Simmons 2008). As mothers can respond to social cues and alter their offspring, it is feasible that they may respond to cues of the likelihood of sperm competition to ensure their offspring are competitive. Within Chapter 4 I test for maternal effects on male traits that could influence the outcome of sperm competition.

1.7 Proteomics

Proteomics is the large scale study of the proteins within biological organisms. Technical advances and an increase in the availability of genomic information have resulted in an explosion in the use of proteomic techniques within biological studies.

1.7.1 Common techniques

Mass spectrometry (MS) is commonly used for proteomic analysis (Aebersold and Mann 2003). There are generally five steps required to analyse samples of proteins (briefly discussed here, but see Groß 2011). First, proteins are broken into peptides using a digestive enzyme, this is frequently trypsin. Separation of proteins can occur prior to digestion, using electrophoresis (SDS-PAGE or 2D-PAGE). Alternately, or in addition, peptides can be separated, according to their size and hydrophobicity, using high performance liquid chromatography (HPLC). The peptides are then ionised, this is normally done through electrospray ionisation (ESI) or matrix-assisted laser desorption/ionisation (MALDI). Charged peptides are then able to move within

the vacuum of the MS machine, attracted to the oppositely charged mass analyser. The mass/charge (m/z) ratio of each peptide is measured within the MS machine. Often, tandem MS (or MSMS) is performed. This method incorporates a fragmentation stage. After initial analysis of the precursor ion, this peptide is fragmented and the resulting ions are also analysed. The data obtained from the MS analysis can then be used to identify the proteins within the sample, by matching to a database of predicted peptide masses based on known protein sequences. Although there are various methods available to perform each stage of this procedure, the optimal technique will depend on the aims of the experiment.

Proteomic methods of accurate protein quantification are rapidly improving (Bantscheff, Schirle *et al.* 2007). There is a growing array of available techniques that fall into three categories; labelled, targeted and label-free. A brief comparison of these can be found in Table 1.1. Labelled protocols involve the incorporation of stable isotopes, often 15N or 13C, into a biological sample. This is commonly done within cell cultures, as it is relatively simple to alter the growth media to include only heavy versions of essential amino acids (such as heavy lysine). Stable isotope labelling by amino acids in cell culture (SILAC, Ong, Blagoev *et al*. 2002) is a useful tool for analysing relative differences in protein abundance between cell lines. Multiple amino acids can be used, creating predictable peptide mass shifts within different cultures, which can then be combined for MS analysis. Relative differences in the abundances of specific peptides can then be inferred based on the proportions of peptides at each mass. Similar techniques, based on the ratios of signal intensities between peptides of the same sequence with different masses, include iTRAQ (isobaric tag for relative and absolute quantification), iCAT (isotope coded affinity tag) and metabolic labelling of whole organisms. With respect to mammalian reproduction, metabolic labelling techniques have been useful for characterising the ejaculated proteins (Dean, Findlay *et al*. 2011) and their rates of turnover (Claydon, Ramm *et al.* 2012).

Targeted quantitative proteomics techniques can be used for absolute quantification of specific proteins within a sample. This technique aims to account for the variability in shotgun proteomics techniques by targeting specific peptides for further fragmentation and analysis, this method is commonly known as selected reaction monitoring (SRM, or multiple reaction monitoring, MRM, Lange, Picotti *et al*. 2008). This method requires some preliminary analysis. Firstly, candidate proteotypic peptides for the protein of interest are identified by initial shotgun analysis and computed predictions. These must be specific and unique to the target protein, and must behave consistently during MS analysis. Absolute quantification can then be achieved by adding isotopically labelled synthetic peptides and comparing the relative intensity of the heavy and light peptides (discussed within Lange, Picotti *et al*. 2008).

Within this thesis, label-free techniques are used. Methods of label-free quantitation generally use either spectral counting or peak ion intensity as a measure of protein abundance (Old, Meyer-Arendt *et al.* 2005; Bantscheff, Schirle *et al.* 2007; Patel, Thalassinos *et al.* 2009). Label-free techniques allow complex biological samples to be analysed relatively simply, as there is no need for the addition of labelled isotopes or preliminary analysis of targeted peptides. They have also been shown to be sensitive and accurate, particularly in calculating relative differences in the abundance of proteins within a sample (Old, Meyer-Arendt *et al.* 2005). Label-free methods were chosen as I wanted to measure broad differences in the reproductive proteomes of house mice according to their environmental conditions.

1.7.2 The use of proteomics to study reproductive proteomes

Scientists studying molecular events leading to fertilisation are faced with a challenging environment to study. Transcriptome information is often, at best, a modest indicator of protein expression (Ghazalpour, Bennett *et al.* 2011). This is further complicated by the dynamic environment of the female reproductive tract

Table 1.1: Table comparing common approaches to quantitative proteomic.

Method	Pros	Cons
Stable isotope labelling (e.g. SILAC, iTRAQ, ICAT)	• High-throughput • Relatively sensitive • Potential for relative comparisons of multiple samples	• Labels can be expensive • Analysis can be complex • Better suited to cell cultures
Targeted proteomics (e.g. SRM, MRM)	• Can be absolute • Highly sensitive and reproducible • Suitable for low abundance proteins	• A lot of preliminary work • Low throughput
Label-free methods (e.g. spectral counting, peak intensity comparisons)	• High-throughput • Relatively simple • Effective at finding large differences	• Relative • Less refined/accurate • Less effective for low abundance proteins

throughout the oestrus cycle (Horvat, Vrčić *et al.* 1992; Weidong, Clement *et al.* 1995; Mariani, Souto *et al.* 2000; Seytanoglu, Georgiou *et al.* 2008). In addition, post translational modifications of proteins can affect their activity (Sylvester, Morales *et al.* 1991). The recent advances in proteomics techniques, particularly with regard to quantitative proteomics, have made it an indispensable tool in biological research, including in the field of reproductive science (for a review of common proteomic techniques and their use in reproductive biology studies see Wright, Noirel *et al.* 2012).

Numerous discovery studies have qualified the proteins present in the gametes (for example in sperm: Stein, Go *et al.* 2006; Peddinti, Naduri *et al.* 2008; Asano, Nelson *et al.* 2010; Brewis and Gadella 2010 and Belleannée, Labas *et al.* 2010 for a review including the maternal environment see Arnold and Frolich 2010). This has led to investigations that were not possible with previously available technology. For instance, changes occurring within the porcine oviduct throughout the oestrus cycle (Seytanoglu, Georgiou *et al.* 2008) and in response to gametes (Georgiou, Sostaric *et al.* 2005) have been documented. Further, a comparative approach has been used to describe the seminal plasma of domestic ungulate species (Druart, Rickard *et al.* 2013) and has successfully shown adaptive evolution of ejaculate proteins within rodents (Ramm, McDonald *et al.* 2009).

1.7.3 Proteomics within behavioural ecology

Recently, there has been increasing interest in the application of proteomics within the field of behavioural ecology (Karr 2007; Diz, Martinez-Fernandez *et al.* 2012; Valcu and Kempenaers 2014). It is recognised that mRNA transcripts are not representative of protein abundance (Vogel and Marcotte 2012). Further, measures of the proteome are more likely to be representative of the phenotype than the genome (Diz, Martinez-Fernandez *et al.* 2012) and so, as natural selection occurs at a phenotypic level, protein level adaptations may be missed using other techniques.

The reproductive proteome is particularly exciting to study, due to the prevalence of sperm competition and sexual conflict, and the rapid co-evolution of male and female traits. Proteomics studies have allowed identification of seminal fluid proteins that interact with the female on a molecular level, mediating female behaviour and fertilisation (Wolfner 2007; Marshall, Huestis *et al.* 2009; Laflamme and Wolfner 2013). There is also evidence that males can alter the quantities of specific proteins within their ejaculate, according to the level of competition or female mating history (Wigby, Sirot *et al.* 2009; Sirot, Wolfner *et al.* 2011). Although proteomics methods are used increasingly within this field, there are areas that remain unexplored. This is particularly true within mammals. Phenotypic plasticity within the reproductive proteome of house mice is investigated here within Chapters 2 – 4.

1.8 Thesis overview

This thesis can be broadly considered in two sections. The first, including Chapters 2, 3, and 4, use the house mouse as a model to investigate phenotypic plasticity as an adaptive response to sperm competition risk, in various forms. Chapter 2 considers ejaculate investment according to social status, testing the prediction for the non-random roles model of seminal fluid production. Female plasticity in response to environmental cues of the likelihood of multiple mating is tested in Chapter 3. Effects of the maternal environment prior to pregnancy, indicating high or low multiple mating, on male offspring is investigated in Chapter 4.

The second section of this thesis considers evolutionary adaptations to sperm competition within two groups of mammals; rodents and ungulates. Testes and associated epididymides were collected for a comparison of testicular architecture (Chapter 5) and epididymal proteins (Chapter 6).

Chapter 2
Social status and ejaculate investment in the house mouse.

2.1 Chapter overview

House mice live in a complex social environment. In this chapter I investigate how males of contrasting social status invest in their ejaculate production.

Male social status has been linked to success in sperm competition, with dominant males typically having an advantage over subordinates. Theory predicts that subordinate males should invest more in ejaculates to compensate for this. However, it is currently unknown if both sperm and seminal fluid components of the ejaculate are tailored according to male social status. Here I present results of

quantitative proteomics analyses to compare the ejaculate components of dominant and subordinate male house mice (*Mus musculus domesticus*). The seminal vesicles are substantial accessory sex glands in rodents that secrete the majority of seminal fluid proteins, including those that coagulate inside the female to form the copulatory plug. My proteomic analysis of seminal vesicle fluid reveals that subordinate male mice produce proportionally more SVS2, a protein essential in copulatory plug formation. Despite having significantly smaller seminal vesicle glands, and so less total protein available, subordinate males also produce copulatory plugs of equivalent size to those of dominant males. Since dominant males produce and ejaculate more sperm than subordinates, increased investment in SVS2 production may help subordinate males to limit an otherwise strong disadvantage under sperm competition. My findings thus reveal that males invest differently in both sperm and seminal fluid components of the ejaculate according to their social status, with important implications for competitive outcomes.

2.2 Introduction

Ejaculates, a complex mixture of sperm and seminal fluid components, are costly to produce (Dewsbury 1982). Males are therefore expected to invest prudently in ejaculates according to their resources, opportunities and likely success in sperm competition (Tazzyman, Pizzari *et al.* 2009; Parker and Pizzari 2010). Optimal sperm investment strategies have been predicted extensively by sperm competition games models (Parker 1990; Parker, Ball *et al.* 1997; Parker and Pizzari 2010; Parker, Lessells *et al.* 2013) (also see Chapter 1). More recently, Cameron *et al.* (2007) have produced a total ejaculate model, offering predictions for investment in both sperm and seminal fluid.

In species with a hierarchical social system, a male's role within sperm competition is typically predictable. Dominant males are likely to have a competitive advantage, for example by mating at an optimal time relative to ovulation, placing them in a favoured role for achieving fertilisation success. In this case, the sperm competition

games model of non-random roles offers the most relevant predictions of optimal investment strategy (Parker 1990). The non-random roles model predicts that a male mating in a disfavoured role should increase sperm investment to partly compensate for their disadvantage in sperm competition (Parker 1990; Ball and Parker 2003). Unfortunately, the Cameron et al (2007) model does not offer predictions for non-random roles. It does, however, agree with the sperm competition games model of a loaded raffle with regards to sperm investment; that males within the dis-favoured role should consistently invest more in sperm than those in the favoured role (Parker 1990; Cameron, Day *et al.* 2007). The Cameron et al (2007) model of total ejaculate investment predicts the benefit a male can gain by investing more in their seminal fluid production. This could be particularly important for species that produce a copulatory plug from seminal fluid components. In this instance, increased investment in seminal fluid may lead to production of a larger plug, creating a competitive advantage. Plug forming seminal fluid proteins could therefore have an additional value of increased investment, particularly for the disfavoured male as the female is likely to mate again with a more dominant male. Logically, the Cameron et al (2007) model predicts that males in the disfavoured role will increase energy expenditure on seminal products, compared to when only sperm will affect the degree of disadvantage, whilst maintaining a high investment in sperm.

Empirical studies have tested some of these predictions within various species. Dominant males of non-mammalian species have been shown to produce or ejaculate fewer sperm than subordinates (Liljedal and Folstad 2003; Pizzari, Cornwallis *et al.* 2003). By contrast, studies of rodents have found subordinate males invest less in sperm production than dominant males (Koyama and Kamimura 2000; Lemaitre, Ramm *et al.* 2012; Schradin, Eder *et al.* 2012) . With regards to seminal fluid, the only empirical evidence to date suggests that subordinate males decrease their investment. The seminal vesicles, the major accessory sex glands in rodents, are smaller in subordinate bank voles, suggesting a lower investment in seminal fluid production (Lemaitre, Ramm *et al.* 2012). Bank voles plastically alter the size of their seminal vesicle glands according to the level of competition (Lemaitre, Ramm *et al.*

2010), which suggests that the seminal vesicle secretion has an important role in competitive mating, although there is no evidence for this within house mice (Ramm and Stockley 2009a). The ability of male Drosophila to plastically invest in seminal fluid protein production, according to the level of competition (Wigby, Sirot *et al.* 2009), shows the potential for strategic investment in specific seminal proteins. House mice may strategically alter the proteins within their seminal vesicle secretion, regardless of no differences in organ mass. It is possible that males alter their seminal fluid proteins according to their social status. This is particularly likely in species that produce a copulatory plug from components of the seminal fluid. In these species the disfavoured males could gain a large advantage by investing more in the production of the copulatory plug. Here, the house mouse (*Mus musculus domesticus*) is used as a model to test this.

The house mouse has a complex hierarchical social system in which females prefer dominant, territory holding males (Bronson 1979; Wolff 1985; Coopersmith and Lenington 1992; Mossman and Drickamer 1996; Rich and Hurst 1998). Accordingly, dominant males attain the most copulations (Wolff 1985) and sire the most litters (DeFries and McClearn 1970). However females often choose to mate with more than one male resulting in a moderate level of multiple paternity in natural populations (Dean, Ardlie *et al.* 2006). Subordinate males of laboratory strains produce fewer sperm, which are less motile, than dominant males (Koyama and Kamimura 1998: Koyama and Kamimura 2000, but see Bates, Maechler *et al.* 2014 regarding the use of laboratory strains to study the effects of male social hierarchy). It is as yet unknown if house mice alter seminal fluid protein production according to their social status. The dominance hierarchy and moderate level of sperm competition of house mice make them an ideal model to test the current theory on overall ejaculate investment according to sperm competition roles.

The first part of this study measures physiological differences between dominant and subordinate males, as well as proteomic differences in their seminal vesicle secretions. The primary aim is to determine whether males will strategically alter the

production of specific proteins within their seminal vesicle secretion, testing the prediction that subordinate males may increase production of those that form the copulatory plug and could therefore offer a competitive advantage. Differences in overall investment in ejaculate production are also measured, specifically the size of the seminal vesicles and the number of sperm within the epididymis. The reduced sperm production of subordinate males may be due to physiological constraints from being in a state of chronic stress (Blanchard, Sakai *et al.* 1993). Therefore, within this study, faecal corticosterone is measured as a biomarker of chronic stress.

The second part of this study aims to determine whether males have different ejaculate allocation strategies, according to their social status and associated level of expected mating success. It is predicted that males will allocate ejaculates according to their expected reproductive opportunities (Tazzyman, Pizzari *et al.* 2009). House mice are able to adapt their ejaculates in response to the social environment. In the presence of rivals, house mice strategically ejaculate fewer sperm, independent of differences in the size of the copulatory plug (Ramm and Stockley 2007). To test whether males will also ejaculate according to their social status, both the number of sperm within the female tract and the size of the copulatory plug are measured. Each male was mated with two females to test allocation across sequential ejaculates and sperm depletion. Male mating behaviour is also analysed, as it is known to vary according to competition (Preston and Stockley 2006). Combined, these studies test whether males will invest differently in ejaculate production according to their social status, and whether this corresponds with differences in ejaculate allocation. Quantitative proteomic analysis allows differences in individual ejaculate proteins to be investigated.

2.3 MethodsExperiment 1) Differences in the production of ejaculate components according to social status.

2.3.1 Subject mice

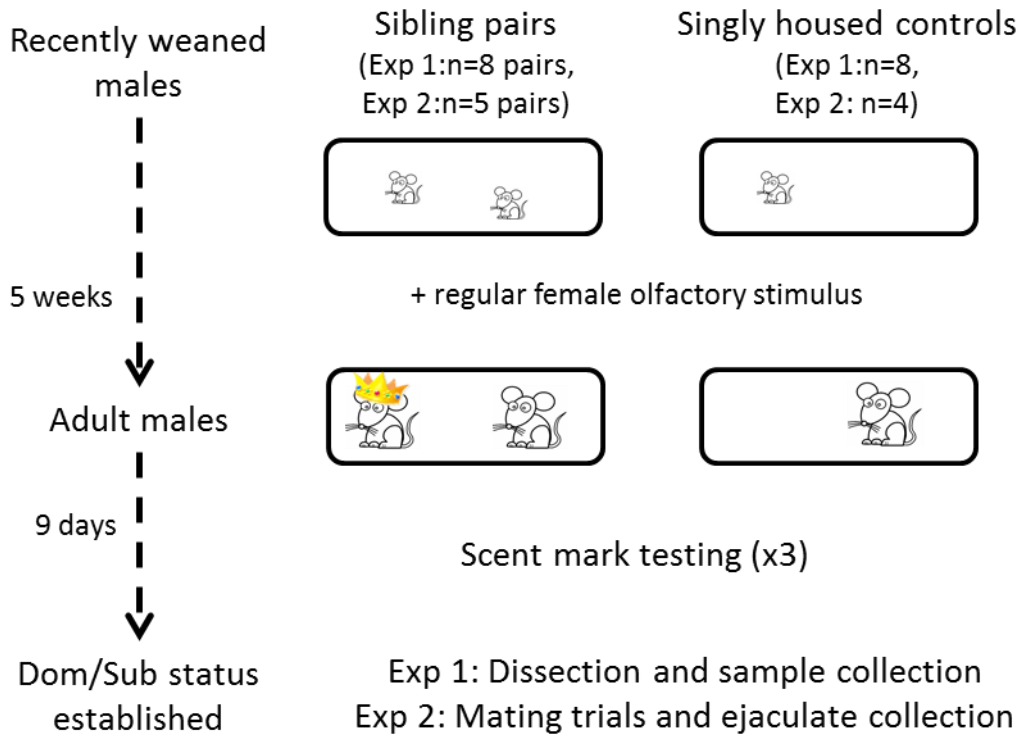
Male house mice (*M. musculus domesticus*) (N=24) were taken from third generation litters of an outbred colony, founded by wild mice captured from local populations in Cheshire. Pups from these litters were weaned at 26 days old. Subject males were taken when all pups had been housed in single sex sibling groups for at least 24 hours (age: mean ± s.e. 27.63 ± 0.41 days).

All animals were housed in wire lidded standard M3 cages with polypropylene bases (48cm x 15cm x 13cm: North Kent Plastics Cages ltd, Kent, UK) on Absorb 10/14 substrate with shredded paper nest material and cardboard enrichment. Food (5002 certified rodent diet, lab diet, St. Louis) and water were provided *ad libitum.* Lights were maintained on a reversed phase light:dark cycle of 12:12h (lights on at 20:00 hours) so that all handling was performed during the dark phase under red lights.

2.3.2 Forming dominance relationships

From weaning, subject males (n=16) were housed in sibling pairs to establish dominance relationships. Sibling pairs were used in order to minimise the likelihood of aggression whilst also reflecting a natural situation, whereby littermates are yet to disperse. Furthermore, this creates a balanced, paired design for later statistical analysis. A control group (n=8) were singly housed but otherwise treated in the same manner as the subject males. To reduce the effect of genetic differences, males in the control group were taken from the same litters as the paired males. To stimulate normal sexual development, soiled bedding from unrelated females was added to each cage once per fortnight (Vandenbergh 1971). This was maintained for the duration of the study.

Figure 2.1: Brief overview of the experiment and methods for forming dominant subordinate pairs.



The study lasted for 5 weeks, at which point a dominance relationship had been established and all males were sexually mature. Within this time, some of the pairs had to be split due to signs of aggression. Small scuffles are common within group housed mice, after cage cleaning or temporary separation, as mice re-affirm their status. As male mice can become very aggressive when competing for dominance (DeFries and McClearn 1970), the behaviour of subject males was monitored closely. Pairs were split if they did not settle within the first 30 minutes of being re-introduced to a clean cage, or if any physical marks appeared on either of the males. At no point during the experiment was fighting allowed to escalate and no males came to any physical harm, beyond small marks, before being split. Of the 8 pairs, 5 were separated before the end of the study. To maintain the dominance relationship, after pairs had been split, the males' cages were swapped around each day (i.e. the dominant male transferred into their subordinate sibling's cage and vice versa). This ensured they would regularly encounter fresh scent marks of their partnered male, but did not allow the males to interact.

2.3.3 Assessing the presence of a dominance relationship

To assign social status, after 3 weeks of paired housing the scent marking behaviour of each male was tested. Dominant males are known to deposit more scent marks than subordinates (Rich and Hurst 1998). Ultraviolet visualisations of scent marking patterns is commonly used to test social status in house mice (Desjardins, Maruniak *et al.* 1973; Drickamer 2001). Prior to testing, one male of each pair had a small patch of fur clipped from their hindquarters to allow individual identification. All other males were handled equivalently. To test scent marking behaviour, each male was placed individually in a cage lined with Benchkote. To stimulate scent marking, 10µl of urine, from a pooled source of unrelated wild males, was streaked onto the Benchkote. After 45 minutes the mice were returned to their home cages. The Benchkote was scanned using a UV scanner (InGenuis EPI UV kit, Syngene, Synoptics Ltd, UK) coupled to a computer using GeneSys software (Syngene, Synoptics Ltd, UK). The number of individual marks, greater than 20 pixels in size, were counted using image J (Rasband 1997-2012). A consistent pattern was found within all pairs

throughout 3 consecutive scent mark tests, taken over 9 days (see Figure 2.2 and Figure 2.3). In all cases, the male that deposited the most scent marks was assumed to be dominant. This was consistent with my own opinions of each male's behaviour.

Preputial glands secrete olfactory cues into the urine of house mice and are known to be larger within dominant males than subordinates (Bronson and Marsden 1973; Barnett, Dickson *et al.* 1980). After dissection, differences in the weight of the preputial glands provided further confirmation of the established dominance relationships. Dominant males had significantly larger preputial glands than subordinates ($t = 2.93$, $df = 7$, $P = 0.02$).

2.3.4 Faecal corticosterone concentration

Corticosterone is a steroid hormone associated with the stress response in mammals. It is relatively easy and non-invasive to measure the levels of this hormone from faecal samples (Touma, Palme *et al.* 2004). To determine whether male social status lead to differences in corticosterone release, I collected faecal samples from each male prior to dissection and analysed their corticosterone concentration. I used an M3 cage base with lid and an upturned M3 cage base on top, to collect faeces. Subjects were placed onto the cage lid, under the upturned cage bottom. This allows the faecal matter to fall through the bars of the cage lid for collection within the lower cage. After 1 hour, each subject was removed and returned to their home cage.

After collection, all droppings were frozen at -20°C prior to performing an enzyme-linked immunosorbent assay (ELISA) to determine the concentration of corticosterone within the faecal matter, using a standard method (Dusek, Bartos *et al.* 2010). In order to analyse the faecal corticosterone concentration, faeces were defrosted and methanol was used to extract the soluble hormone from the faecal matter. After overnight agitation in 90% methanol, each sample was centrifuged (20 minutes at 1800 rpm). The supernatant was removed, dried, re-suspended in 100% menthanol

Figure 2.2: Typical scent marking patterns from (a) dominant and (b) subordinate male house mice. To analyse scent marking behaviour, males were placed individually into empty cages lined with benchkote for 45 minutes. The benchkote was collected and scanned using a UV scanner to create these images, in which the deposited urine marks can be seen as dark patches or streaks.

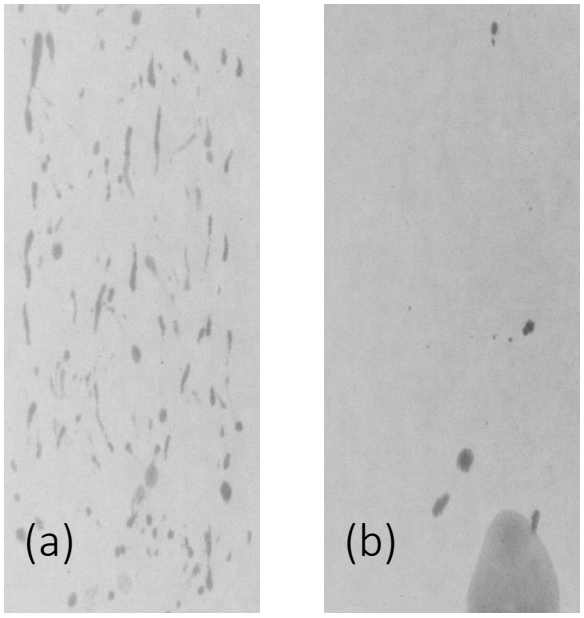
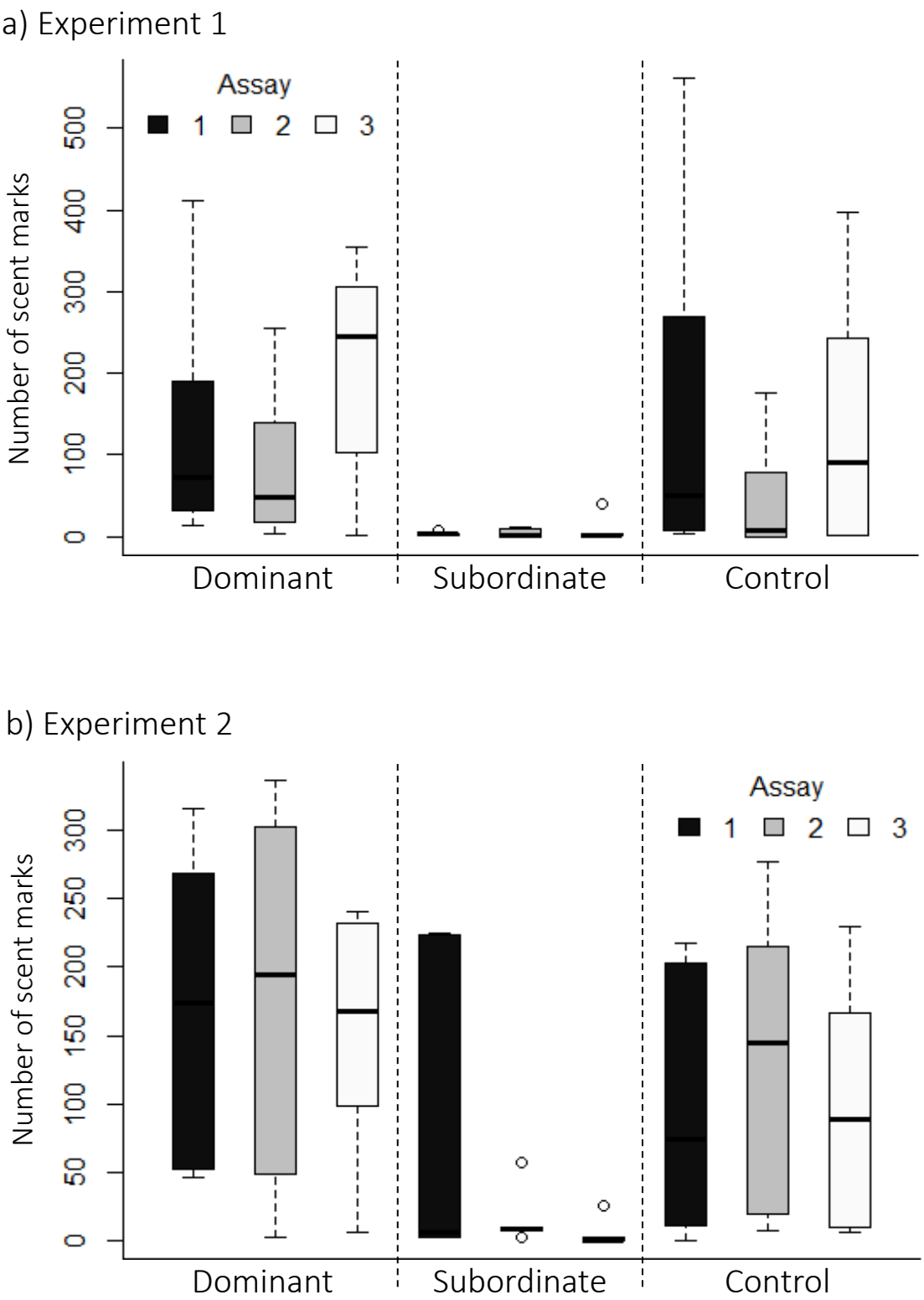


Figure 2.3: Boxplot of the scent mark counts following three scent mark assays for dominant, subordinate and singly housed control males within a) experiment 1 and b) experiment 2.



and frozen at -20°C before further analysis. For the ELISA, Nunc MaxiSorb 96 well plates were coated in corticosterone antibody overnight at 4°C. The following day, 50µl of defrosted sample and 50µl of corticosterone horseradish peroxidase (CC-HRP), was added to each well of the plate. The plate was left for 2 hours at room temperature in the dark before 100µl of substrate (H202, ABTS and substrate buffer) was added to each well. After 15 minutes a microplate reader with SkanIt software (Thermo) was used to read the plate at 405nm. Corticosterone concentrations were calculated based on the optical density of the standards values providing a concentration curve from which sample values could be compared.

2.3.5 Measuring overall reproductive investment

When the males were 2 months old they were sexually mature and a clear dominance relationship had been established within each pair. Reproductive organs were collected from each male, blind to the male's social status, to measure differences in their investment in reproductive traits. Males were killed humanely using an overdose of halothane gas followed by cervical dislocation. Within 30 minutes post-mortem, reproductive tissue was dissected from each male and epididymal sperm counts performed. The testes, preputial glands, coagulating glands and the right seminal vesicle glands were weighed individually and frozen whole at -20°C. The left seminal vesicle glands were squeezed until empty and the contents frozen for further analysis. Epididymal sperm was isolated by macerating the right cauda epididymis on a plastic Petri dish in 100µl of 1% citrate solution. After 2 minutes a further 900µl of 1% citrate was added and a pipette was used to mix the sperm suspension, before collection. An Improved Neubauer haemocytometer was then used to perform sperm counts according to standard protocols (Heineman 2002). Briefly, 10µl of the sperm suspension was added to each side of a haemocytometer. This was left to settle for 15 minutes in a humid box, before counts were performed using a Leica DM1000 light microscope.

2.3.6 Tryptic digestion of seminal vesicle contents

The seminal vesicle secretion is a highly viscous, protein rich substance. Prior to proteomic analysis, defrosted samples were diluted to approximately 50mg/ml with 50mM ammonium bicarbonate solution, before using a pestle and vortexing until attaining a homogenous solution. A Coomassie plus protein assay was used to accurately measure the protein concentration of each sample. Following a standard protocol, 100µg of protein within a total final volume of 200µl was digested using trypsin. Briefly, proteins were denatured by RapiGest SF Surfactant (Waters) at 80°C for 5 minutes, to assist with enzymatic digestion. The disulphide bonds in the sample were reduced and then alkylated by incubation with Dithiothreitol (60°C 10min) followed by Iodoacetamide (RT 60 minutes in the dark). Trypsin (0.2mg/ml, Sigma-Aldrich) was added and the sample incubated overnight at 37°C. After 12 hours, 1M hydrochloric acid and additional trypsin (0.1mg/ml) was added and left to incubate for a further 4 hours to ensure complete digestion. To precipitate any residual protein, each sample was incubated at 37°C with trifluoroacetic acid for 45min. These samples were centrifuged at 13500rpm and 4°C for 90 minutes and the supernatant decanted into low – bind Eppendorfs. The digests were centrifuged at 13500rpm and 4°C for a further 90 minutes, and 10µl of each digest visualised using SDS-PAGE, to ensure complete removal of the protein.

2.3.7 Tryptic digestion of coagulating glands

The coagulating glands are far smaller than the seminal vesicle glands, and contain a small volume of fluid. To ensure a substantial quantity of protein was available for analysis, the glandular tissue including contents were digested and analysed. Prior to tryptic digest, each gland was homogenised using a minilyse (Bertin) bead disrupter. Briefly, individual coagulating glands were added to 2ml vials containing 0.5mm diameter glass beads and 50mM ammonium bicarbonate, to make an approximate 200mg/ml dilution, according to the defrosted weight. Each sample was homogenised at 5000rpm for 30s, followed by 30s on ice, 10 times. Homogenised samples were transferred to an Eppendorf by creating a small hole in the bottom of

each vial, placing this over an Eppendorf and centrifuging at 5000rpm for 15 minutes at 4°C. To wash remaining proteins from the beads, enough 50mM ammonium bicarbonate to make the final dilution approximately 100mg/ml, according to the defrosted weight, was added to the sample vials before a second centrifuging. For each sample, all of the collected supernatant was vortexed and a Coomassie plus protein assay performed to accurately measure the protein concentration. Trypsin digests were then performed on 100µg of protein, within a final volume of 200µl, as described previously.

2.3.8 Mass-spectrometric analysis

Prior to HPLC-MSMS analysis, pH paper was used to ensure each sample was acidic, and so suitable for MS analysis. The seminal vesicle secretion samples were diluted 300 fold with 97:3:0.1 HPLC grade water:MeOH:TFA. Such a dilution was used due to the low complexity and broad dynamic range of this sample, with only 8 proteins accounting for over 90% of the total. Investigative tests suggested 1 in 300 would be an optimal dilution. At this dilution the high abundance proteins do not appear to overload the detectors, which would result in underestimating the quantity of the highly abundant proteins, whilst inhibiting detection of the low abundance proteins. As the coagulating gland sample included tissue proteins, these samples only required a 1 in 2 dilution.

The tryptic peptides were resolved, over a linear organic gradient of 3-40% buffer B (0.1% formic acid in acetonitrile), using a nanoACQUITY (Waters) ultra-performance liquid chromatography system. A 50 minute gradient was used for the relatively simple seminal vesicle secretion samples whereas the coagulating gland samples were resolved over a 90 minute gradient. The HPLC system was coupled to an electrospray ionisation source and an LTQ Orbitrap Velos (Thermo) mass spectrometer, acquiring high resolution mass data in a data-dependent manner (Makarov, Denisov *et al.* 2006). The top 20 most intense peptides in each MS scan were selected for fragmentation for MSMS analysis.

2.3.9 Label–free quantification

Progenesis LC-MS software (NonLinear dynamics) was used to analyse the raw HPLC-MSMS data and provide label–free relative protein abundances. This software aligns raw data from the HPLC-MSMS runs according to retention time and m/z values. The automatic alignment for each run was manually checked and re-aligned where necessary until confident that matching peptides had been correctly aligned. After all peptide ions were matched, those with charge states of +1 and >+9 were excluded before creating an aggregate file (.mgf) of the raw data for the peptides ranked 1-3 at each peak. This aggregate .mgf file was searched using a local Mascot server (v 2.3.01) against a protein database of reviewed UniProt *Mus musculus* entries with additional unreviewed ejaculate specific entries of proteins identified elsewhere (Ramm, McDonald *et al.* 2009). Mascot search parameters were set at 10ppm peptide tolerance and 0.5Da MSMS tolerance, with one mis-cleave and a fixed cysteinyl carbamidomethylation and variable oxidation of methionine modification accounted for. The Mascot search results were imported back into Progenesis LC-MS as an .xml file and protein identifications assigned to each peptide peak. Proteins with at least 3 unique peptides were quantified by comparing relative ion intensities for each peptide within each individual sample. Data were then analysed as described in Section 2.3.16.

Experiment 2) Differences in mating behaviour, copulatory plugs & ejaculates, over 2 matings, according to social status

2.3.10 Subject males & forming a dominance relationship

Male house mice (*M. musculus domesticus*) (N=15) were taken from fifth generation litters of the same outbred colony as the males within experiment 1. Pups were weaned at 26 days old and subject males were taken when all pups had been housed in single sex sibling groups for at least 24 hours (age: mean ± s.e. 30.4 ± 0.21 days). Due to low numbers of males within the available litters it was only possible to pair 5 sets of siblings, with a further 5 males taken from the same litters as singly housed

controls. Husbandry and housing were kept consistent with the previous experiment as this successfully resulted in the establishment of a dominance relationship within sibling pairs. Scent marking behaviour was tested, as in experiment 1, to ensure a dominance relationship had been established mature prior to beginning the mating trials.

2.3.11 Subject females

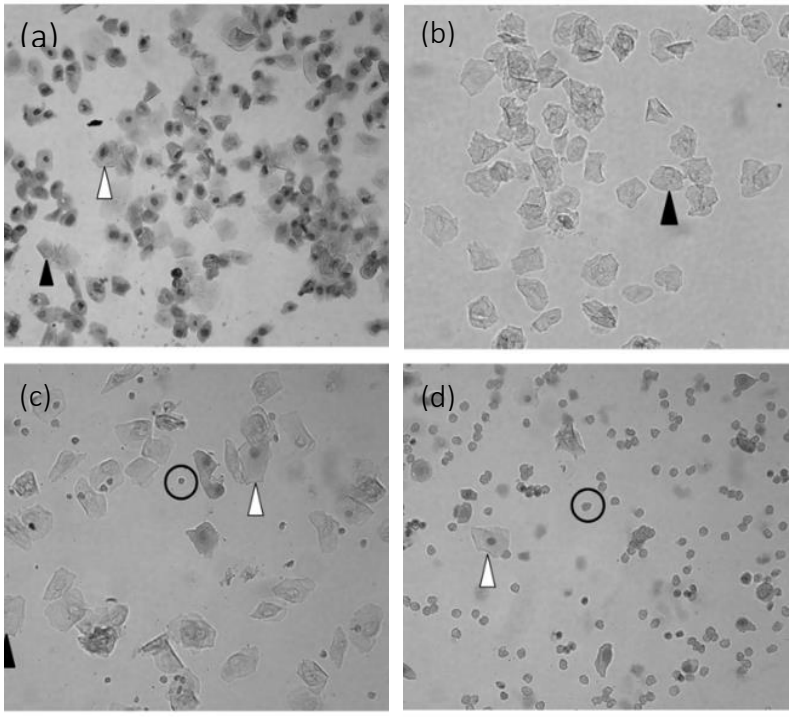
An outbred stock of 7-8 week old, sexually naïve, ICR females (N=80) was purchased from Harlan UK to test the mating behaviour of the subject males. ICR females were used as this placid strain is known to be good for breeding, making successful mating more likely. Further to this, using a laboratory strain reduces the genetic differences between the females and, being an albino strain, makes the behavioural observations easier. The females arrived 2 weeks before mating trials would begin, allowing time for them to habituate to the reversed light cycle and to ensure a normal oestrus cycle, stimulated by male bedding (Koyama 2004).

One week prior to mating trials, vaginal swabs were taken from each female, over 5 consecutive mornings, to check for normal oestrus cycling and to estimate on which days each female was likely to be receptive to mating (Hotchkiss and Vandenbergh 2005). A 1µl sterile plastic swab was used to collect vaginal cells, which were smeared onto a glass slide with 10µl of 0.1% methylene blue and a coverslip added. Cells were viewed under a light microscope to determine oestrus stage (see Figure 2.4). Oestrus testing was performed every 3 weeks during the mating trials to ensure females were cycling normally so that the females being paired were likely to be receptive.

2.3.12 Mating trials

After a dominance relationship had been established within the sibling pairs, mating behaviour was recorded to analyse differences according to social status. Each mating trial was performed within an enclosure (80cmx 60cmx60cm) containing a water bottle, food pellets and a tunnel for shelter. First, a male was released into the

Figure 2.4: Images of vaginal cytology, at 200x magnification, at each stage of oestrus. Taken from Byers *et al.* 2012 and used as a reference for oestrus testing. Cell types identified: - nucleated epithelial (white arrow), cornified epithelial (black arrow) and leukocytes (circle). Stages:- a) pro-oestrus, b) oestrus, c) metoestrus and d) dioestrus.



enclosure to habituate for 10 minutes. A female was then placed within a perspex tube into the enclosure for a further 10 minutes, allowing the female to habituate and the mice to interact. The female was then released and the pair observed remotely for mating activity. Mice were observed remotely, via a CCTV feed to the next room, for mating activity. Each trial was also recorded to DVD using a Panasonic recorder (DMR-EX79) for later analysis, outlined below.

If no mating behaviour had been observed within 30 minutes of pairing the mice, the female was removed and replaced with a second female. The second female was allowed to habituate within a handling tunnel for 10 minutes prior to being released with the male. A third female was tried in the same manner if no mating occurred within 30 minutes. If, after 30 minutes with the third female, there was no mating the male was placed back in his home cage to be trialled on a different day.

House mouse mating behaviour is characterised by a series of intromissions, whereby a male will mount the female and perform a number of thrusts, but not ejaculate, before dismounting. On the final intromission a male will ejaculate, signified by the pair becoming immobile, often tipping over, for a prolonged period (between 8-20 seconds) (Estep, Lanier *et al.* 1975). This makes the end of the copulation relatively easy to determine. In this study, I watched each mating until ejaculation had occurred, noting the time of each intromission.

To compare ejaculate depletion and remating rates, data was collected on two successive matings for each male. After a male had mated successfully the first time, he was paired with up to three oestrus females in the same manner, each day, until a second successful mating occurred.

2.3.13 Analysis of mating behaviour

The recorded successful mating attempts were analysed to quantify the number, duration and rate of thrusts and intromissions, as well as differences in each male's ejaculation behaviour. A timer programme developed by a colleague (J Halstead) was

used to manually navigate the DVD file to each time point that an intromission had been recorded when watching the behaviour live. The counts and durations, for all mating behaviours, were measured for analysis.

2.3.14 Collection of the ejaculate

To compare the ejaculate allocation of each male, immediately after ejaculation the copulatory plugs and uterine contents were collected from the female. After ejaculation had occurred, the female was killed humanely, using halothane gas followed by cervical dislocation. Each female was weighed and measured before dissection of the reproductive organs. The copulatory plug was removed from the vagina and weighed, before storing at -20°C. The uterus, containing the rest of the ejaculate, was isolated from the cervix to the oviducts and placed in a plastic Petri dish. Incisions were made along the length of the uterine horns to assist with washing all of the sperm from the uterus. The uterus was placed in an Eppendorf and 200µl of phosphate buffered saline (PBS) solution used to rinse the Petri dish. The ejaculate solution was added to the Eppendorf, along with the uterus, which was vortexed on the lowest speed for 30 seconds to dislodge the sperm from the uterine horns. The supernatant was pipetted from the tissue and three further individual 100µl PBS washes were performed in the same manner, using low speed vortexing for 30 seconds to dislodge the sperm before collecting the fluid. The sperm washed from the uterus was therefore collected in a final volume of 500µl PBS. The number of sperm within the uterine wash was then counted using an improved neubauer haemocytometer as previously described (see Section 2.3.5). The uterine washes were then stored at -20°C prior to proteomic analysis.

2.3.15 Proteomic analysis of the ejaculate

To quantify differences in the proteins within the fluid section of the ejaculate, the sperm samples from the uterine washes of mated females were analysed using a similar methodology as described for the analysis of the seminal vesicle secretion. Briefly, the homogenous solution was assayed to determine the protein

concentration using the Coomassie plus protein assay before a trypsin digest was performed on 50µg protein in a final volume of 200µl. Due to a change of supplier, the Trypsin used here was purchased from Promega, but still used at the same concentration (0.2mg/ml with a 0.1mg/ml top up). The tryptic digests were diluted 1:1 with 97:3:0.1 HPLC grade water:MeOH:TFA and then resolved over a 90 minute linear organic gradient using ultra performance liquid chromatography (Waters nanoAcquity) coupled with tandem mass spectrometry using the LTQ Orbitrap Velos (Thermo).

2.3.16 Data analysis

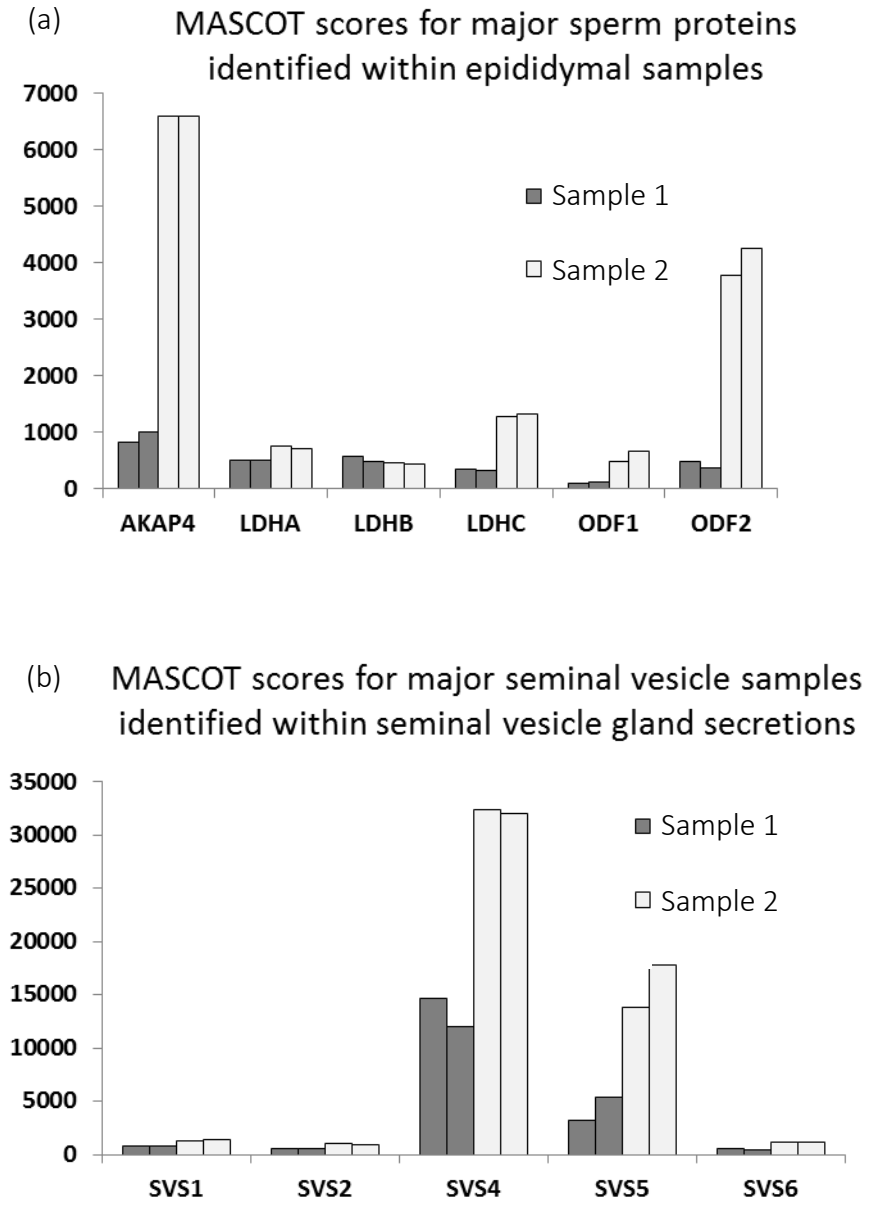
All data analysis was performed in R (v 3.1.0) (R-Development-Core-Team 2011). Data were transformed as appropriate. Alternatively, non-parametric analysis was used if normalisation of the data was not possible. Paired t-tests were deemed appropriate to compare values for the dominant and subordinate males as this allows for littermates being similar. When t-tests were unsuitable, GLMMs were used to model the data using the lmer and lm4 packages in R (Osadchuk, Salomacheva *et al.* 2010; Dean, Findlay *et al.* 2011). This allowed the data distributions to be fitted to binomial or Poisson error structures, as appropriate, as well as accounting for the random effect of genetic similarities between siblings. The influence.ME (Kolde 2013) and Outliers (Dusek and Bartos 2012) packages were also used to test for outliers or data values of particular influence where necessary.

Proteomic analysis using Progenesis LC-MS provided identifications and relative quantifications of the proteins within the samples analysed here. As each data set produced was slightly different, each was processed in a slightly different manner prior to statistical analysis. For all data, only proteins that had been quantified based on 3 or more unique peptides were included for further analysis, as these provide the most reliable quantitative results. The proteomic analysis of coagulating glands quantified proteins from the whole gland, as a result a large amount of cellular proteins were included within the data set. To measure differences in only the secreted proteins, I limited analysis to only proteins that had been previously

identified within the coagulating gland secretion (Dean, Clark *et al.* 2009). Considering ejaculate proteins, small sample sizes limited the analysis according to social status, as there were too few individuals within each group, for either the first or second matings, to perform statistical tests. Data from matings 1 and 2 were therefore combined. To account for the variability of different mass spectrometry runs, the raw data from all runs were combined, and manually aligned, for Progenesis LC-MS analysis. Statistical analysis was performed on the whole data set and on a reduced set of proteins known to be male derived and within the ejaculate. The shortened set of male specific proteins included those identified by Dean *et al.* (1986; 2009) and others known to be sperm or seminal fluid specific.

Preliminary investigations suggested that the level of technical variation would be lower than biological variation, and so technical replicates would not be required. This initial analysis was performed on seminal vesicle and epididymal protein samples collected from two wild male house mice. Tryptic digests and proteomic analysis using LC-MSMS was performed in a manner consistent with the data collected within this chapter. For each sample the LC-MSMS analysis was performed twice, in order to test technical repeatability of the mass spectrometry. Protein identifications were performed using MASCOT and the MASCOT scores used as an indicator of repeatability and abundance (Figure 2.5). Although MASCOT scores are based on the probability that such a protein match would have occurred by chance (given as -10logP of the probability, so that large scores relate to confident matches), and therefore do not technically relate to abundance, they generally offer a good indication during preliminary analyses. Absolute quantification of proteins was not possible within Progenesis LC-MS, due to technological constraints. Therefore, analysis of the experimental data is considering relative abundances and differences in overall composition. To standardise across samples the label – free quantification data was transformed into the proportion of each protein within each sample prior to statistical analysis, according to Equation 2.1.

Figure 2.5: Plots of the MASCOT scores taken from preliminary analyses to determine technical reproducibility of LC-MSMS. Two biological samples were analysed using LC-MSMS twice. Each bar represents an individual replicate for one of the major (a) sperm or (b) seminal vesicle proteins.



Equation 2.1: Mathematical notation of the formula used to convert normalised protein abundance (Nab) to proportional protein abundance (Pab) for each protein (P) within each sample of n proteins.

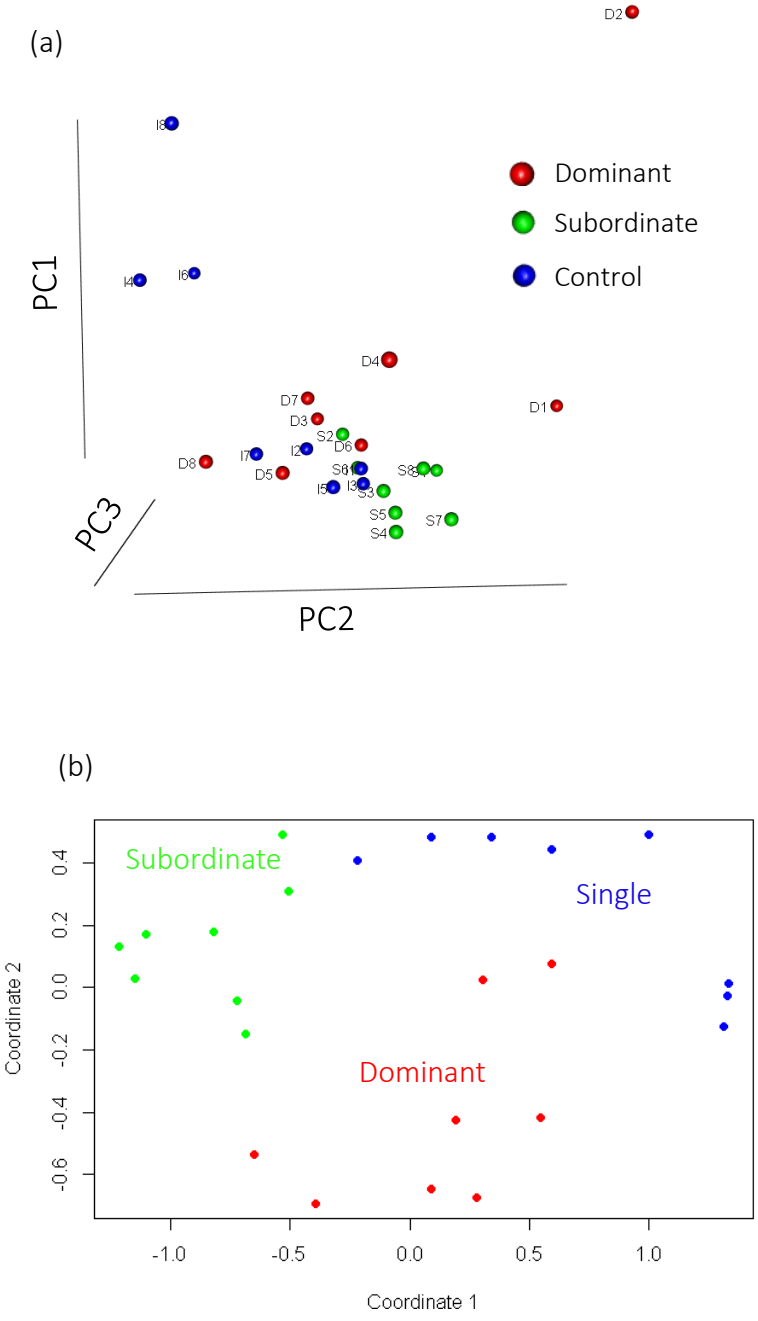
$$Pab_{Px} = \frac{Nab_{Px}}{\sum_{n} P}$$

Preliminary analysis of the data was performed using principle components analysis (PCA). PCA is a common statistical technique that simplifies data into principle components that explain decreasing amounts of the variance. This is an unsupervised approach and was not particularly effective at grouping the seminal vesicle protein data according to social status (Figure 2.6). However, differences in the protein composition of a sample are likely to be subtle and so a supervised approach may be more effective at finding differences according to social status. A supervised approach was also considered as it should mask some of the similarities which are due to relatedness of siblings within the treatment groups. Random forest (RF) analysis was tested to see how effectively this could classify the data, and therefore to see how well it could identify subtle differences in protein composition across the different groups. Initial tests showed that the RF approach was far more effective than PCA at grouping the data (Figure 2.6) and so, in addition to further reasons detailed below, was chosen as the preferred method of data analysis.

RF, a classification tool which uses ensemble learning to analyse non-parametric, complex biological data sets, is becoming increasingly popular (reviewed in Qi 2012). RF has successfully been used to classify microarray data (DNA microarray: Statnikov, Wang *et al*. 2008, Díaz-Uriarte and Alvarez de Andres 2006, and tissue microarray: Shi, Seligson et al. 2005), to study single nucleotide polymorphisms (SNPs) for genome wide association studies (GWAS, Botta, Louppe *et al*. 2014, Lunetta, Hayward *et al*. 2004) and for classifying proteomics data (Izmirlian 2004).

RF is a robust non-parametric method of data analysis suited to analysing high-dimensional proteomics data, with a high number of predictor variables and low n number. It uses a random subset of data to create, and then train, an ensemble of classification trees. RF then uses the remaining samples, not used in the training stage, to test the ability of the trees to correctly classify the samples. Variable importance measures are then given to each predictor variable (protein abundance) indicating how accurately trees classify the sample when trained using that variable.

Figure 2.6: (a) Plot of the first three principle components from a Principle Components Analysis (PCA) of the Progenesis LC-MS abundance values, normalised within the software, for proteins within the seminal vesicle secretion. Plot (b) is a multi-dimensional scaling plot taken from a random forest (RF) analysis using the same data.



As well as being an efficient method of data classification, another benefit of using RF techniques is these variable importance scores that offer a reliable indication as to the importance of each protein for correctly classifying the data. This information can inform further analysis of specific proteins, which may show the greatest differences between samples. The Party and RandomForest packages in R were used to perform this analysis (Gilbert 2000; Breiman 2001; Good and Coon 2006; Strobl, Hothorn *et al.* 2009).

When analysing compositional data, centred log ratio (CLR) transformation is generally recommended to account for the non-independence of data points (Aitchison 1986; Aitchison and J. Egozcue 2005). However, there is some debate over whether to transform data prior to using RF analysis (Ranganathan and Borges 2011). Here, I performed the analysis on transformed and non-transformed data. There were very minor differences between these methods but the most optimal analyses, with the lowest classification errors, was generally for the CLR transformed data. Where this was not the case, results from both transformed and untransformed data are presented. The RF approach is, inherently, random. To account for this, each RF model was performed 1000 times. The classification accuracy and variable importance scores reported are the averages of the 1000 runs. To determine significance values for the classification scores of each model, a bootstrapping approach was used. To do this, the model was again replicated 1000 times but for each run the classification was randomised. The results were then used to quantify the proportion of times the classification error was the same or better than that given by the original model, therefore predicting the likelihood of getting that classification error by chance.

2.4 Results

Male physiology and ejaculate production

2.4.1 Scent marking behaviour

Dominant males are known to deposit more scent marks than subordinates (see Figure 2.2 for examples of scent mark patterns). Within each pair, scent marking behaviour was compared three times to confirm that a dominance relationship had been established. For the first study, one male within each pair consistently deposited significantly more scent marks in all 3 assays (dom vs. sub: test 1 v = 36, *P* = 0.008; test 2 v = 36, *P* = 0.008; test 3 v = 28, *P* = 0.022). Within the second experiment, the scent marking behaviour was less consistent for one of the five pairs of males. By the third test a significant difference in the number of scent marks deposited was found within all pairs (v = 21, *P* = 0.031), confirming that a dominance relationship had been established.

Control, singly housed males were also tested and there was no difference in the number of scent marks deposited between the dominant and control males. Control males also deposited significantly more than the subordinate within both batches of males (1^{st}: v = 21, *P* =0.036, 2^{nd}: v = 0, *P* = 0.031). See Figure 2.32 for scent mark counts from all males.

2.4.2 Reproductive physiology

There was no difference in body mass between dominant and subordinate males (dom vs. sub df = 7, t = 0.54, *P* = 0.603). Neither was there a difference in testes mass (dom vs. sub df = 7, t = -0.14, *P* = 0.90) (see Figure 2.7). Despite this, dominant males had significantly more sperm within their epididymides than both the subordinate and singly housed control males (dom vs. sub df = 7, t = 0.14, *P* = 0.004; dom vs. control df = 7, t = -2.80, *P* = 0.026; sub vs. control df = 7, t = 2.33, *P* = 0.05) (see Figure 2.8). This agrees with previous studies that have found clear differences in sperm counts between male mice, regardless of testes mass (Ramm and Stockley

2009a). Dominant males also had significantly larger seminal vesicles, both absolutely and when accounting for body mass (dom vs. sub df = 7, t = 3.71, *P* = 0.034). Differences in the total quantity of protein within the seminal vesicles could not be tested due to the difficulty in dissecting the entirety of the seminal vesicle contents. The protein concentration of the seminal vesicle secretion was measured and subordinate males had a higher protein concentration than dominant males (dom vs. sub df = 7, t = -3.96, *P* = 0.005). All of the tryptic digests were performed on 100µg of protein, to control for these differences. See Table 2.1 for reproductive physiology data.

2.4.3 Faecal corticosterone

Overall, there was no difference between the faecal corticosterone concentrations of dominant and subordinate males. However, by the time the faeces had been collected most of the pairs (5 of 8) had been split and so these males were singly housed. Taking this into account within the analysis reveals a clear interaction effect. The subordinate males that were still housed within the same cage as their dominant sibling had significantly higher levels of corticosterone than their dominant brother (t = -2.20, *P* = 0.048) (see Figure 2.9).

Figure 2.7: Boxplots of male physiology according to social status: a) body mass, b) testes mass, c) epididymal mass.

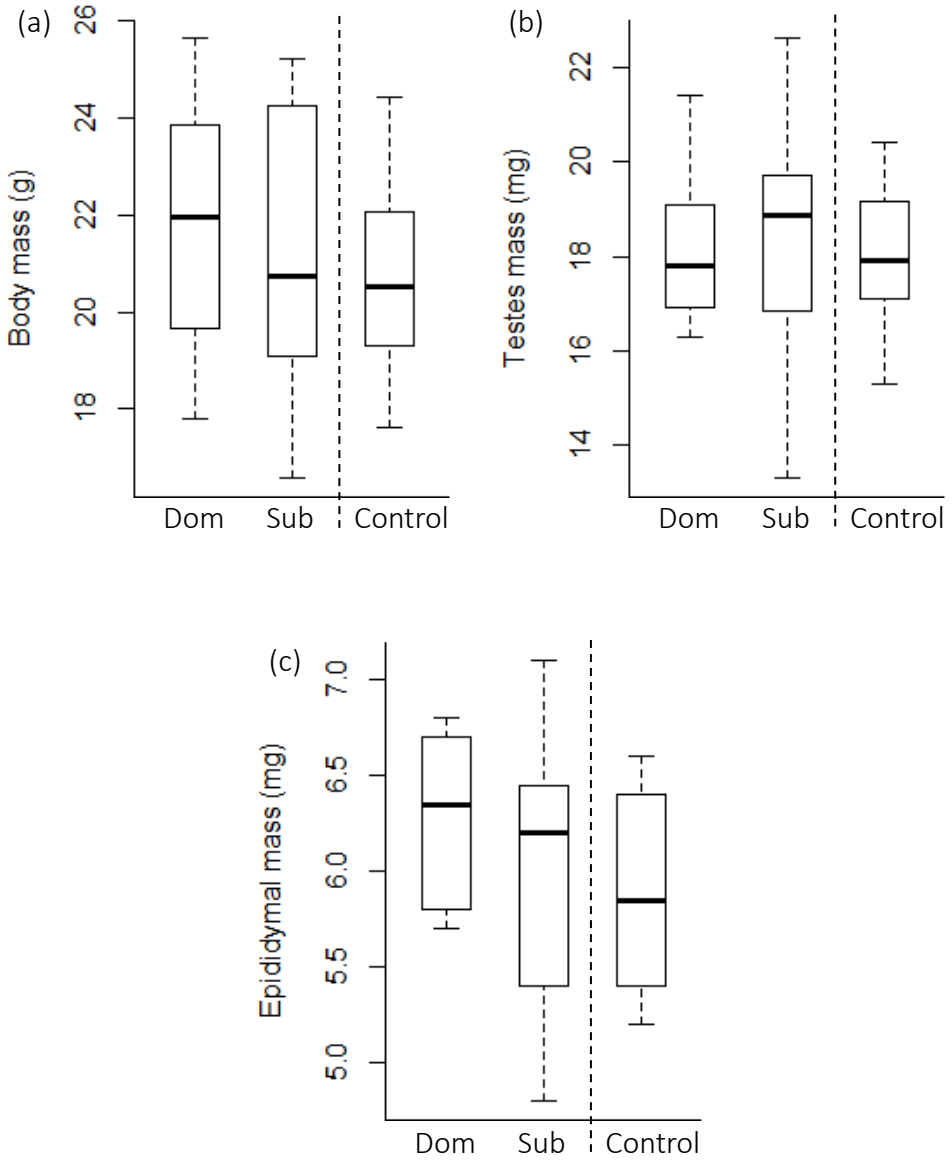


Figure 2.8: Boxplots of male reproductive physiology in relation to male social status: a) preputial gland mass, b) seminal vesicle mass and c) epididymal sperm count (* $P < 0.05$).

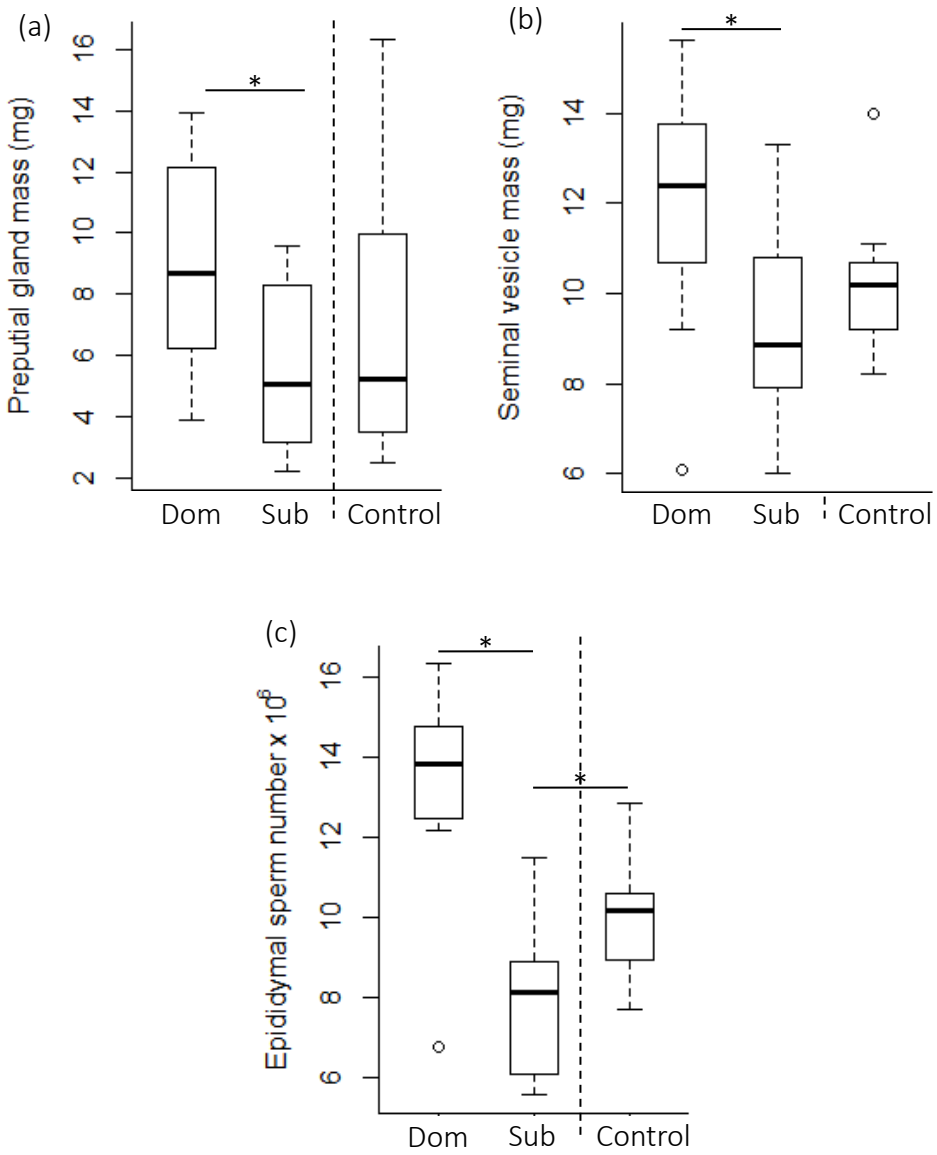
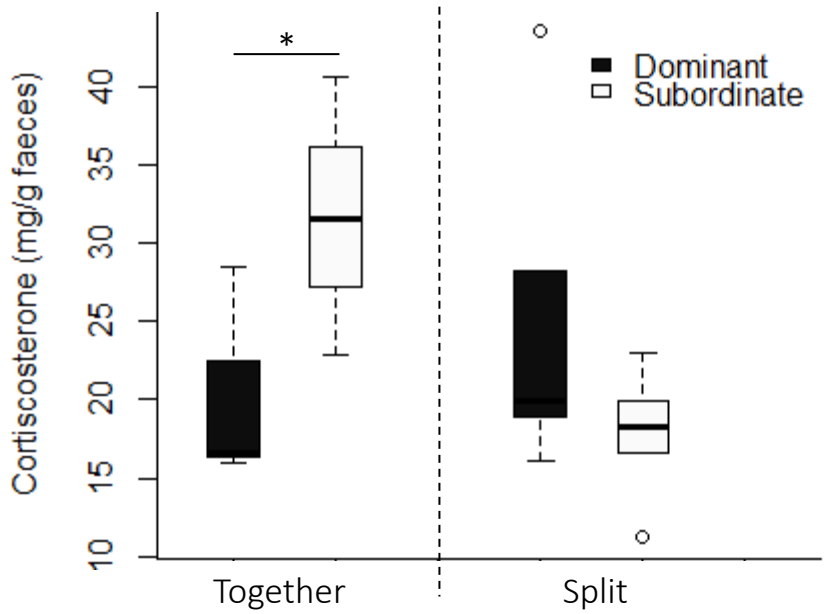


Table 2.1: Results from the analysis of male reproductive morphology according to social status. Analysis of differences between dominant and subordinate males was performed using a paired t-test. Comparisons of control and dominant or subordinate males were performed using a mixed model with "litter" included as a random factor. The results of analysis of the residual values for each organ are included, to show the relative differences between the size of these between males.

	Dominant		Subordinate		Control		Dominant v. Subordinate			Control v. Dominant			Control v. Subordinate		
	Mean	**s.e.**	**Mean**	**s.e.**	**Mean**	**s.e.**	**df**	**t**	***P***	**df**	**t**	***P***	**df**	**t**	***P***
Body mass (g)	21.81	0.98	21.24	1.09	20.74	0.77	7	0.54	0.603	15	-0.8	0.454	15	0.05	0.960
Preputial glands (mg)	90	13	56	10	70.25	16.7	7	2.93	0.022	15	-1.9	0.085	15	1.64	0.122
residuals ~ BM							7	3.9	0.006	15	-1.7	0.114	15	2.07	0.056
Testes (mg)	181.6	6.23	183.4	9.69	180	5.81	7	-0.1	0.896	15	-0	0.993	15	-0.3	0.754
residuals ~ BM							7	-0.5	0.652	15	0.55	0.589	15	-0.5	0.620
Epididymides (mg)	62.75	1.64	60	2.66	58.88	1.91	7	0.87	0.415	15	-1.3	0.221	15	0.31	0.758
residuals ~ BM							7	0.84	0.430	15	-1.1	0.301	15	0.32	0.752
Seminal vesicles (mg)	119.3	10.7	93	8.03	103	6.33	7	2.62	0.034	15	-2.4	0.003	15	1.78	0.095
residuals ~ BM							7	3.71	0.008	15	-2.9	0.011	15	2.27	0.038
Epididymal sperm count	13.1	1.03	7.89	0.7	10	0.55	7	4.28	0.004	15	-4.6	0.000	15	3.92	0.001

Figure 2.9: Boxplot of faecal corticosterone concentrations for dominant and subordinate male pairs which were housed together or had been separated (* $P < 0.05$).



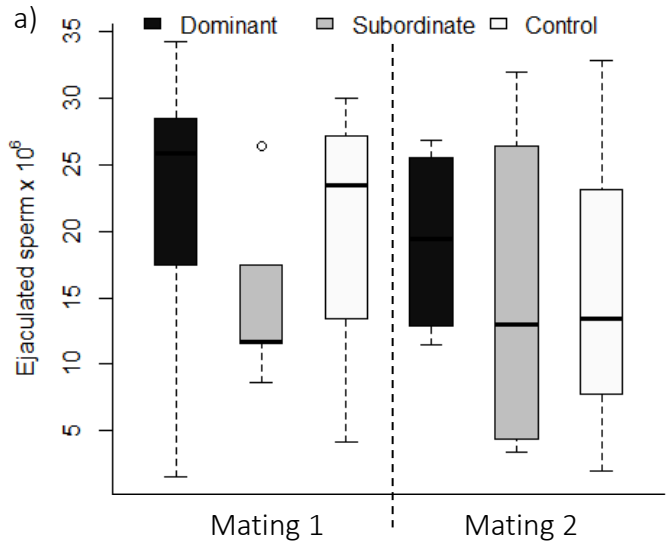
2.4.5 Copulatory plug size

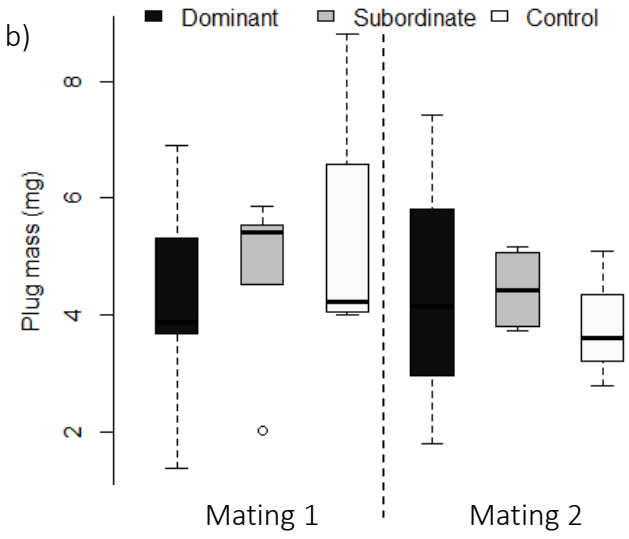
There was no effect of social status on copulatory plug size. There was also no difference in plug size between the first and second copulations. Copulatory plug size did correlate with the number of trials before mating occurred. For both the first and second copulations, males that took longer to mate produced larger plugs (1st mating: $F_{1,9} = 21.17$, $P = 0.001$; 2nd mating $F_{1,9} = 6.95$, $P = 0.027$). Further analysis of mating behaviour can be found in Chapter 4.

2.4.6 Number of sperm within ejaculates

The number of sperm within a first and second ejaculate from dominant, subordinate and control males was compared. Analysis of just the paired males showed that dominant males ejaculated significantly more sperm than subordinates in the first mating (t = -4.75, df = 3, $P = 0.018$). This was still true when data from both matings were combined, and mating included as a fixed effect within the model (t = -2.42, df = 10, $P = 0.036$) (see Table 2.2). As the aim of this experiment was to compare the number of sperm ejaculated by dominant and subordinate males, it is reasonable that this was analysed separately. When control males were also included in the model there was no effect of social status. This may be due to the much broader range of values within the control group masking the effect (see Figure 2.10). The broad range of values seen within the control males encompasses those of the dominant and subordinate males. This may therefore be a result of natural variation in the dominance of singly housed male mice. When considering all males, the number of ejaculated sperm correlated with the number of trials before the second, but not the first, copulation occurred (1st mating: $F_{1,12} = 0.49$, $P = 0.498$; 2nd mating $F_{1,9} = 6.71$, $P = 0.029$). Further analysis of mating behaviour can be found in Chapter 4.

Figure 2.10: Boxplots of a) The number of ejaculated sperm and b) the copulatory plug mass from the first and second matings of dominant, subordinate and control males.





2.4.7 Mating behaviour

The mating duration, measured as the time between the first intromission and ejaculation, was significantly longer for dominant males (t = -2.86, df = 18, *P* = 0.01). For all males, the duration of the second mating tended to be shorter than the first (t = -2.04, df = 17, *P* = 0.057) (see Figure 2.11). The range of mating durations observed was unexpected. One dominant male mated for 4.5 hours, whereas two of the subordinate males ejaculated during their first intromission. Prior to analysis, data was log transformed to account for this variation. Both dominant and control males performed more intromissions and more thrusts per copulation than subordinate males (see Table 2.2). However there was no difference in the number of thrusts per intromission, the proportion of the copulation spent intromitting or the duration of the ejaculation.

Each male was trialled with up to three females per session, until a successful mating occurred (see Section 2.3.5). There was a significant interaction effect of social status and mating on how many trials it took before copulation. Dominant males took fewer trials to mate the first time, but a greater number of trials before the second mating, than the subordinate and control males (see Figure 2.12).

2.4.8 Consecutive matings: Ejaculate allocation and mating behaviour

Analysing the males of each status individually showed that dominant males took a greater number of trials before the second mating than the first (df = 3, t = -4.64, *P* = 0.019). For each male type, dominant, subordinate, and control, there was no difference in the copulatory plug mass, the number of ejaculated sperm, the number of intromissions or the mating duration between the first and second matings (see Table 2.3)

Figure 2.11: Boxplots of the a) mating duration, from first intromission to ejaculation, and b) number of intromissions performed within the first and second matings of dominant, subordinate and control males.

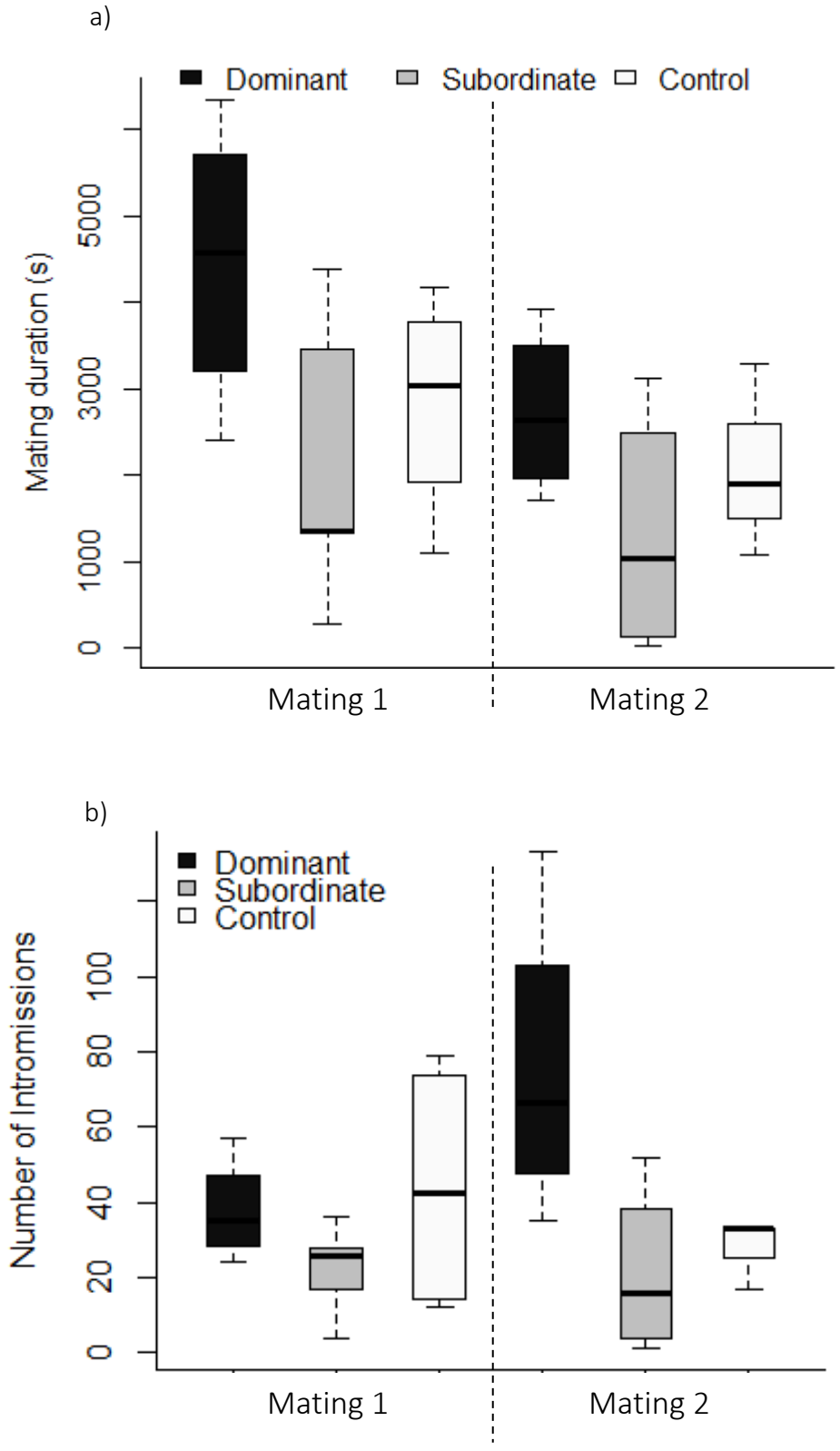


Figure 2.12: Boxplot of the number of females a male was tried with prior to a successful first or second mating of dominant, subordinate and control males.

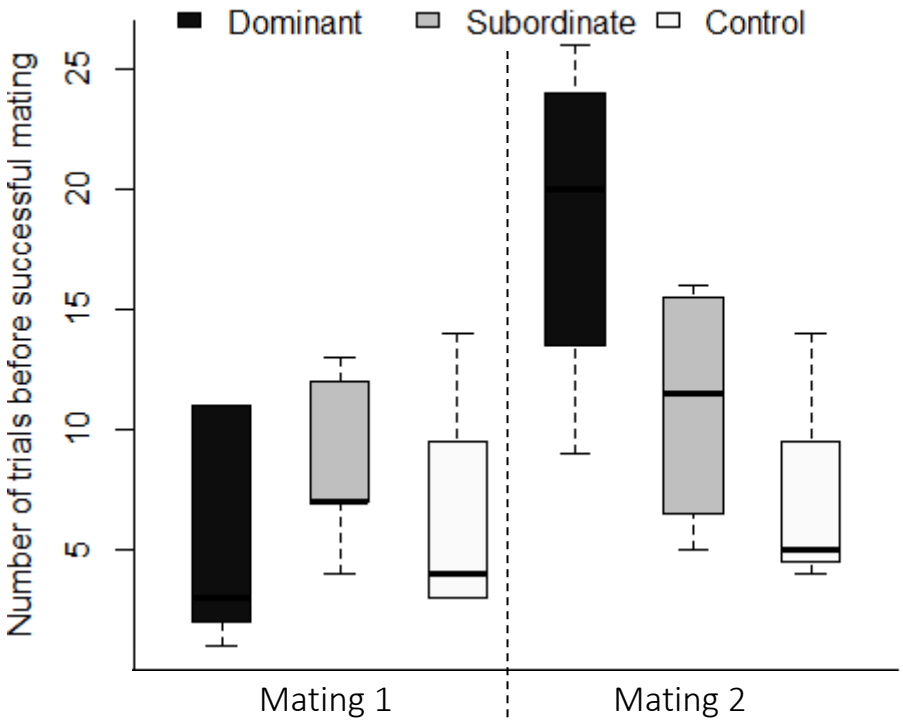


Table 2.2: Results from the analysis of ejaculate size and mating behaviour within male house mice according to their social status. Results presented are from mixed effect models incorporating litter as a random effect. * modelled using a GLMM with family = Poisson to account for count data.

			Dominant v. Subordinate		
			df	**t/z**	***P***
Ejaculated sperm					
	Mating 1	Status effect	3	-4.76	0.018
	Both matings	Status effect	10	-2.42	0.036
		Mating effect	10	-1.38	0.2
Plug size					
	Both matings	Status effect	17	0.32	0.75
		Mating effect	17	-0.62	0.54
Mating duration					
	Mating 1	Status effect	7	-2.46	0.044
	Both matings	Status effect	17	-3.06	0.007
		Mating effect	17	-2.04	0.057
Number of females tried*					
	Mating 1	Status effect		1.77	0.076
	Both matings	Status effect		1.66	0.097
		Mating effect		5.39	<0.001
		Interaction		-3.06	0.002
Number of intromissions*					
	Mating 1	Status effect		-10.14	<0.001
	Both matings	Status effect		-4.23	<0.001
		Mating effect		6.7	<0.001
		Interaction		-4.16	<0.001

Table 2.3: Results from the analysis of ejaculate size and mating behaviour comparing the first and second matings of dominant, subordinate and control mice. The results presented are from paired t-tests, accounting for the repeated measures.

		Mating 1 vs Mating 2		
		df	**t/z**	***P***
Ejaculated sperm	Dominant	3	0.25	0.82
	Subordinate	3	0.084	0.94
	Control	2	0.96	0.44
Plug size				
	Dominant	3	-0.04	0.97
	Subordinate	3	0.36	0.74
	Control	2	1.84	0.21
Mating duration				
	Dominant	3	1.38	0.26
	Subordinate	3	0.88	0.44
	Control	2	1.47	0.28
Number of Intromissions				
	Dominant	3	-0.01	0.99
	Subordinate	3	-0.02	0.99
	Control	2	1.71	0.23
Number of females tried				
	Dominant	3	-4.64	0.019
	Subordinate	3	-1.07	0.36
	Control	2	-1.73	0.23

Figure 2.13: Relationship between the number of females a male was trialled with prior to successfully mating and the number of sperm ejaculated in the a) first and b) second mating.

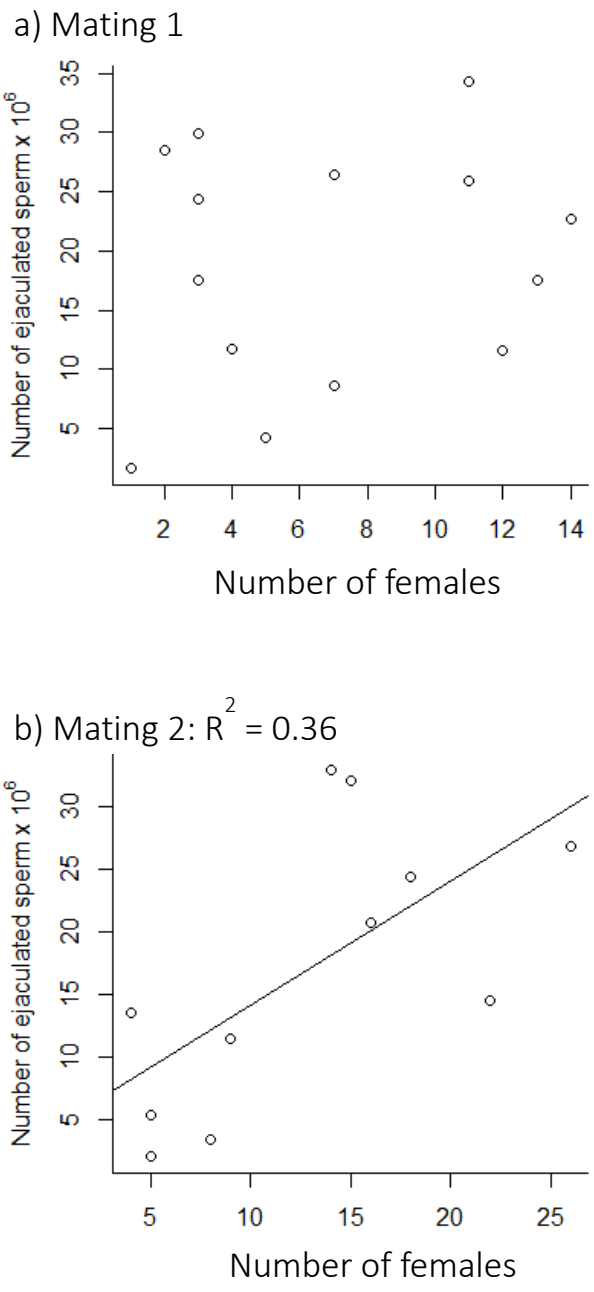
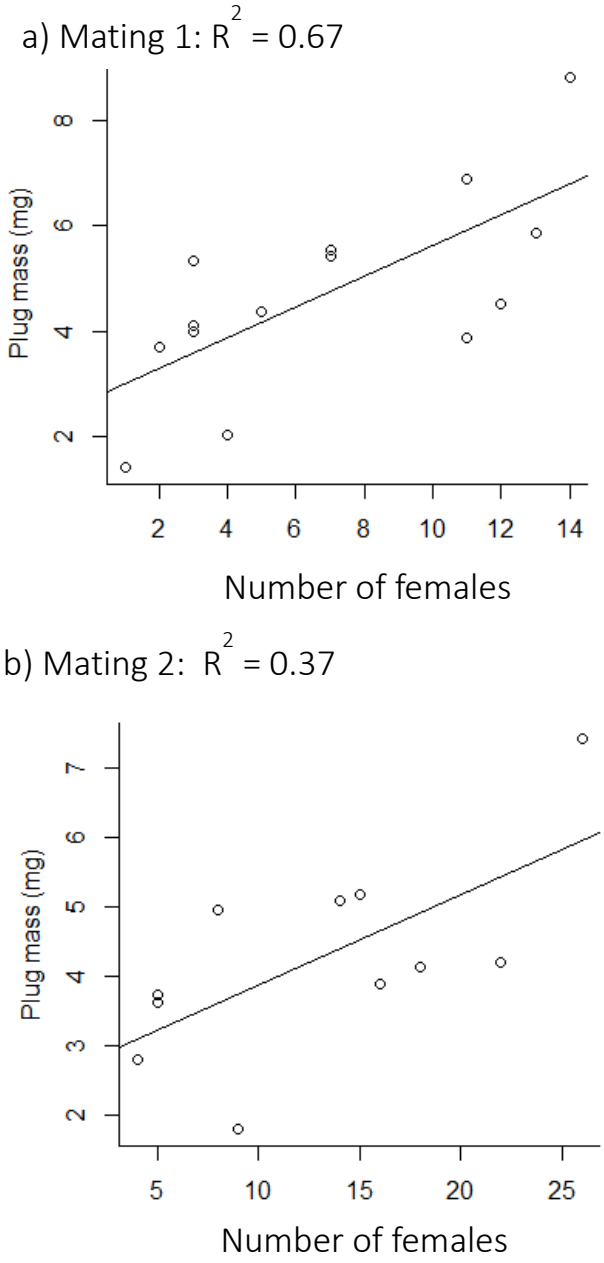


Figure 2.14: Relationship between the number of females a male was trialled with prior to successfully mating and the size of the copulatory plug produced in the a) first and b) second mating.



2.4.9 Seminal vesicle protein production

Differences in the relative proportions of 58 proteins within the seminal vesicle secretion were analysed using random forest (RF) methods. RF analysis classified the untransformed data, according to social status, slightly better than the transformed data (untransformed: 83.32% correct, CLR transformed 78.63% correct). This prediction accuracy was unlikely to be achieved by chance (bootstrapping analysis of 1000 replicates, untransformed: $P < 0.001$, CLR: $P = 0.001$). Although the overall accuracy was slightly better using the untransformed data, the CLR model more accurately classified dominant and subordinate males (see Table 2.5) and so variable importance results from both models are presented (see Table 2.6 and Table 2.7).

RF analysis of untransformed proteomic data from the seminal vesicle secretions found a total of 12 proteins had significant variable importance scores and were therefore important for classifying the data correctly according to social status. Within the CLR transformed model, 16 proteins had significant variable importance scores. Of the proteins with significant variable importance scores, 7 were significant within both models. One of this 7 was the highly abundant protein SVS2. Further analysis revealed that SVS2 accounted for a significantly larger proportion of the subordinate males' seminal vesicle contents than the dominants ($t = -3.24$, $df = 7$, $P = 0.014$) (see Figure 2.15, Figure 2.16 and Figure 2.17).

2.4.10 Coagulating glands

A total of 868 proteins were identified within the coagulating gland samples, of which 77 were also found within the Dean *et al.* (2009) analysis of the coagulating gland secretion. The shortened list of 77 proteins known to be secreted was used for further analysis. RF analysis accurately classified the data with respect to social status 94% of the time (22 out of 24 correct, bootstrapping analysis of 1000 replicates: $P < 0.001$). Five proteins had significant variable importance scores (see Table 2.8). The most abundant of these, SVS3, is a component of the copulatory plug

primarily secreted by the seminal vesicle gland. The coagulating gland secretions of subordinate males contained a significantly smaller proportion of SVS3 than found within the coagulating glands of dominant males (df = 7, t = -3.46, *P* = 0.002), or control males (df = 7, t = -3.83, *P* = 0.001) (see Figure 2.18).

Figure 2.15: (a) Progenesis LC-MS abundances (normalised within the software) and (b) Proportional abundances of the major seminal vesicle proteins within the seminal vesicle secretions of dominant, subordinate and control males. Bars represent mean ± sem.

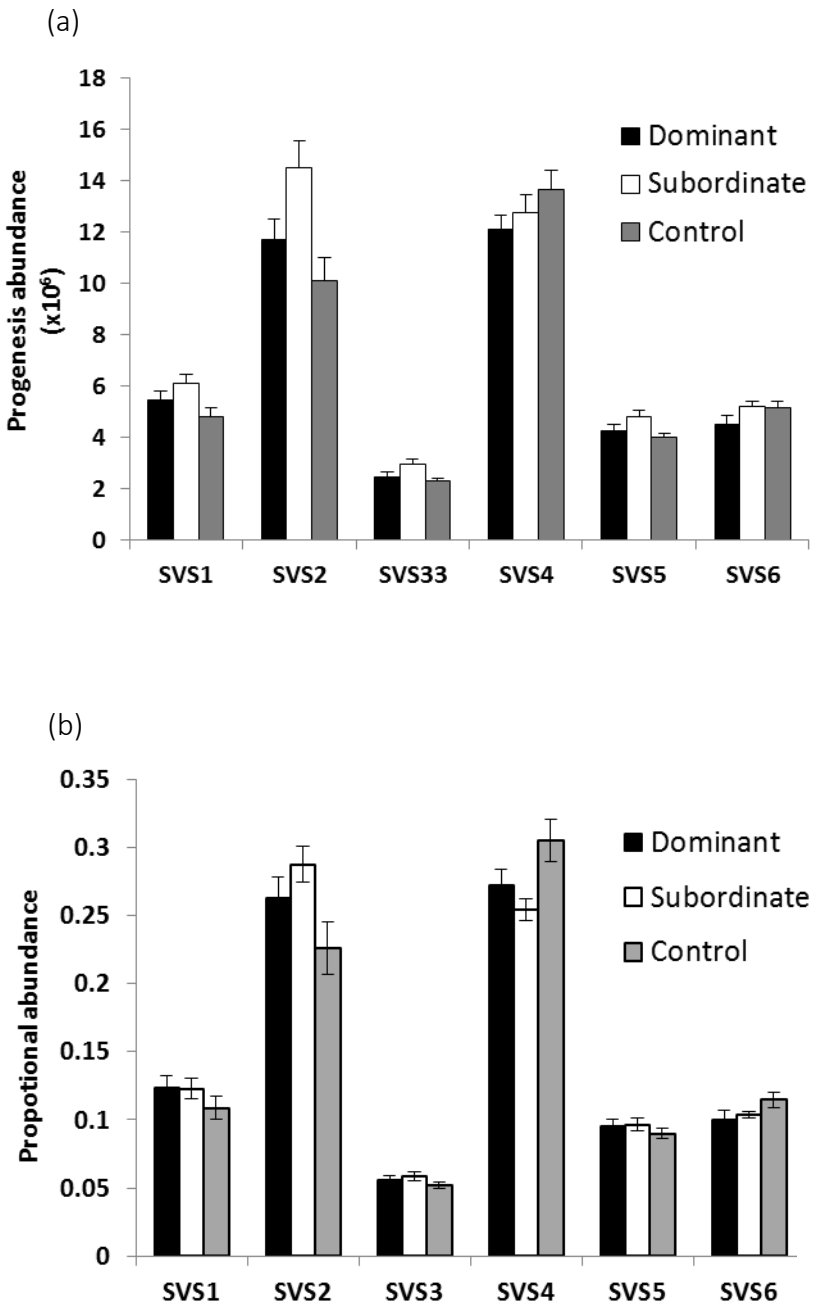


Figure 2.16: Plots of the Progenesis LC-MS abundances of four major seminal vesicle secretory (SVS) proteins within dominant, subordinate and control male seminal vesicle secretions. Each grey point represents the value for one individual. The black circles and lines represent the mean values.

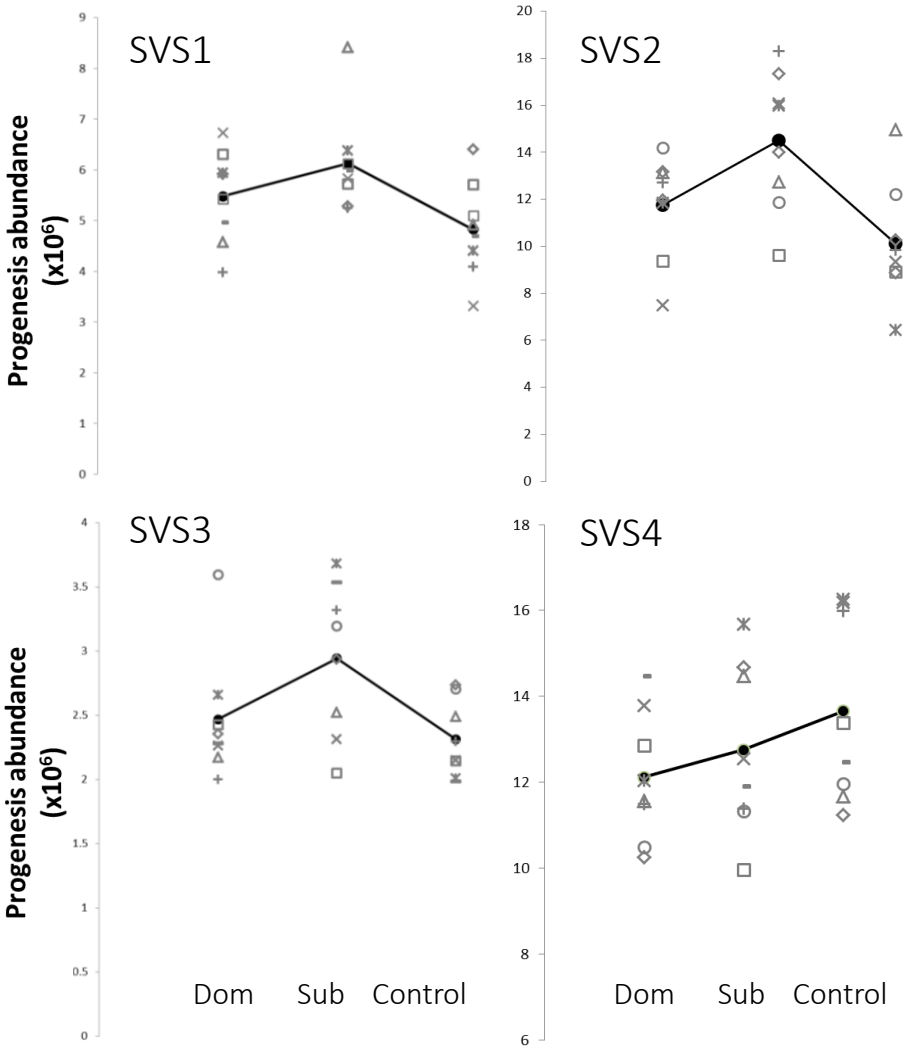


Table 2.4: Results of paired t.tests on the major seminal vesicle secretory (SVS) proteins comparing the normalised Progenesis LC-MS abundance data from dominant and subordinate male seminal vesicle secretion samples.

Protein name	t	*P*
SVS1	-1.19	0.27
SVS2	-2.19	0.06
SVS3	-1.95	0.09
SVS4	-0.63	0.54
SVS5	-3.65	0.008
SVS6	-1.54	0.17

Table 2.5: Table of prediction accuracy scores from random forest analysis of proteomic data. Scores are based on the number of samples correctly classified following 1000 iterations of each random forest model.

Sample	Dom	Sub	Control	Overall
SV secretion - CLR	87.4%	97.3%	51.2%	78.6%
SV secretion -not CLR	68.9%	100%	81.1%	83.3%
Coagulating glands – all proteins	99.2%	87.5%	100%	95.6%
Coagulating glands – 77 proteins	95.9%	87.5%	98.6%	94.0%
Ejaculate	85.4%	99.9%	57.2%	97.7%
According to mating	**1st**	**2nd**		
Ejaculate	96.0%	99.9%		97.7%

Table 2.6: Table of variable importance scores (Vimp) for proteins significantly important, having a Vimp higher than the absolute lowest value, in classifying the samples correctly, according to social status, following random forest analysis of CLR transformed proteomic data from the seminal vesicle secretion. Each score is a mean of 1000 iterations of the random forest model. Accession numbers marked with an * indicate proteins important within this and the non-transformed model.

Accession	Protein name	Mean abundance (%)	Vimp (x100)	95% CI
Q06890*	Clusterin	0.011	1.244	1.23 - 1.26
Q8R2E9*	ERO1-like protein beta	0.019	0.733	0.72 - 0.74
Q9Z0J0*	Epididymal secretory protein E1	0.003	0.692	0.68 - 0.70
Q62216*	SVS2	25.86	0.459	0.45 - 0.47
Q05186*	Reticulocalbin-1	0.003	0.437	0.43 - 0.45
P58252*	Elongation factor 2	0.025	0.418	0.41 - 0.43
Q920F6	Structural maintenance of chromosomes protein 1B	0.975	0.393	0.39 - 0.40
P30933	SVS5	9.389	0.318	0.31 - 0.33
P09411	Phosphoglycerate kinase 1	0.058	0.251	0.24 - 0.26
P81117	Nucleobindin	0.213	0.190	0.18 - 0.20
Q6WIZ7	SVS1	11.829	0.180	0.17 - 0.19
Q8VI13	SVS3	5.523	0.163	0.16 - 0.17
P10126	Elongation factor 1-alpha 1	0.003	0.156	0.15 - 0.16
Q6ZWY9*	Histone H2B type 1-C/E/G	0.002	0.151	0.15 - 0.17
Q8C0D4	Rho GTPase-activating protein 12	0.018	0.107	0.10 - 0.11
Q64356	SVS6	10.607	0.096	0.09 - 0.10

Table 2.7: Table of variable importance scores (Vimp) for proteins significantly important, having a Vimp higher than the absolute lowest value, in classifying the samples correctly, according to social status, following random forest analysis of relative quantitative proteomic data from the seminal vesicle secretion. Each score is a mean of 1000 iterations of the random forest model. Accession numbers marked with an * indicate proteins important within this and the CLR-transformed model.

Accession	Protein name	Mean abundance (%)	Vimp (x100)	95% CI
P58252*	Elongation factor 2	0.025	0.853	0.84 - 0.86
Q9Z0J0*	Epididymal secretory protein E1	0.003	0.639	0.63 - 0.65
Q06890*	Clusterin	0.011	0.515	0.51 - 0.52
P24369	Peptidyl-prolyl cis-trans isomerase B	0.004	0.438	0.43 - 0.45
P05213	Tubulin alpha-1B chain	0.724	0.307	0.30 - 0.31
Q09098	Prostate and testis expressed protein 4	0.977	0.259	0.25 - 0.27
Q62216*	SVS2	25.86	0.180	0.17 - 0.19
Q8R2E9*	ERO1-like protein beta	0.019	0.160	0.15 - 0.17
Q8BND5	Sulfhydryl oxidase 1	0.326	0.156	0.15 - 0.16
Q8CEK3	Serine protease inhibitor kazal-like protein	2.06	0.148	0.14 - 0.15
Q6ZWY9*	Histone H2B type 1-C/E/G	0.002	0.144	0.14 - 0.15
Q05186*	Reticulocalbin-1	0.003	0.140	0.13 - 0.15

Figure 2.17: Boxplot of the centred log ratio (CLR) transformed proportional abundance of SVS2 within seminal vesicle protein secretions of dominant, subordinate and control males.

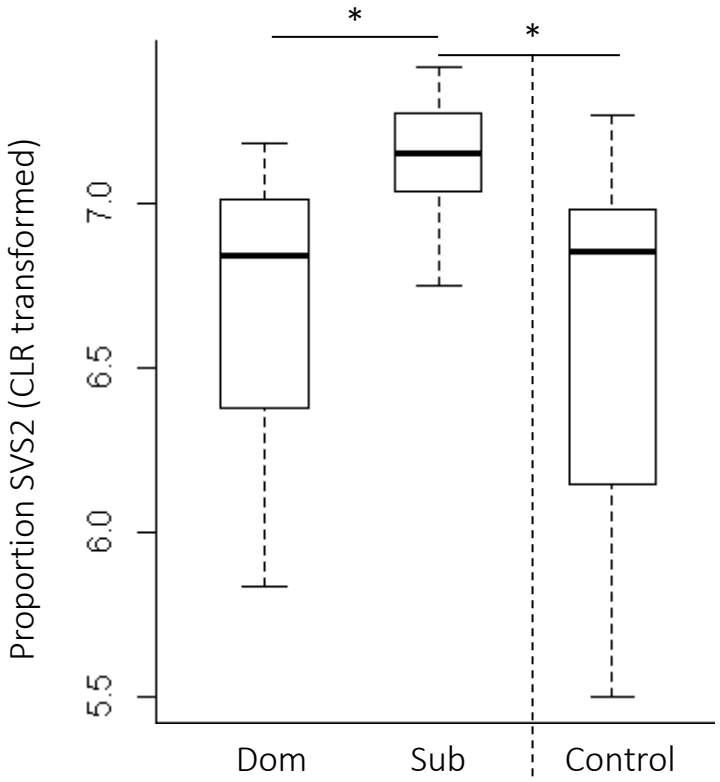
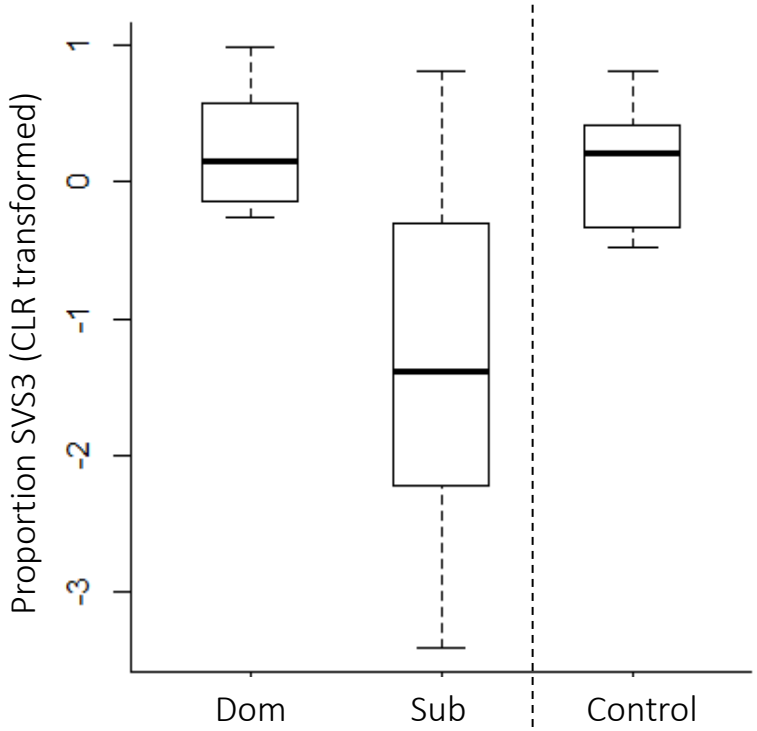


Table 2.8: Table of variable importance scores (Vimp) for proteins significantly important in classifying the samples correctly, according to social status, following random forest analysis of the reduced set of 77 proteins quantified using proteomic analysis of the coagulating glands. Each score is a mean of 1000 iterations of the random forest model.

Accession	Protein name	Mean abundance (%)	Vimp (x100)	95% CI
Q8VI13	SVS3	0.125	0.614	0.60 - 0.62
P06151	L-lactate dehydrogenase A	0.887	0.477	0.47 - 0.49
Q9CZS1	Aldehyde dehydrogenase X	0.0002	0.431	0.42 - 0.44
P01887	Beta-2-Microglobulin	0.041	0.218	0.21 - 0.22
P17156	Hspa2	0.014	0.218	0.21 - 0.23

Figure 2.18: Boxplot of the centered log ratio (CLR) transformed proportional abundance of SVS3 within coagulating gland protein secretions of dominant, subordinate and control males.



2.4.11 According to social status

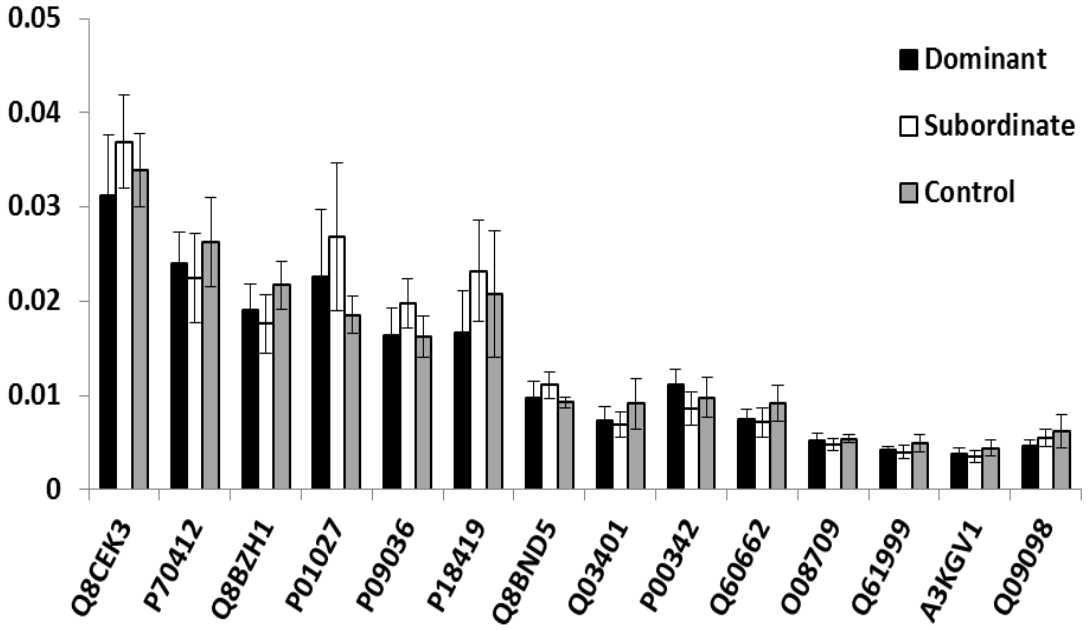
Analysis of ejaculate proteins according to social status was limited by the small number of individuals within each group. To account for this, the data from both the first and second matings were pooled as described within Section 2.3.16. Differences between the first and second ejaculates could therefore be masking some of the effect of social status. A total of 682 proteins were identified, 59 of which could be confidently considered male specific after cross referencing with other studies (Huck, Lisk *et al.* 1986; Dean, Clark *et al.* 2009) and previous work. RF analysis of just the 59 proteins only classified the data correctly, with respect to social status, 43.2% of the time (10 out of 25 correct, bootstrapping analysis of 1000 replicates: $P = 0.36$). Analysis of all 682 proteins had a prediction accuracy of 82.7% (20 out of 25 correct, bootstrapping analysis of 1000 replicates: $P < 0.001$). From the analysis of the full data set, there were 21 proteins that had significant variable importance scores (see Table 2.9 and Figure 2.20). Most of these were very low abundance proteins, limiting the confidence of further analysis as this could be due to slight differences in the mass spectrometry analysis of the samples from the first and second matings, as these were performed at different times. The proportions of the most abundant ejaculate proteins, grouped according to social status, are shown in Figure 2.19.

2.4.12 According to first / second mating

Following RF analysis, prediction accuracy for classifying the first compared to second ejaculates was better when using the reduced data set of 59 male specific proteins (97.7% correct for 682 proteins, compared to 100% correct when using 59 proteins, bootstrapping analysis of 1000 replicates: $P < 0.001$ for both). As using the reduced data set also removes the proteins derived from the female, this is the analysis presented here. In total, 20 proteins had significant variable importance scores (see Table 2.10). Of these, SVS4 and PATE4 (also known as SVS7) were relatively abundant and found in significantly greater proportions within the second

mating (SVS4: t = 2.75, df = 21, P = 0.012, PATE4: t = 5.16, df = 21, P < 0.001) see Figure 2.20 and Table 2.11. Care should be taken when interpreting the results of data that has been pooled from separate mass spectrometry analyses. This is discussed within Section 2.5.3.

Figure 2.19: Proportions of the most abundant proteins within the ejaculate according to social status. Serum albumin, which accounts for approximately 30% of all ejaculate proteins identified, is excluded. Bars represent mean ± sem.



Accession	Protein name
Q8CEK3	Serine Protease Inhibitor Kazel-like
P70412	CUZD1
Q8BZH1	TGM4
P01027	Complement C3
P09036	SPINK3
P18419	SVS4
Q8BND5	Sulfhydryl oxidase 1
Q03401	CRISP1
P00342	L-Lactate dehydrogenase C
Q60662	A-Kinase Anchor Protein 4
O08709	Peroxiredoxin-6
Q61999	Outer dense fiber protein 1
A3KGV1	Outer dense fiber protein 2
Q09098	PATE4

Table 2.9: Table of variable importance scores (Vimp) for proteins significantly important in classifying the samples correctly, according to social status, following random forest analysis of relative quantitative proteomic data for the 682 ejaculate proteins identified. Each score is a mean of 1000 iterations of the random forest model.

Accession	Protein description	Mean abundance%	Vimp (x100)	95 %CI
P57780	Alpha-actinin-4	0.0062%	0.082	0.079 - 0.085
O54782	Epididymis-specific alpha-mannosidase	0.0038%	0.056	0.054 - 0.059
Q62000	Mimecan	0.0083%	0.054	0.052 - 0.057
Q9DBJ1	Phosphoglycerate mutase 1	0.0187%	0.049	0.046 - 0.052
Q99JY9	Actin-related protein 3	0.0295%	0.047	0.044 - 0.050
Q91XA2	Golgi membrane protein 1	0.0036%	0.045	0.042 - 0.047
P09103	Protein disulfide-isomerase	0.0045%	0.042	0.040 - 0.045
Q8BH61	Coagulation factor XIII A chain	0.0041%	0.040	0.037 - 0.042
O70251	Elongation factor 1-beta	0.0086%	0.038	0.036 - 0.040
P47753	F-actin-capping protein subunit alpha-1	0.0013%	0.033	0.031 - 0.035
P10107	Annexin A1	0.0625%	0.033	0.030 - 0.035
Q9Z183	Protein-arginine deiminase type-4	0.0032%	0.033	0.030 - 0.035
Q9Z1Q5	Chloride intracellular channel protein 1	0.0062%	0.030	0.028 - 0.031
P06869	Urokinase-type plasminogen activator	0.0215%	0.029	0.027 - 0.032
A2BE28	Protein LAS1 homolog	0.5373%	0.026	0.024 - 0.028
Q6PDI5	Proteasome-associated protein ECM29 homolog	0.0103%	0.025	0.023 - 0.027
Q08879	Fibulin-1	0.0085%	0.024	0.022 - 0.027
P20918	Plasminogen	0.0153%	0.024	0.022 - 0.026
Q63836	Selenium-binding protein 2	0.0100%	0.023	0.021 - 0.026
Q99JI6	Ras-related protein Rap-1b	0.0424%	0.023	0.021 - 0.025
Q8BG05	Heterogeneous nuclear ribonucleoprotein A3	0.0067%	0.023	0.021 - 0.025

Table 2.10: Table of variable importance scores (Vimp) for proteins significantly important in classifying the samples correctly, according to first or second mating, following random forest analysis of relative quantitative proteomic data for the reduced set of 59 ejaculate proteins. Each score is a mean of 1000 iterations of the random forest model.

Accession	Description	Mean abundance (%)	Vimp (x100)	95% CI
Q3UN54	Secreted seminal-vesicle Ly-6 protein 1	0.15%	3.664	3.64 - 3.69
Q6NY15	Testis-specific gene 10 protein	0.01%	2.559	2.54 - 2.58
Q09098	PATE4	1.02%	2.419	2.40 - 2.44
P15265	Sperm mitochondrial-associated cysteine-rich protein	0.22%	2.316	2.30 - 2.34
P08228	Superoxide dismutase [Cu-Zn]	0.13%	2.182	2.16 - 2.20
P45700	Mannosyl-oligosaccharide 1,2-alpha-mannosidase IA	0.04%	1.606	1.59 - 1.62
P06869	Urokinase-type plasminogen activator	0.04%	1.526	1.51 - 1.54
Q60736	Zona pellucida sperm-binding protein 3 receptor	0.13%	1.080	1.07 - 1.09
Q62522	Zona pellucida-binding protein 1	0.06%	0.983	0.97 - 1.00
P35487	Pyruvate dehydrogenase E1 component subunit alpha, testis-specific form, mitochondrial	0.19%	0.871	0.86 - 0.88
C4P6S0	Sperm head and tail associated protein	0.03%	0.770	0.76 - 0.78
Q03401	Cysteine-rich secretory protein 1	1.51%	0.713	0.70 - 0.72
P18419	SVS4	3.82%	0.328	0.32 - 0.34
Q62216	SVS2	0.07%	0.310	0.30 - 0.34
P35700	Peroxiredoxin-1	0.14%	0.259	0.25 - 0.27
P30933	SVS5	0.08%	0.255	0.25 - 0.26
O08976	Probasin	0.03%	0.250	0.24 - 0.26
Q9DA48	Sperm acrosome membrane-associated protein 1	0.03%	0.172	0.17 - 0.17
Q9Z0J0	Epididymal secretory protein E1	0.33%	0.165	0.16 - 0.17
P00015	Cytochrome c, testis-specific	0.02%	0.076	0.07 - 0.08

Figure 2.20: Plot of CLR transformed abundances of proteins with significant variable importance scores following random forest analysis predicting 1st or 2nd mating. The data for dominant, subordinate and control males has been pooled here as there were no identifiable differences between these groups. Proteins are presented in order of the difference between 1st and 2nd mating (biggest increase – biggest decrease). Proteins to the left of the plot proportionally increased in 2nd mating, proteins to the right proportionally decreased. Circles represent mean ± sem.

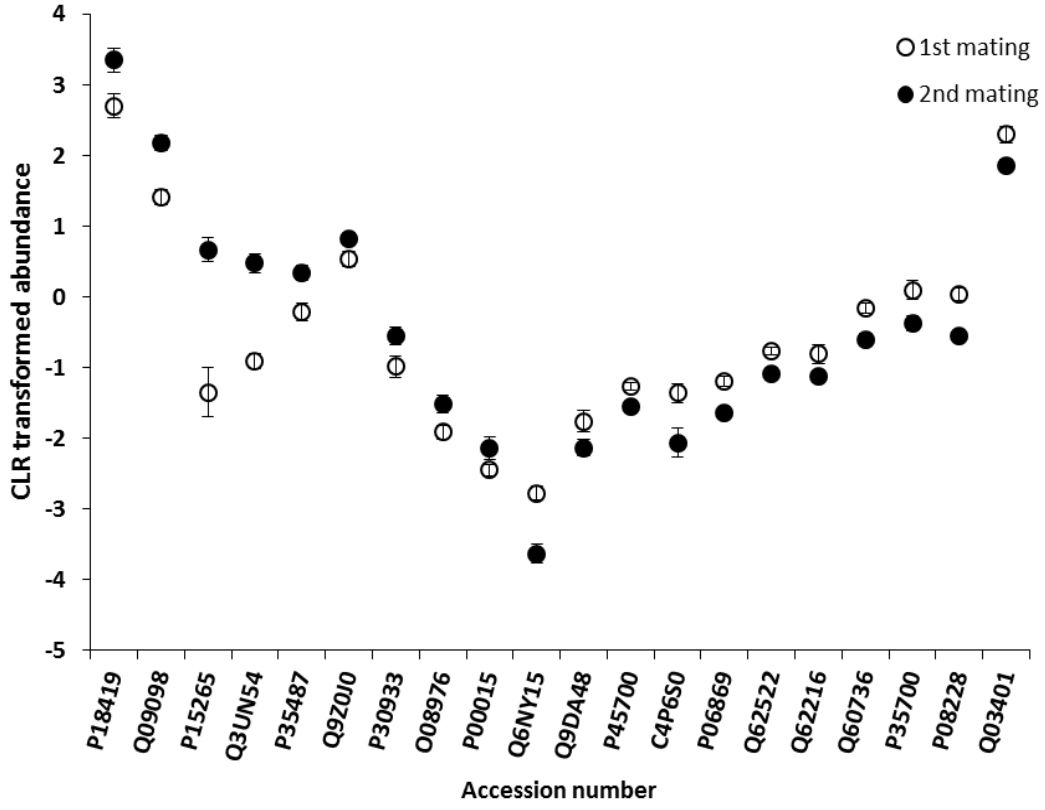


Table 2.11: The proportional abundance of proteins deemed significantly important to classifying the data correctly, according to first or second mating, following random forest analysis.

Accession	Description	M1	M2	M1-M2
P18419	SVS4	2.98%	4.88%	-1.90%
Q09098	PATE4	0.74%	1.37%	-0.63%
P15265	Sperm mitochondrial-associated cysteine-rich protein	0.09%	0.38%	-0.30%
Q3UN54	Secreted seminal-vesicle Ly-6 protein 1	0.07%	0.25%	-0.18%
P35487	Pyruvate dehydrogenase E1 component subunit alpha, testis-specific form, mitochondrial	0.16%	0.23%	-0.07%
Q9Z0J0	Epididymal secretory protein E1	0.32%	0.35%	-0.03%
P30933	SVS5	0.07%	0.09%	-0.02%
O08976	Probasin	0.03%	0.03%	-0.01%
P00015	Cytochrome c, testis-specific	0.01%	0.02%	-0.01%
Q6NY15	Testis-specific gene 10 protein	0.01%	0.00%	0.01%
Q9DA48	Sperm acrosome membrane-associated protein 1	0.03%	0.02%	0.01%
P45700	Mannosyl-oligosaccharide 1,2-alpha-mannosidase IA	0.05%	0.03%	0.01%
C4P6S0	Sperm head and tail associated protein	0.04%	0.02%	0.02%
P06869	Urokinase-type plasminogen activator	0.05%	0.03%	0.02%
Q62522	Zona pellucida-binding protein 1	0.07%	0.05%	0.02%
Q62216	SVS2	0.08%	0.05%	0.03%
Q60736	Zona pellucida sperm-binding protein 3 receptor	0.15%	0.09%	0.07%
P35700	Peroxiredoxin-1	0.18%	0.10%	0.08%
P08228	Superoxide dismutase [Cu-Zn]	0.16%	0.09%	0.08%
Q03401	Cysteine-rich secretory protein 1	1.91%	1.00%	0.91%

2.5 Discussion

This study shows that males alter their ejaculate investment decisions according to their social status. Previous studies have tended to focus on differential investment in sperm (Koyama and Kamimura 1998; 2000; Liljedal and Folstad 2003; Pizzari, Cornwallis *et al.* 2003; Montrose, Harris *et al.* 2008; Lemaitre, Ramm *et al.* 2012). Studies of rodent species, however, did not support predictions of sperm competition theory; that disadvantaged subordinate males should compensate by increasing investment in sperm production (Parker 1990). Here, investment in the entire ejaculate, as well as mating behaviour, was tested. Proteomics analysis uncovers that male house mice can plastically alter the composition of their seminal vesicle secretion. This supports more recent theory that considers the importance of seminal fluid components as well as sperm (Cameron, Day *et al.* 2007). This theory predicts that for species in which seminal fluid components can greatly affect fertility, such as those that produce a copulatory plug, males within a disfavoured role may gain a greater advantage through increasing their seminal fluid output. The results presented here support this, as subordinate males produce proportionally more SVS2, a major component of the copulatory plug. Subordinate males are disadvantaged by having significantly smaller seminal vesicle glands than the dominant males, yet despite this produce an equivalently sized copulatory plug. Combining analysis of ejaculate production with mating behaviour and ejaculates gives a comprehensive picture of male reproductive investment for a species in which seminal fluid can have a large impact on fertility.

2.5.1 Ejaculate investment varies according to social status

The major novel finding of this study is that male house mice can plastically alter the composition of their seminal fluid. Subordinate males are shown to produce proportionally more SVS2 within their seminal vesicle glands. SVS2 is a major component of the copulatory plug, being readily cross-linked by transglutaminase (Williams-Ashman 1984; Huck, Lisk *et al.* 1986; Clark and Galef 1998). Further, SVS2 impacts fertilisation success by acting as a decapacitation factor and improving

sperm survival in the uterus (Kawano and Yoshida 2007; Montrose, Harris *et al.* 2008). Previous studies have shown rapid divergence of SVS2 within rodents (Ramm, Oliver *et al.* 2008; Dean, Clark *et al.* 2009; Ramm, McDonald *et al.* 2009), and it's homologue semenoclotin within primates (Gandelman, Vom Saal *et al.* 1977). This protein is therefore likely to be highly important for improving fertility, particularly within competitive matings.

An additional finding from proteomic analysis of the accessory sex glands was the increased proportion of SVS3 found within the coagulating glands of dominant mice. SVS3 is another component of the copulatory plug, primarily produced by the seminal vesicle glands (Lin, Luo *et al.* 2002). As SVS3 only accounts for a small proportion of the coagulating gland secretion (≈0.1%), this is less likely to be functionally significant than the differences found for SVS2. Regardless, the classification accuracy given by the RF analysis supports that there are significant differences between the coagulating gland secretions of males depending on their social status. When also considering that the males within the dominant and subordinate pairs are siblings, these findings are not just due to genetic variation but instead are a response to differences in their social status.

Experiment 1 also measured differences in the overall physiology of males of differing social status. Subordinate males had smaller seminal vesicle glands and produced fewer sperm, therefore showing a lowered overall reproductive investment in ejaculates. The decreased investment in sperm production agrees with previous studies in laboratory strains of house mice (Koyama and Kamimura 1998; 2000). There is also evidence from a different rodent species, the bank vole, that dominant males have larger seminal vesicle glands (Lemaitre, Ramm *et al.* 2012). Here, considering the seminal vesicle secretion in addition to the overall gland size, I found that subordinate males' seminal vesicle secretion has a higher protein concentration. This does not suggest that subordinate males produce an equivalent total amount of seminal proteins, however it is possible that differences in the

weight of the seminal vesicles may not entirely represent differential investment in seminal protein production.

Despite the difference in seminal vesicle size of dominant and subordinate males, there was no difference in the size of the copulatory plugs they produce, in either the first or second matings. It is possible that the observed differences in the composition of the seminal vesicle secretion enable the subordinate males to produce a plug equivalent to the dominant males; unfortunately this could not be tested directly here. Although there were no differences in copulatory plug size, dominant males ejaculated more sperm than subordinate males. This was also found previously in the bank vole, as subordinate males ejaculated fewer sperm and there was no difference in the size of the copulatory plug (Lemaitre, Ramm *et al.* 2012).

Dominant males needed fewer trials prior to their first successful copulation, but more trials in between ejaculations. The size of the copulatory plug correlated with the number of trials before a male would successfully mate, for both the first and second copulations. In contrast, the number of sperm within the ejaculate was only correlated with the number of trials prior to the second mating. Dominant males may have taken longer in between ejaculations in order to replenish their sperm reserves, as sperm take longer to produce than the seminal fluid proteins (Claydon, Ramm *et al.* 2012). The extended break between copulations may also be due to differences in the mating behaviour of dominant and subordinate males.

Although there was no difference in the pattern of behaviour within each copulation, such as intromission rate and ejaculate duration, dominant males mated for significantly longer and performed more intromissions and thrusts. Male house mice are known to adapt their mating duration according to the level of local competition (Preston and Stockley 2006). Males mating in the presence of rivals ejaculate sooner than those with no competition. Subordinate males may be ejaculating more rapidly to avoid being cuckolded by a dominant male. Dominant male golden hamsters continue to copulate with long intromissions even after their sperm stores have depleted (Huck, Lisk *et al.* 1986). A long mating could act as a form of mate guarding.

Here, the dominant male could have been employing this approach. A longer mating duration and more thrusts correlates with a greater number of ejaculated sperm (see Chapter 4). An increased number of thrusts may function to stimulate males to release more sperm. However, it is also possible that males with higher status also have greater sperm reserves and are able to monopolise the female for longer. Regardless, it is clear that dominant males invest a greater amount in each mating than the subordinate males, which may account for their reluctance to remate.

Combined, this analysis of mating behaviour, ejaculate production and allocation, supports current, relevant theory (Cameron, Day *et al.* 2007). There is an extreme bias in fertilisation success rates of dominant and subordinate house mice, with dominant males siring around 90% of offspring in a semi-natural environment (DeFries and McClearn 1970). Current theory predicts that in this instance, with a large level of bias, subordinate males should invest less overall in their ejaculate production (Cameron, Day *et al.* 2007). Of the energy invested in ejaculate production, sperm competition models predict subordinate males should allocate most of this towards sperm production, in order to compensate for their disfavoured role (Parker 1990; Cameron, Day *et al.* 2007). There is growing evidence, however, that this is not true within rodent systems (Koyama and Kamimura 1998; 2000; Lemaitre, Ramm *et al.* 2012). In house mice, components of the seminal fluid produce the copulatory plug. The presence of a plug can have a large impact on fertility (Dean 2013). Therefore, within this system, a male can gain a greater advantage in increasing investment in seminal fluid production than producing more sperm. Current theory also accounts for differential investment being predicted by the degree with which a male can gain from increased investment. The results here are both in agreement with theory (Cameron, Day *et al.* 2007), and previous empirical studies (Lemaitre, Ramm *et al.* 2012). Differences in the composition of the seminal vesicle secretion highlight that comparing gross production measures, such as gland size, may mask differences in the relative production of specific proteins that could have functional significance to a male's fertilisation success.

Overall, these results show that dominant and subordinate males exhibit distinct reproductive phenotypes. Dominant males have a greater overall investment in ejaculates, producing more sperm, more seminal fluid and mating for a longer duration. However, subordinate males may gain a greater advantage through investing in copulatory plug formation than increasing sperm production. Producing a greater proportion of SVS2 may enable subordinate males to produce a large copulatory plug, despite differences in seminal vesicle mass.

2.5.2 Potential for plasticity in social status

Differences in sperm production were found, despite no difference in testes mass. This has been found previously when house mice plastically increase their sperm production due to an elevated risk of sperm competition (Ramm and Stockley 2009a). Subordinate male house mice could have the opportunity to be a dominant, territory holding male in the future and maintaining testes mass may allow males to rapidly increase sperm production when required.

It has been suggested that subordination stress physiologically inhibits sperm production in rodents (Blanchard, Sakai *et al.* 1993). This was not tested here, as understanding the physiological mechanisms of differential investment was not the primary aim of the study. However, corticosterone levels are commonly measured as a biomarker of stress as they can be tested non-invasively (Touma, Palme *et al.* 2004). Corticosterone is a mammalian stress hormone, generally found to be increased in subordinate male house mice (Louch and Higginbotham 1967; Ely and Henry 1978; Keeney, Jessop *et al.* 2006) but see (Koyama and Kamimura 1998). Here, I found that subordinate males still housed with their dominant sibling had highly elevated faecal corticosterone levels. If stress is the main cause of supressed reproductive investment, the physiological indicator of chronic stress is rapidly reduced to normal levels after males are separated. Despite this, behaviour and reproductive investment of males varied according to social status. These results show that co-housing of male siblings is stressful for the subordinate male. Separation reduces this stress, however this does not lead to a rapid change in

reproductive investment of either dominant or subordinate males to a level akin to being a single male.

2.5.3 The use of proteomics analysis

Using emerging proteomics techniques has allowed novel questions to be answered within this study. As this was a new application of this technology there were inevitably some methodological challenges. Some caution should be taken when interpreting the results from the ejaculate data. There were too few samples available to look for differences according to social status within ejaculates from each males' first or second mating. As a result, data from the first and second ejaculates had to be pooled. These samples had been digested and analysed using mass spectrometry separately. The acquisition of MS data is, to some extent, subject to random variation in the detection of peptide ions (Good and Coon 2006). Progenesis LC-MS software can account for this variation between samples that have been analysed within a close time frame. However, it is unknown how effective this method is at accounting for samples digested and analysed separately. Regardless, there were considerable differences between the first and second mating, which are unlikely to be accounted for by technical variation alone. Furthermore, the proteins that show the greatest difference are SVS4 and PATE4 (SVS7). These proteins are produced within the seminal vesicle glands, which show a rapid rate of protein turnover (Claydon, Ramm *et al.* 2012). It could be expected that seminal vesicle derived proteins, which are produced rapidly, would be found in higher proportion within the second ejaculate. See Chapter 4 for further analysis of ejaculate protein composition.

Additional testing gave me confidence in the difference in the proportion of SVS2 found within the seminal vesicle secretions of dominant and subordinate male mice. Visualisation of the protein composition using SDS-PAGE suggests variation in the thickness of the SVS2 band according to social status, although this was not measured as it would not be accurate. The result was confirmed by performing proteomics analysis on whole seminal vesicle samples, tissue and secretion, from

each male. This data is not reported here as the results were far more variable due to technical difficulties. Seminal vesicle secretion in solution precipitates after freezing, which I was unaware of. After homogenising, samples were frozen prior to performing a tryptic digest for proteomics analysis. On defrosting, unfortunately the samples were no longer homogenous and an irreversible precipitate had formed. The proteomics analysis revealed large levels of variation in the relative abundance of proteins, which was far beyond that seen within the analysis of the seminal vesicle secretion. Despite this, SVS2 was still consistently found in higher proportions within the subordinate males. However, the results from the whole sample analysis are not reported here due to a lack of consistency.

2.5.4 Control males

Singly housed males were analysed as a reference throughout this study, to determine whether dominant and subordinate males increased or decreased their investment in reproductive traits relative to an individual male. The control male could be expected to behave similar to a dominant male, as they are dominant within their own territories. However, as the dominant and subordinate males are housed in a pair they may respond to cues of sperm competition, which are known to alter sperm production and ejaculate allocation in house mice (Ramm & Stockley 2007, Ramm & Stockley 2009). Results of the initial study fitted with these predictions, as control males deposited more scent marks than the subordinate male (therefore acting as a dominant) but produced fewer sperm and smaller seminal vesicles than the dominant males (due to a lack of competition). Considering the seminal fluid proteins, the pattern observed in the abundances of the major seminal vesicle proteins (**Figure 2.15**) is less predictable, with control males behaving like neither the subordinate or the dominant male. Although only speculative, it is of note that all of the copulatory plug forming proteins (SVSI – III, Lin, Luo *et al*. 2002) tend to be in lower abundances within the control males. It is possible that control males invest less in copulatory plug production as they have not been housed in a competitive environment. Based on very small sample sizes (n=4) there was no significant difference in the copulatory plug mass from the control males compared

to the paired males, although the plug from control males tended to have a lower mass (see Figure 2.10).

2.6 Conclusions

This study combines behavioural and proteomic techniques to show that dominant and subordinate male house mice exhibit distinct reproductive phenotypes. A novel application of emerging techniques has shown that subordinate male house mice may compensate for lowered overall investment in ejaculate production, by increasing the proportion of specific seminal fluid proteins that form the copulatory plug. This study also confirms the importance of considering the entire ejaculate, beyond sperm numbers, when studying investment strategies. In species that produce a copulatory plug from their seminal fluid this is particularly important, as differential investment in specific proteins can greatly affect fertilisation success.

Chapter 3
Plasticity in female behaviour and reproductive traits according to the social environment

3.1 Chapter overview

The number of mates a female could encounter will vary according to the local population. Here, I test whether the likelihood of multiple mating causes female house mice to alter their behaviour or physiology.

Males and females have conflicting interests over reproduction. This conflict is often intensified due to sperm competition, as males adapt to compete against rival males. A greater risk of sperm competition causes males to alter sperm production, sperm

quality and mating behaviour. Females may therefore adapt to counteract the males' increased fertilising ability, to reduce the incidence of embryolethal polyspermy and maintain control over paternity.

Proteins within the oviduct act to mediate fertilisation and sperm selection. Here I test if females alter their production of these proteins according to social experience, as this could be an important mechanism mediating cryptic female choice. I used proteomics to analyse the oviduct secretion of female house mice in relation to their exposure to males. I created two treatment groups: high and low. In the "high" group females had regular interactions with three different males, whereas females in the "low" group had fewer interactions with only one male. The interactions were controlled to allow visual, olfactory and tactile stimulation, but no opportunities for mating.

I found no difference in the oviduct protein secretion of female house mice according to their exposure to males, and no difference in their gross uterine morphology. Instead, the oviduct secretion of siblings was found to be more similar, showing a strong genetic influence. I also found that females from the high likelihood of multiple mating group spent more time investigating male odour and had a larger anogenital distance, although this did not translate into differences in mating success.

3.2 Introduction

Sexual conflict occurs due to the differing interests of the sexes with regards to reproduction (Parker 1979; Arnqvist and Rowe 2005; Parker 2006) (see Chapter 1, Section 1.5). Males aim to improve their reproductive success by increasing their number of mates (Bateman 1948). As mating is more costly for females, they are

expected to be more selective, aiming to mate with only the highest quality males (Trivers 1972). Further to this, mating is often harmful to females (Fowler and Partridge 1989; Johnstone and Keller 2000; Shi and Murphy 2014) and polyspermy, when more than one sperm attempts to fertilise one egg, is commonly embryolethal (Gilbert 2000). This creates a co-evolutionary arms race as males and females adapt to counter the conflicting interests of the other sex.

Sperm competition and cryptic female choice heightens this conflict by increasing selection on reproductive traits. Sperm competition is the competition between males to fertilise a given set of ova (Parker 1970). Diverse male adaptations due to sperm competition are well documented across all taxa (see Chapter 1). This places greater selection on females to adapt in order to maintain control over paternity and reduce the incidence of polyspermy (Birkhead, Møller *et al.* 1993). Cryptic female choice occurs when females selectively alter a male's fertilisation success (Thornhill 1983; Eberhard 1996). Cryptic female choice, combined with sperm competition between males, and sexual conflict between the sexes, drives rapid rates of co-evolution (see Figure 1.1, adapted from Arnqvist and Rowe 2005).

Within rodents, male adaptations to sperm competition are well documented (see Chapter 1). Experimental evolution studies, maintaining separate monogamous and polygamous selection lines, have shown that male house mice (*Mus musculus*) maintained with high levels of sperm competition produce more sperm, which have a greater motility and gain greater success in competitive matings (Firman and Simmons 2010; Firman and Simmons 2011). The same authors also tested females from the same selection lines. Females from the polygamous lines produced more defensive ova, that had lower fertilisation rates when using IVF techniques, than those from the monogamous lines (Firman, Gomendio *et al.* 2014). Increased ova defensiveness has also been found across species within the *Mus* genus that have differing levels of sperm competition (Martín-Coello, Benavent-Corai *et al.* 2009). Here, the effect of sperm competition on ova defensiveness was as strong as it was

on sperm competitiveness (Martín-Coello, Benavent-Corai *et al.* 2009). Thus, sexual conflict causes adaptation of the female, as well as male, gametes.

Along with evolutionary responses to selection pressures, individuals exhibit plasticity in reproductive traits according to the level of competition within their local population. Phenotypic plasticity occurs when an individual alters physiological or behavioural traits in response to social or environmental cues (Via and Lande 1985). Plasticity in response to risk of sperm competition has been well described within male rodents. According to the level of competition, males can alter mating behaviour, sperm production, accessory gland size and ejaculate allocation (delBarco-Trillo and Ferkin 2004; delBarco-Trillo and Ferkin 2006; Preston and Stockley 2006; Ramm and Stockley 2007; Vaughn, delBarco-Trillo *et al.* 2008; Ramm and Stockley 2009a; Ramm and Stockley 2009b; Lemaitre, Ramm *et al.* 2010). This increased investment in male traits most likely leads to additional mating costs for the female, due to sexual conflict. There is evidence that female house mice can plastically alter their ova defensiveness according to the likelihood of multiple mating (Firman and Simmons 2013). Here, female house mice received olfactory cues and interactions, but not copulations, from either 1 or 10 males over 8 weeks, creating low and high risk of sperm competition environments. Using IVF assays, the authors found that ova collected from females in the high risk group were more defensive, resulting in a lower fertilisation rate than those from females in the low risk group (Firman and Simmons 2013). This is the first example of plasticity in female reproductive traits, in response to the social environment.

Here, I use the house mouse to investigate plasticity in female behaviour, physiology and oviduct proteins in response to the likelihood of multiple mating. There is a moderate level of sperm competition within natural populations of house mice (Dean, Ardlie *et al.* 2006), which fluctuates according to population density and sex ratio (Firman and Simmons 2008). Males in a high risk of sperm competition population produce more sperm (Ramm and Stockley 2009a). When mating with cues from competitors, males perform faster and repeated ejaculations (Preston and

Stockley 2006) and also ejaculate fewer sperm (Ramm and Stockley 2007). Females in populations with high levels of sperm competition may receive more sperm from individual males, due to repeated ejaculations, as well as being more likely to mate multiply. Females are expected to respond to this to reduce the risk of polyspermy (Frank 2000). Previous studies have found females can alter ova defensiveness (Martín-Coello, Benavent-Corai *et al.* 2009; Firman and Simmons 2013; Firman, Gomendio *et al.* 2014). As well as variation in ova defensiveness, females could also alter their behaviour and reproductive physiology in order to maintain control over paternity. Here, I test this within the house mouse

Within this study I also investigate changes within the oviduct. The mammalian oviduct is the site of fertilisation (Buhi, Alvarez *et al.* 1997). It also provides short term sperm storage and mediates sperm function and selection (Suarez and Pacey 2006; Suarez 2008b; Holt and Fazeli 2010). This complex tube is known to secrete specific proteins that can alter sperm binding, penetration and fertilisation rates (Martus, Verhage *et al.* 1998; Buhi, Alvarez *et al.* 2000; Kouba, Abeydeera *et al.* 2000; Buhi 2002; McCauley, Buhi *et al.* 2003; Killian 2004; Lyng and Shur 2009; Hanaue, Miwa *et al.* 2011). Oviduct-specific glycoprotein (OGP) is a protein secreted by the oviduct and commonly found across mammals (Martus, Verhage *et al.* 1998; McCauley, Buhi *et al.* 2003; Lyng and Shur 2009). Within the house mouse this protein is known to mediate sperm binding to the egg coat protein, ZP3 (Lyng and Shur 2009). Many other proteins within the oviduct are likely to be important for in sperm selection and binding, however OGP is the only well characterised protein to date. Females may alter their oviduct protein production in order to reduce the incidence of polyspermy and maintain control over paternity. Quantitative proteomics techniques are used here to compare the oviduct secretions of females that have been housed under differing levels of male interaction. Differences found within this study may help to highlight additional proteins that are functionally important for mechanisms of cryptic female choice.

3.3 Methods

3.3.1 Subjects & housing

Female house mice (*M. musculus domesticus*) (N=32) were housed in environments simulating a low or high likelihood of multiple mating. Subject females were from of an outbred colony founded by wild mice captured from local populations in Cheshire. Fourth generation litters were weaned at 28 days old. At around 5 weeks old (age: mean ± s.e. 35.13 ± 0.23 days) pairs of sibling females were placed into enclosures (1mx1mx90cm). There were enough litters containing four females to spread pairs of siblings evenly, so that each treatment group consisted of eight pairs of siblings from the same eight litters. Each enclosure contained three open M3 cages with lids containing food and water placed to the side, loose bedding and two cardboard shelters.

The males used to provide stimulus were 5 week old, virgin males (N=32). Adult males, which had previously bred successfully (N=16), were used later in the experiment, to analyse mating behaviour of the test females.

3.3.2 Male stimulus

For 12 weeks, a combination of male odour and controlled interactions was used to create environments representing either a high or low likelihood of multiple mating. Females in the high group encountered three different males each week, and those in the low group only one. On the Monday, Wednesday and Friday of each week high group females had one male placed within one of the cages in their enclosure for 4 hours. A different male was used on each day, but the same three males were used each week. For females in the low group, on one day each week one male was placed into a cage in their enclosure for 4 hours. On the other two days a cage was closed for 4 hours, with no male inside. This protocol allowed males and females to fully interact, without allowing copulation to occur. The cages remained in the enclosures after the male had been removed, giving females a constant interaction

with the odour of the stimulus males. The cage that each male was placed in was rotated so that males would countermark the scent of the previous male and so each cage could be perceived as part of a mixed population, as opposed to an individual male's territory.

After 12 weeks of enclosure housing, females were placed individually into clean M3 cages containing soiled male bedding. This was taken from the cage of the male the female would later be paired with. The females were then removed and enclosures cleaned to clear the scent of males that had previously been within the enclosure. Females were returned to their home enclosure, remaining within their individual cages so that they could be individually identified. All females were oestrus tested (as described in Chapter 2, Section 2.10). The first female to be found in oestrus was killed and reproductive tissue samples were dissected, to analyse reproductive morphology. The other female was used to test behavioural interactions and maternal effects. This experimental design allowed physiological and behavioural differences between the females of each treatment group to be tested, along with looking for evidence of maternal effects (see Chapter 4).

3.3.3 Collecting reproductive tissue samples

Oestrus testing occurred each morning until all females had been either killed or paired. The first female of each pair shown to be in oestrus, or either of the two if both were in oestrus, was killed humanely using an overdose of halothane followed by cervical dislocation. Samples were collected when females were in oestrus as the oviduct secretions can vary throughout the oestrus cycle (Buhi 1996; Abe and Hoshi 2007; Seytanoglu, Georgiou *et al.* 2008). On days in which multiple females were to be dissected, each was killed individually so that samples were consistently collected within half an hour after death. Each female was weighed and the ano-genital distance (AGD) measured using callipers. Two measurements were made, the first was the distance between the vagina and anus; this is the most common AGD. The second measurement was taken between the anus and the urethral orifice (see Figure 3.1). Prior to analysis, the AGD was divided by the body mass of the female to

give the anogenital distance index (AGDI) value. This approach is commonly used to account for allometry (Vandenbergh and Huggett 1995; Hotchkiss and Vandenbergh 2005).

After external measurements had been collected, the uterine tracts and ovaries were dissected from the female. These were placed on clean benchkote, next to a ruler and photographed using a digital camera in order to calculate further measurements later. The tissue below the cervix was removed and the rest of the tract weighed. The top of each uterine horn was isolated and placed onto a glass slide. Excess uterine tissue was trimmed and 10µl of PBS was added to the remaining oviduct and ovary, to prevent drying out. Using a dissecting microscope (Leica), the ovaries and oviducts were teased apart. Both the left and right oviducts, along with the PBS, were collected and frozen at -20°C prior to further analysis. The ovaries were collected and stored in the same manner.

3.3.4 Measuring the uterine horn

Image J was used to measure the length and width of the uterine horns. For each digital image, a 1cm length on the ruler was measured and used to scale further measurements of that image. Measurements were taken of the length of each uterine horn and the uterine body. To measure uterine horn width, two values were taken and averaged for each horn (see Figure 3.2).

3.3.5 Proteomic analysis of oviduct samples

Oviduct samples were defrosted and washed in ammonium bicarbonate to isolate the secreted proteins from tissue proteins. To do this, the paired oviduct samples were removed from the freezer and 50µl of 50mM ammonium bicarbonate was added to each Eppendorf. Samples were left at room temperature for 20 minutes before being vortexed at full speed for 30 seconds. The supernatant was removed from the tissue samples. A Coomassie plus protein assay was performed on the supernatant to determine the protein concentration. Tryptic digests of 50µg of protein, in a 200µl final volume, were then performed as described in Chapter 2

(Section 2.3.7) for each sample. The digested peptide samples were diluted 1:1 with 97:3:0.1 HPLC grade water:MeOH:TFA and analysed using HPLC-MSMS, over a 90 minute organic gradient, as described within Section 2.3.8. Protein identification and label-free relative quantification was performed using MASCOT and Progenesis LC-MS as described in Section 2.3.9.

Figure 3.1: External genitalia of female house mice showing the ano-genital distance (AGD) measurements taken using a caliper (measurement error ±0.25mm). Adapted from Cook 1965(Cook 1965).

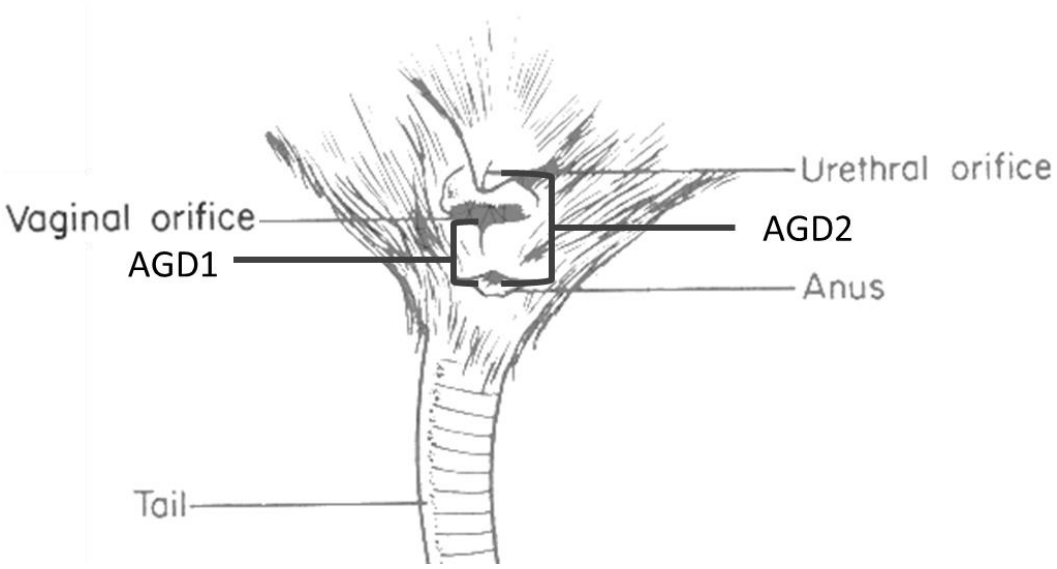
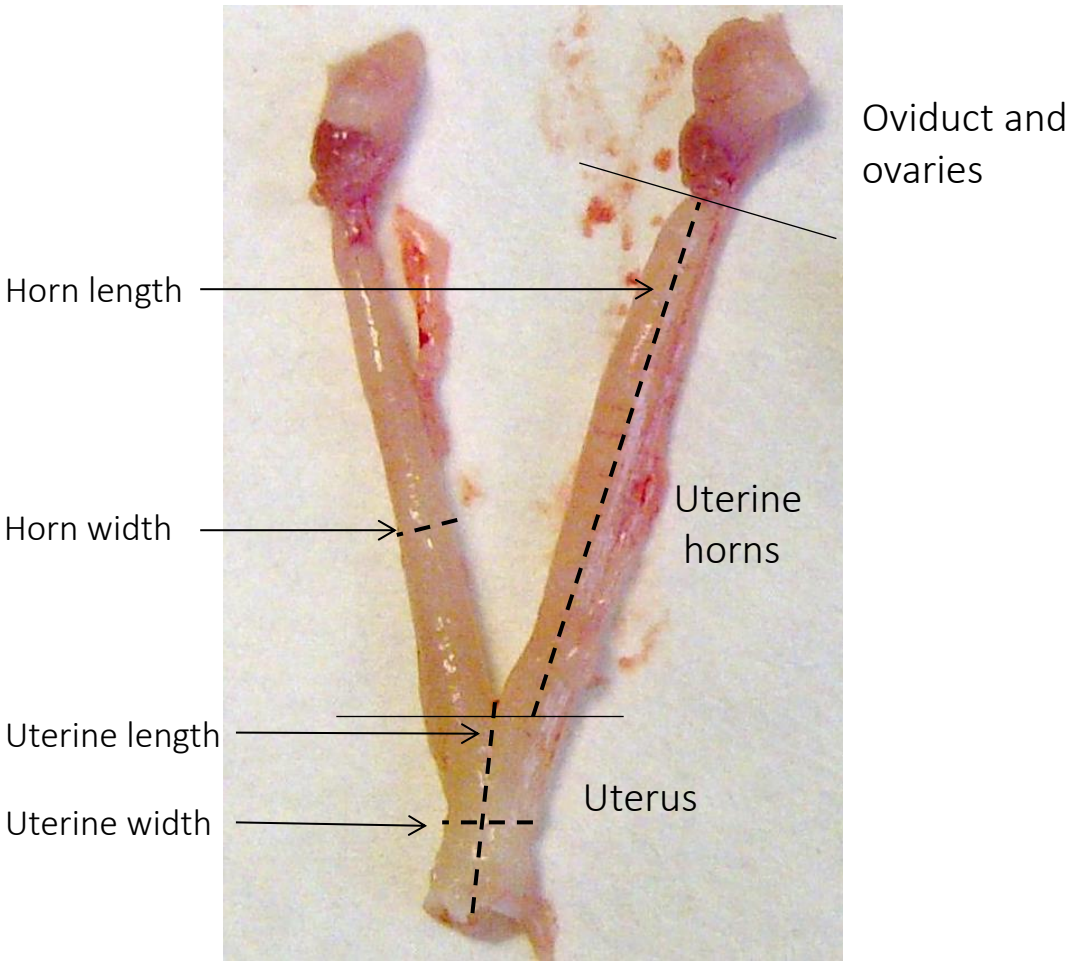


Figure 3.2: An image of a female reproductive tract from a house mouse, outlining the measurements taken. For every sample, two measurements were taken for each trait and an average used. The calculated measurement error, based on the repeatability of the measurements of these traits, was 4 ± 0.85%.



3.3.6 Testing behavioural interactions with males

One female from each enclosure pair was used to test for treatment group effects on the behaviour of females when interacting with males. After the first oestrus female had been removed from an enclosure, for the collection of reproductive tissue samples, the remaining female was oestrus tested each day. When this second female was in oestrus, she was weighed and released into the enclosure. As the behaviour was to be filmed, only a minimal amount of shelter was initially provided. Each enclosure contained an open nest box, separate nest box lid, the female's open cage with lid, and some scattered shredded paper bedding. At 1500h a male was placed into the enclosure within his M3 cage. After 10 minutes the male was released into the enclosure, the cage and open lid were left in to provide extra shelter. Each enclosure was filmed for one day from the time the male was placed into the enclosure. The next day, a cardboard nest box and additional shredded paper bedding was added. After a further 5 days, when each female would have had an oestrus cycle, the male was removed from the enclosure.

3.3.7 Video analysis

Behavioural interactions were quantified within the first hour after the male was released. Each enclosure was filmed using a DVD recorder (Panasonic DMR-EX79) set up remotely. An in house timer programme was then used to measure both the frequency and duration of a number of behaviours within the first hour of the DVD recording. The behaviours measured were: time spent together, female approaching the male, male approaching the female, sniffing, male chasing female, female sniffing or investigating the male cage, and time spent apart. Mating behaviour was not measured here due to unforeseen limitations in the experimental set up. In addition, only six pairs successfully mated to produce litters (three from each treatment group).

3.3.8 Data analysis

The behavioural and physiological data was analysed as described in Chapter 2. Here, using GLMMs to analyse the behavioural data tended to result in overdispersion. This occurs when the variance is greater than expected for a standardised distribution (Clark, Karpiuk *et al.* 1993). Ben Bolker's overdisp_fun() function was used to test for overdispersion within each model. Including an individual-level random effect can account for this by fitting the data in a similar manner to a lognormal-Poisson distribution (Zielinski, Vandenbergh *et al.* 1991). For the data analysed here that approach was not effective. Instead the data were log transformed and LMMs used as this analysis appeared to model the data effectively.

A total of 853 proteins were identified and quantified within the oviduct samples. Comparing all identified proteins with those known to be functional and present within the oviduct secretion (Buhi, Alvarez *et al.* 2000; Drickamer, Robinson *et al.* 2001; Lyng and Shur 2009) allowed me to trim this list to 91 proteins. This data set, along with the simplified list of 91 proteins that are known to be secreted by the oviduct, was analysed using Random Forest (RF) analysis as described in Chapter 2 (Section 2.16). Additionally, to test whether the protein complement of the oviduct samples was more similar within sibling pairs than treatment groups, I performed an unsupervised RF analysis on the CLR transformed data set of 91 proteins using the Random Forest package (Breiman 2001) in R (v.3.1.0) (R-Development-Core-Team 2011). With the unsupervised RF approach a grouping variable is not specified. Each RF model produces a matrix of proximity scores. These values range from 0-1 and correspond to how similar each sample is to the other samples; with a value of 1 meaning the samples are identical. I performed 1-sample t-tests on the proximity scores provided by the unsupervised model, to determine whether the score between sibling samples was significantly higher than the scores proximity scores of that individual and non-sibling samples (the other 14 individuals). This would therefore indicate to what extent genetic differences affected the protein composition of the oviduct secretion. The proximity scores according to treatment group were also analysed using a t-test for comparison.

In order to visualise the variation within the data set I produced heat maps using the pheatmap package in R (Kolde 2013). Prior to producing the heatmaps, data was CLR transformed and then standardised, with a mean of 0 and standard deviation of 1, to make them more visually comparable.

3.4 Results

3.4.1 Proteomic analysis of the oviducts

A total of 853 proteins were identified within the oviduct samples, 91 proteins were known to be functional and present within the oviduct secretion (Buhi, Alvarez *et al.* 2000; Drickamer, Robinson *et al.* 2001; Lyng and Shur 2009). Random forest (RF) analysis was not able to classify either the large, or reduced, data sets according to treatment group. In all analyses, of both CLR transformed and non-transformed data, the prediction accuracy was always 50% as each model gave all samples the same classification. Variable importance scores and bootstrapping analysis of this data is not presented here as it would not be informative. To visualise variation within the data sets I created heatmaps of the standardised values from the CLR transformed abundances (Figures 3.3 and 3.4). These suggest that the samples were grouping according to siblings as opposed to treatment groups.

The experimental design was balanced so that each female had a sibling within the other experimental group. To test whether the oviduct secretions were more similar to siblings than non-siblings, regardless of treatment group, I analysed the proximity scores from an unsupervised RF model. Using one-sample t-tests I found that, for 7 of the 8 sibling pairs, proximity scores were significantly larger between siblings than unrelated individuals (Table 3.1). A high proximity score indicates that the sibling samples were much more similar than the non-sibling samples. In contrast, the proximity scores were not different according to whether individuals were from the same or different treatment group ($t = 0.20$, $df = 118$, $P = 0.84$).

3.4.2 Female reproductive morphology

Females from the high and low likelihood of multiple mating environments did not differ in body mass by the end of the treatment ($t = -0.39$, $df = 7$, $P = 0.71$). There was also no difference in uterine measurements, the mass of the clitoral or adrenal glands, or the uterine mass according to treatment group (see Table 3.2). There was

a trend for the anogenital distance index (AGDI) to be larger in females from the high treatment group (t = 1.96, df = 7, *P* = 0.09). The AGDI measure including the external genitalia, was significantly larger in the "high" treatment group females (t = 2.73, df = 7, *P* = 0.030)(see Figure 3.1 and Section 3.3.3).

3.4.3 Behaviour when interacting with a novel male

There was no difference in the amount of time females spent with the male according to treatment group (t = 0.03, df = 7, *P* = 0.98). Females from the high treatment group spent significantly longer within the male's home cage (t = -2.42, df = 7, *P* = 0.046) and significantly more time either sniffing, or being sniffed by, the male (t = -3.07, df = 7, *P* = 0.018). There were no other significant differences in behaviour. Considering the raw data, males tended to approach and chase females from the low treatment group more often than those in the high treatment group (see Table 3.3).

Figure 3.3: Heat map of the centred log ratio (CLR) transformed and then standardised protein abundances of all proteins identified within the oviduct following proteomic analysis. CLR transformed abundances were standardised to have a mean of 0 within each sample (black on the scale). Positive values (green) have a higher abundance than the mean, and negative values (red) are less abundant than the mean.

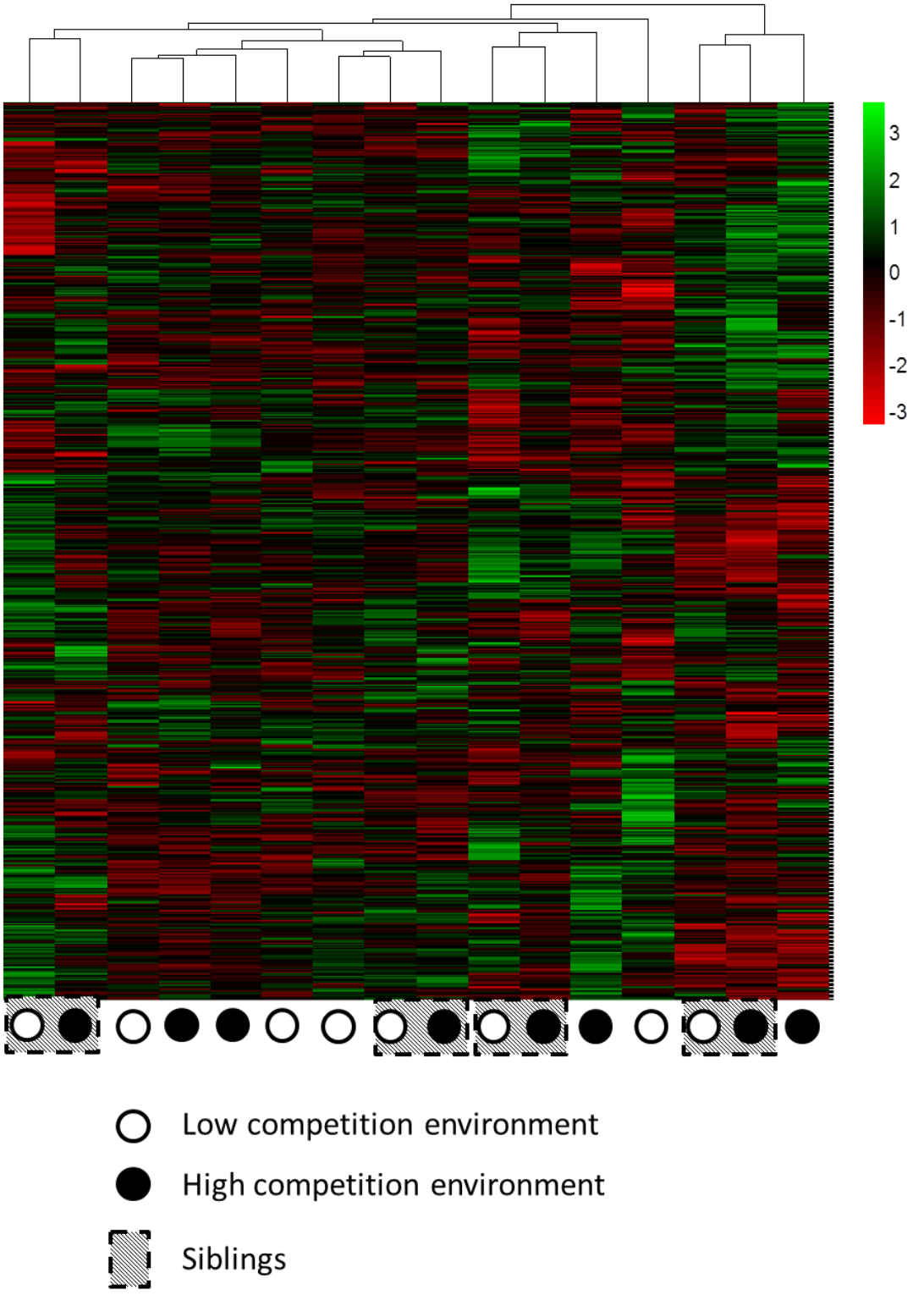


Figure 3.4: Heat map of the CLR transformed and then standardised protein abundances of 91 proteins identified within the oviduct, known to be present in oviduct secretions, following proteomic analysis. CLR transformed abundances were standardised to have a mean of 0 within each sample (black on the scale). Positive values (green) have a higher abundance than the mean, and negative values (red) are less abundant than the mean.

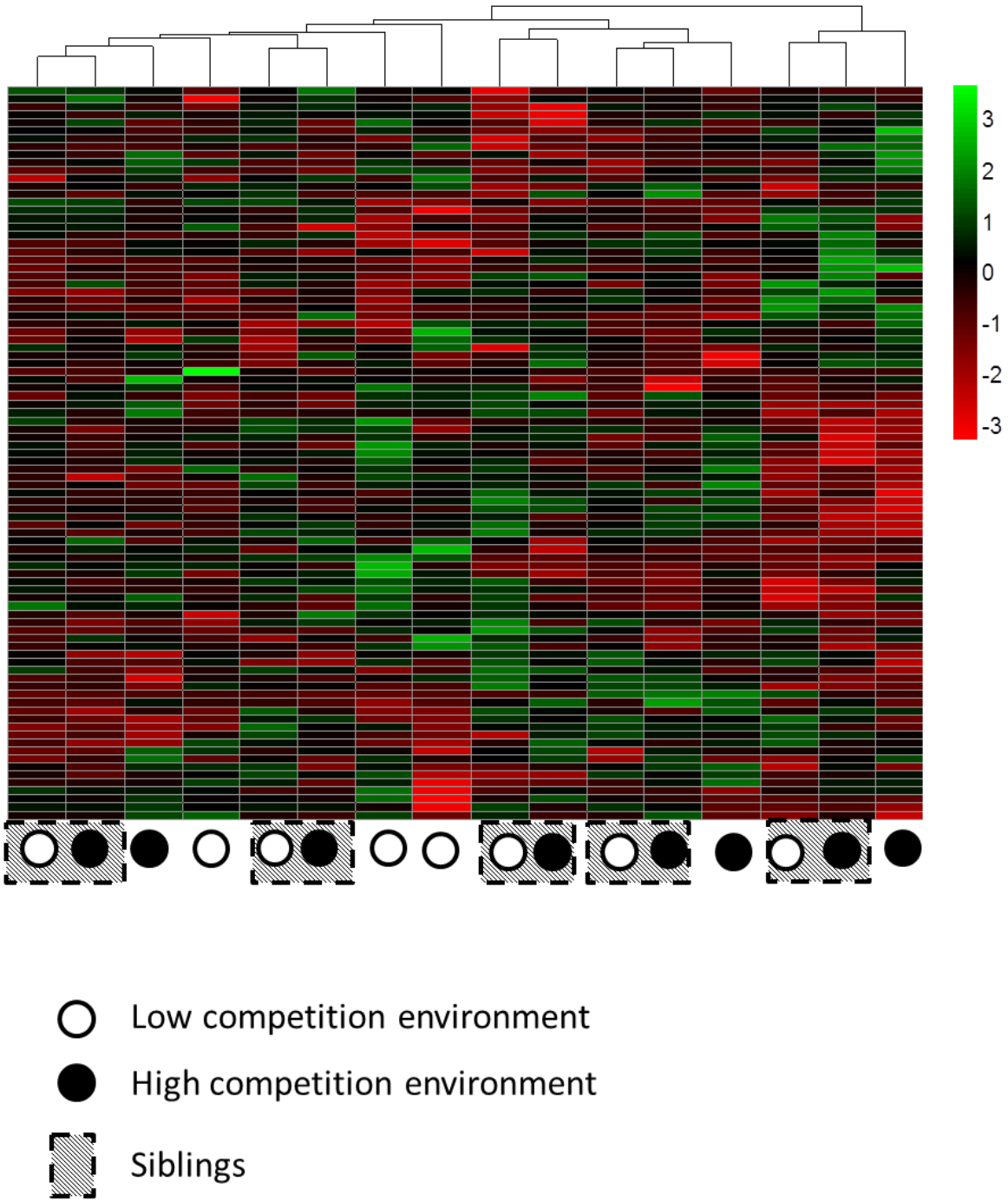


Table 3.1: Table of proximity scores taken from an unsupervised random forest model. These were used to analyse how similar siblings are to each other, compared to non-sibling females, using a 1-sample t-test.

	Proximity scores					
Female pair	Between siblings	Mean non-sib	95% CI non-sib	t	df	*P*
1/2	0.434	0.274	0.24-0.31	-10.79	13	<0.0001
3/4	0.273	0.262	0.21-0.32	-0.42	13	0.34
6/7	0.498	0.226	0.17-0.28	-9.83	13	<0.0001
7/8	0.340	0.285	0.25-0.32	-3.65	13	0.0015
9/10	0.420	0.281	0.24-0.32	-8.05	13	<0.0001
11/12	0.355	0.288	0.26-0.32	-5.04	13	0.0001
13/14	0.422	0.281	0.25-0.31	-8.48	13	<0.0001
15/16	0.443	0.272	0.23-0.31	-9.77	13	<0.0001

Table 3.2: Female reproductive morphology measurements taken from females from high and low likelihood of multiple mating treatment group environments. Results presented were analysed using a linear mixed effects model, including litter as a random factor and body mass as a covariate to account for allometry. Where body mass was not required within the model, results from a paired t-test are instead presented as these are equivalent (indicated by *).

Group effect from mixed model including: covariate = Body mass
random effect = Litter

	High risk of multiple mating		**Low risk of multiple mating**		**Group effect**		
	Mean	**s.e.**	**Mean**	**s.e.**	**df**	**t**	***P***
Body Mass (g)	16.18	1.06	16.76	0.82	7	1.30	0.235*
Clitoral glands (mg)	4.29	0.83	3.29	0.44	6	-1.38	0.218
Adrenal glands (mg)	11.34	0.91	12.50	1.15	6	0.84	0.433
AGDI (arcsine square root transformed)	0.32	0.02	0.29	0.02	7	1.96	0.090*
AGD2I (arcsine square root transformed)	0.49	0.03	0.42	0.02	7	2.73	0.030*
Uterine body width	0.33	0.04	0.41	0.04	6	2.35	0.263
Uterine horn length	1.56	0.11	1.48	0.09	6	-0.63	0.551
Uterine horn width	0.13	0.02	0.17	0.02	6	1.19	0.280
Tract mass (mg)	134.88	27.24	172.45	18.46	6	1.16	0.291

Table 3.3: Behaviour when interacting with a novel male of females from high and low likelihood of multiple mating treatment group environments. Results presented were analysed using a linear mixed effects model, including litter as a random factor. Data were transformed where required.

	High risk of multiple mating		Low risk of multiple mating		Group effect		
	Mean	**s.e.**	**Mean**	**s.e.**	**df**	**t**	***P***
Time together							
Total (s)	204.33	35.11	206.01	39.68	7	0.03	0.976
Frequency	94.50	17.19	84.88	16.42	7	-0.45	0.666
Female approach							
Total (s)	14.99	6.79	14.14	4.56	7	-0.10	0.919
Frequency (log transformed)	18.38	9.13	15.75	3.51	7	0.77	0.467
Sniffing							
Total (s)	196.24	35.81	110.90	37.91	7	-3.07	0.018
Frequency (log transformed)	60.13	9.14	47.00	10.44	7	-1.54	0.165
Female to male cage							
Total (s) (log transformed)	585.85	83.16	461.76	71.49	7	-2.42	0.046
Frequency	71.00	8.99	64.88	9.04	7	-0.53	0.611
Chasing							
Total (s)	34.39	13.69	51.55	16.74	7	0.85	0.425
Frequency	13.38	3.57	22.13	4.02	NA	1.59	0.16
Male approach							
Total (s)	33.45	10.03	53.18	15.58	7	2.08	0.076
Frequency	40.25	10.34	54.63	11.23	7	1.36	0.217
Nothing							
Total (s)	2527.87	90.13	2689.26	119.33	7	1.48	0.181
Frequency	70.22	2.50	74.96	3.41	7	0.00	1.000

3.5 Discussion

3.5.1 Proteomic analysis of the oviduct

The oviduct is vital in moderating sperm behaviour and fertilisation (Suarez and Pacey 2006; Suarez 2008b; Holt and Fazeli 2010). It is feasible that females could plastically alter their oviduct environment according to social cues of high mating rates; however I found no evidence for this. The oviduct environment is known to alter in pigs due to reproductive cycle and nutrition (Novak, Treacy *et al.* 2002; Novak, Almeida *et al.* 2003; Seytanoglu, Georgiou *et al.* 2008). The potential for plasticity in the mouse oviduct remains relatively unknown. As the composition of the oviduct secretion can influence early embryo development (Leese, Hugentobler *et al.* 2008), functional limitations may reduce the potential for variation. Furthermore, as components of the seminal fluid can alter later maternal signalling to the embryo (Bromfield, Schjenken *et al.* 2014), females expecting to mate multiply may respond to this after mating. Overall, this study highlights the limited amount of knowledge of the variation in protein secretions of the oviduct. A greater fundamental understanding of the proteins within the female reproductive tract, and their interactions with seminal fluid and sperm, would greatly assist further studies of female plasticity.

Although no treatment effect was found, there was a strong genetic influence on the oviduct secretion. Siblings had more similar oviduct protein secretions than non-siblings. This may have implications for fertilisation outcomes based on genetic incompatibility of mating partners. Females may mate multiply to reduce the risk of their ova being fertilised by a genetically incompatible male (Zeh and Zeh 1996; 1997; Jennions and Petrie 2000). Mating multiply may also reduce the likelihood of inbreeding effects (Stockley, Searle *et al.* 1993), particularly in species that do not have the capacity for kin recognition. In each case, mating multiply can potentially allow sperm selection of the most genetically compatible male to occur. Therefore,

the female may benefit from the molecular environment within the oviduct being strongly linked to genotype.

It is certainly possible that other mechanisms could increase how selective the oviduct is, including subtle plasticity in the oviduct secretions. Oviductal-specific glycoprotein modulates sperm binding to the zona-pellucida (McCauley, Buhi *et al.* 2003; Lyng and Shur 2009). This protein was identified within all samples here, however no quantitative difference was found. A more selective method of analysis, collecting only the oviduct secretion, may be more useful to quantify subtle differences in low abundance proteins of interest. This result also gives confidence that the RF analysis is not entirely random and only highlights genuine differences.

3.5.2 Reproductive morphology

No evidence was found for plasticity in gross uterine morphology according to anticipated multiple mating, suggesting that the size of the uterus may not have a significant role in sperm selection. Importantly though, although not measured here, there is still the potential for subtle differences in the folding of the endometrium to influence fertilisation outcomes. In species with sperm storage organs, evolutionary adaptations of female genital morphology are well documented (Presgraves, Baker *et al.* 1999; Miller and Pitnick 2002; Minder, Hosken *et al.* 2005; Brennan, Prum *et al.* 2007; Simmons and Garcia-Gonzalez 2011). Plasticity in female sperm storage organs has been found in response to the larval environment of some invertebrate species (Amitin and Pitnick 2007; Schafer, Berger et al. 2013 but see Baminger and Haase 1999). Within mammals, there is evidence that oviduct morphology has evolved in response to post-copulatory sexual selection (Gomendio and Roldan 1993; Anderson, Dixson *et al.* 2006), however the plasticity of the reproductive tract has not previously been tested. In muroid rodents, there is evidence that the vagina length, penile length and copulatory plug size are linked (Baumgardner, Hartung *et al.* 1982) and selection may therefore be acting on these traits to coevolve. The same study found that the upper reproductive tract correlated only with female body mass (Baumgardner, Hartung *et al.* 1982).

Differences were found in the anogenital distance of female house mice according to treatment group. Anogenital distance (AGD) in female house mice is generally determined by prenatal exposure to exogenous androgens, particularly in response to neighbouring male siblings in utero (Gandelman, Vom Saal *et al.* 1977; Clark and Galef 1998; Ryan and Vandenbergh 2002). Recent evidence shows that the AGD is not fixed at birth (Dusek, Bartos *et al.* 2010) and varies with oestrus stage (Dusek and Bartos 2012). Therefore the plasticity identified here is not unprecedented.

Female house mice born within high density populations have a larger AGD (Zielinski, Vandenbergh *et al.* 1991). Within wild populations of house mice, a greater AGD correlates with more aggressive behaviour (Drickamer 1996), the mechanism linking the two is uncertain however. In populations with a greater level of competition, a larger AGD may indicate an advantageous adaptation. In contrast, a smaller AGD correlates with a greater rate of oestrus and greater chance of pregnancy, and so may be beneficial in females (Drickamer 1996). The result found within this study, that females from the high risk of multiple mating treatment group tended to have a larger AGD, suggests that the AGD of females in the high group may plastically alter through a mechanism that adapts their behaviour to be more competitive. Any reduction in fertility may be less detrimental for the high group females as they are within a population with a high potential mating frequency.

The result shown here is only a trend, however it does add to previous suggestions that the AGD is not fixed at birth and entirely dependent on the in utero environment. This is also the first evidence that this could alter plastically, according to the social environment.

3.5.3 Behavioural differences

The results of the behavioural analyses were suggestive of some relatively weak treatment effects, with females from the low treatment group appearing less willing to interact with the males. As each treatment group produced the same number of litters, differences in this initial response did not lead to differences in mating

success. Further analysis of behaviour after the initial introduction, and at mating, would be needed to determine if the treatment effect remained when females had habituated to the novel male.

3.6 Conclusions

The internal female reproductive tract is a complex organ, and a better understanding of this environment would benefit our understanding of fertility and reproduction greatly. This study suggests genetics has a greater influence on the oviduct proteome than social environment. This may be important for mechanisms of sperm selection to counter the risk of genetic incompatibility. Social environment did influence those aspects of female physiology and behaviour tested here. A high likelihood of multiple mating led to females having a larger AGD. There is little previous evidence for plasticity in this measure. This could be an adaptive response, correlating with increased competitiveness. Females from the high likelihood of multiple mating group also showed more interest in a novel male. Behavioural differences when interacting with a novel male did not result in differential mating success, and so these relatively subtle differences in behaviour may be rapidly overcome.

Chapter 4
Maternal effects due to social experience in the house mouse

4.1 Chapter overview

The maternal environment can have a huge impact on offspring phenotype. In this chapter I investigate whether a mother's social experience prior to pregnancy leads to differences in her offspring.

Maternal effects can benefit offspring by ensuring they will be optimally adapted to the local environment. For example, differences in population density can affect the intensity of local competition for resources and mates, and mothers in high density populations may therefore produce more competitive offspring. By extension of this

idea, in species with sperm competition, females might also influence the reproductive phenotype of their male offspring according to local sperm competition risk. Here, I test this hypothesis for the first time in a model vertebrate, the house mouse. Maternal experience was manipulated under controlled experimental conditions to produce offspring sired under identical conditions with contrasting levels of sperm competition risk (high or low).

Using proteomic analysis I found differences in the seminal fluid composition of male offspring according to the social experience of their mothers. In particular, CUB and zona pellucida-like domain-containing protein 1 (CUZD1) tended to be found in higher proportions within the male offspring of females that were exposed to conditions simulating high sperm competition risk. This protein is produced by the epididymis and assists with fertility. By contrast, maternal social environment did not influence litter size or sex ratio, or the mating behaviour or mass of the reproductive organs of male offspring.

Within this chapter I also present analyses of general trends in mating behaviour and ejaculate composition.

4.2 Introduction

During pregnancy and lactation, mothers can alter the phenotype of their offspring. Any maternal influence on offspring phenotype, which is not a direct result of genetics, is known as a maternal effect (Bernardo 1996; Wolf and Wade 2009). Local environments and populations naturally vary. If mothers can alter the phenotype of their offspring to best suit current conditions, those offspring can gain a greater chance of future survival (Mousseau and Fox 1998a; Mousseau and Fox 1998b).

There is evidence that maternal effects are widespread across diverse taxa, including mammals (Mousseau and Fox 1998b; Ryan and Vandenbergh 2002; Simpson and Miller 2007; Maestripieri and Mateo 2009; Henriksen, Rettenbacher *et al.* 2011). Rodents have been widely used as a model for maternal effects, particularly in

response to nutrition and stress (e.g. Pratt and Lisk 1989; Trombulak 1991; Zielinski, Vandenbergh *et al.* 1991; Kaiser, Kruijver *et al.* 2003; Liang and Zhang 2006, see Chapter 1, Section 1.6.2). Along with in utero effects, maternal behaviour during lactation also influences offspring phenotype. Increased grooming behaviour stimulates epigenetic changes in offspring that alter future response to stress (Weaver, Cervoni *et al.* 2004). To date, the influence of social interactions, representing variation in the level of sexual competition, has not been tested. This may be particularly important within mammals, whereby variation in offspring quality leads to different likelihood of reproductive success, depending on the gender.

It is hypothesised mothers will influence the sex ratio of their litters (Trivers and Willard 1973). The Trivers-Willard hypothesis predicts that high quality females should produce more of the sex that has a greater variability in their reproductive success (Trivers and Willard 1973). Or, in polygamous species, high quality females should produce more sons as a low quality son is likely to produce fewer offspring than a low quality daughter. There is evidence for this within mammals, if the measure of female quality is taken at the time of conception (Clutton-Brock and Iason 1986; Cameron 2004; Sheldon 2004). It is suggested that there is a nutritional mechanism for this, with high levels of glucose at conception potentially causing a male bias (Cameron 2004). Additionally, low social status and high parity cause female rodents to produce fewer sons, (Huck, Pratt *et al.* 1988; Pratt and Lisk 1989), although this may also be a result of nutritional differences.

Thus, mothers can alter their litters and the future success of their offspring by mechanisms in utero or during lactation. This could be particularly important during local fluctuations in population density, which would increase competition for resources, territory and mates (Dantzer, Newman *et al.* 2013). In species with multiple mating, increased competition for mates is also associated with an increased incidence of sperm competition. However, it is unknown if maternal

effects influence male reproductive traits associated with success in sperm competition.

Here, I use the house mouse as a model to test whether male offspring from females that have been in an environment with a male biased sex ratio, and a high level of interaction with males, will differ from offspring of females with only low levels of male interaction. I use a balanced experimental design, such that female littermates are distributed across treatment groups, to limit the influence of genetic effects. I first consider the sex ratio and size of the litters females produced. After weaning, all male offspring are then treated equivalently to limit environmental differences, allowing variation due to maternal environment to be investigated. I examine male mating behaviour and ejaculate composition to determine if maternal environment prior to pregnancy can alter male offspring reproductive phenotype. Further to this, I combine the mating and ejaculate composition data to investigate general patterns of reproduction in the male house mouse.

4.3 Methods

4.3.1 Subject females and litters

In Chapter 3, sibling pairs of female house mice were housed in enclosures (1mx1mx90cm) from weaning until 4 months old. Each enclosure was assigned to be either a high or low competition environment. A combination of odour cues and social interactions was used to create this. At the end of this social experience, one female from each enclosure was paired with a stock male, which had previously bred successfully, to produce a litter. Once paired with a male, there were no further differences in the treatment of the females. Therefore, differences caused by the maternal environment prior to pregnancy, as opposed to experiences during pregnancy, are being tested here. This protocol also limited the chance of abortion due to the Bruce effect (Bruce 1959). After one week, males were removed from the enclosures. As females were paired when in oestrus, and the oestrus cycle of a house mouse averages 4-5 days long, this should have allowed two oestrus events before the male was removed.

Of the 16 females paired immediately after the high or low competition environments, only six produced litters. This was not a treatment group effect; three litters were produced by high group females and three from low. It is possible that breeding was unsuccessful due to external building work causing noise and disruption within the enclosure room. Furthermore, the females that did not produce a litter also tended to have a smaller body mass than those that did (body mass, mean ± sem: produced a litter 18.17 ± 0.93 g; no litter 15.17 ± 0.87 g).

In order to increase the sample size, females were paired again to the same male as before. Females that had not become pregnant within the first breeding attempt were removed from the enclosure. Each female was placed into a clean cage, containing soiled bedding from the male she was partnered with. For females within the high competition group, soiled bedding from the three males she had previously been exposed to was also added to the cage, to maintain a high competition

environment. After 5 days of caged housing, females were released into clean enclosures. To counter the effects of noise, where possible the mice were paired into an enclosure in a different room (8 females, enclosures measured 60cm x 120cm). The other two females were paired in the quietest enclosures available within the first room. Each enclosure contained a clean cage with open lid, a nest box and cardboard enrichment. A thin layer of shredded paper bedding was spread around the enclosure floor, to encourage nesting and provide additional shelter. Each female had 30 minutes to habituate to the new enclosure, before a male was released. Males remained with the females for 2 weeks in order to maximise the chance of successful breeding. Of the 10 breeding pairs, 7 produced a litter in this second attempt.

4.3.2 Measuring litters

Although the litters were produced in two breeding events, all were treated identically. For both breeding groups, after the males were removed, females were monitored closely for signs of pregnancy or parturition. After a female had produced a litter, care was taken to cause minimal disturbance to the mother and pups. The pups were counted when each litter was 3 days old. At this point, the mother and pups were removed from their enclosure and housed in the cage that had been within their enclosure. Enrichment from the enclosure was placed into the cage to maintain the same olfactory environment. Litters remained with their mothers until they were 26 days old, at this point each pup was weighed, sexed and weaned into single sex sibling groups.

4.3.3 Male offspring: mating, ejaculate collection and reproductive physiology

After weaning at 26 days old, male house mice (N=45) were housed in single sex cages with siblings until all litters had been weaned. At this point, brothers were split and each male was housed singly. Soiled female bedding was added to each cage every 2 weeks to stimulate normal sexual development. To test differences in mating behaviour and ejaculate production, each male was mated to an ICR female using

the same method described in Chapter 2. In this instance, however, each male only performed one mating. As described in Chapter 2, ejaculates and copulatory plugs were collected from the female immediately after mating. After each male's successful mating, he was rested for one week before reproductive organs were collected. Each male that mated successfully (N=30) was killed and dissected, blind to the male's mother's treatment, to measure differences in their investment in reproductive traits. Reproductive organs were collected and sperm counts performed as in Chapter 2.

4.3.4 Analysing proteomic differences in the ejaculate

Ejaculates (N=30) were digested using Trypsin before HPLC-MSMS analysis as described in Chapter 2.

4.3.5 Data analysis

Data was analysed as described in Chapters 2 and 3. RF analysis was performed on both the full set of ejaculate proteins identified, containing at least 3 unique peptides for quantification, and a shortened set of 59 proteins that were known to be male specific and found within the ejaculate (Dean, Clark *et al.* 2009; Dean, Findlay *et al.* 2011). I also extracted the proximity scores from an RF unbiased model, as described in Chapter 3. Random forest (RF) models produce a proximity table that gives a score for how similar each individual sample is to all other samples. Such that, each sample has a score of 1 when compared to itself. Scores closer to 0 indicate little to no similarity between samples. Within the previous chapters, litter mates have been balanced across treatment groups. Here, males from 12 litters were available, 6 from each maternal treatment group. Littermates are therefore within the same treatment groups. This reduces the independence of data points, as siblings are likely to be similar, and so I wanted to evaluate whether this would have an impact on the analysis. To do this, I compared the proximity scores for related versus unrelated individuals with those that were within the same versus different treatment groups. Proximity scores were also compared according to litter group

and whether males were doubly or singly housed, as the litters were produced during two attempts (Section 4.3.1) and the data includes that from the dominant and subordinate males within Chapter 2.

Additionally, RF regression analysis was used to test whether the protein composition of the ejaculate correlated with mating behaviour and ejaculate traits. To do this, an RF model with the continuous trait of interest as the dependent variable, and the CLR transformed relative protein abundances was run 1000 times. For each model iteration, variable importance scores of each protein were extracted. Further to this, for each of the 1000 iterations, a linear model of the raw data values of the dependent variable, against the prediction scores from the RF model. An average of the 1000 F values, taken from the 1000 linear models, was used to calculate a *P* value for the regression of the continuous variable against the RF model predictions.

4.4 Results

4.4.1 Proteomic analysis of the ejaculate: Proximity scores

Prior to performing the RF analysis according to maternal treatment group, I first compared the scores within a proximity matrix from an unsupervised RF model on the focused set of 59 male derived ejaculate proteins. This was done to determine whether littermates being within the same treatment group would affect the results due to being more similar to each other. Proximity scores according to relatedness, treatment group, litter group (first or second litters) and housing (double or single) are summarised in Table 4.1. The proximity scores quantify how similar each individual sample is to all of the other individual samples, ranging from 0 – 1, with a score of 1 meaning samples are identical.

Proximity scores were not different according to relatedness, treatment group, batch, or housing when taken from the unsupervised model (Table 4.1). Therefore, these factors are not influencing the composition of the ejaculate in a way that would influence the outcome of analysis according to treatment group. When taken from a supervised RF model, with maternal treatment group given as a predictor, there was again no difference according to litter group or housing. Within this supervised model, there was a significant difference in the proximity scores between individuals in the same treatment group and those in a different treatment group (df = 431.9, t = -11.12, $P < 0.001$). There was also a significant difference between the scores for sibling males compared to non-sibling (df = 41.7, t = 5.77, $P < 0.001$). Although siblings have more similar ejaculate protein composition than non-siblings, the t-value for the difference in proximity scores according to treatment group is larger, suggesting this has a greater effect. Further, although littermates were found within the same treatment groups, males were taken from 12 different litters, 6 in each treatment group. Treatment group effects within further analyses can therefore be viewed as such.

Table 4.1: Proximity scores from supervised and unsupervised random forest (RF) analysis of ejaculate proteins. Supervised model included treatment as the grouping variable.

	Unsupervised RF model		Supervised model	
	Same	Different	Same	Different
Treatment group	0.221	0.219	0.563	0.425
Litter	0.273	0.215	0.631	0.480
Litter group (1st / 2nd)	0.222	0.220	0.493	0.497
Housing (double / single)	0.218	0.216	0.482	0.500

4.4.2 Maternal effects on ejaculate protein composition

RF analysis was performed on proteins identified and quantified from the uterine washes after mating. Initially, all 649 proteins that were quantified using a minimum of 3 unique peptides were included. Over 1000 runs the RF analysis prediction accuracy averaged 80.65%, according to maternal treatment group (24 out of 30 correct, bootstrapping analysis of 1000 replicates: $P < 0.001$: Table 4.2). From this analysis, there were 59 proteins with significant variable importance scores. Many of these were low abundance and likely to have been derived from the female (Table 4.3).

A second RF analysis was also performed for 59 proteins known to be male derived and present in the ejaculate from previous studies (Dean, Clark *et al.* 2009; Dean, Findlay *et al.* 2011), was also performed. This analysis of 59 proteins had an overall prediction accuracy according to maternal treatment group of 77.60% (23 out of 30 correct, bootstrapping analysis of 1000 replicates: $P = 0.002$). Here, 10 proteins were found to have significant variable importance scores (see Table 4.4 and Figure 4.1). Of these, 4 had also been significantly important within the analysis of 649 proteins. The most abundant protein of the 4 that were classified as important within both analyses, was the CUB and zona pellucida-like domain-containing protein 1 (CUZD1). This is known to be produced by the epididymis and has functional significance for fertility (Yamazaki, Adachi *et al.* 2006). There tended to be relatively more CUZD1 within ejaculates from male offspring of the high competition maternal group, although this was not significant ($df = 28$, $t = 1.87$, $P = 0.07$, see Figure 4.2).

4.4.3 Litters

There was no difference in the size, or sex ratio, of the litters according to maternal treatment group. Further, no difference was found in the mean body mass of the weaned offspring (Table 4.5 and Figure 4.3).

4.4.4 Male reproductive physiology & mating behaviour

In total, of 293 attempts there were 30 successful matings recorded; 16 by offspring of the high competition maternal group and 14 from the low. There was no difference in the mass of any sexual organs measured, according to prior maternal experience (Table 4.6). There was also no difference in the mating behaviour, such as the number of thrusts or intromissions, or the size of the ejaculates the males produced (Table 4.7). As there were no differences between the groups, the data from all males was pooled and correlations within the data were analysed to further understand the relationship between mating behaviour and ejaculate allocation.

Table 4.2: Classification accuracy scores following 1000 iterations of a random forest (RF) model of either all identified proteins within the ejaculate, or a reduced set of 59 proteins known to be male derived.

	High	Low	Overall
649 proteins	100.00%	58.53%	80.65%
59 proteins	95.74%	56.86%	77.60%

Table 4.3: Proteins significantly important at classifying the samples according to the treatment group when all proteins identified within the ejaculate were modelled using random forest analysis. * indicates also significant in the model including only proteins known to be male derived.

Accession	Protein Description	Mean	Vimpx100	95% CI
O08976*	Probasin	0.013	0.100	0.10 - 0.10
Q9D8W5	26S proteasome non-ATPase regulatory subunit 12	0.007	0.091	0.09 - 0.10
Q61171	Peroxiredoxin-2	0.031	0.091	0.09 - 0.10
Q8C0M9	L-asparaginase	0.013	0.083	0.08 - 0.09
O54833	Casein kinase II subunit alpha	0.003	0.076	0.07 - 0.08
Q6URW6	Myosin-14	0.015	0.076	0.07 - 0.08
P62908	40S ribosomal protein S3	0.017	0.072	0.07 - 0.08
P28665	Murinoglobulin-1	0.042	0.067	0.06 - 0.07
P03987	Ig gamma-3 chain C region	0.022	0.066	0.06 - 0.07
Q91VR2	ATP synthase subunit gamma, mitochondrial	0.003	0.062	0.06 - 0.06
Q80YW5	B box and SPRY domain-containing protein	0.005	0.056	0.05 - 0.06
P19783	Cytochrome c oxidase subunit 4 isoform 1, mitochondrial	0.024	0.055	0.05 - 0.06
P97315	Cysteine and glycine-rich protein 1	0.175	0.054	0.05 - 0.06
P47911	60S ribosomal protein L6	0.006	0.053	0.05 - 0.06
Q61838	Alpha-2-macroglobulin	0.310	0.050	0.05 - 0.05
P00489	Glycogen phosphorylase, muscle form	0.010	0.048	0.05 - 0.05
Q6NS57	Mitogen-activated protein kinase-binding protein 1	0.004	0.048	0.04 - 0.05
Q9DC69	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 9, mitochondrial	0.019	0.046	0.04 - 0.05
Q8C8R3	Ankyrin-2	0.029	0.045	0.04 - 0.05
O08528	Hexokinase-2	0.008	0.044	0.04 - 0.05
Q9WTI7	Myosin-Ic	0.003	0.042	0.04 - 0.04
P15265*	Sperm mitochondrial-associated cysteine-rich protein	0.082	0.042	0.04 - 0.04
Q8BW94	Dynein heavy chain 3, axonemal	0.208	0.041	0.04 - 0.04
P00405	Cytochrome c oxidase subunit 2	0.011	0.038	0.04 - 0.04
Q62000	Mimecan	0.007	0.038	0.03 - 0.04
Q791V5	Mitochondrial carrier homolog 2	0.014	0.037	0.03 - 0.04
Q8CAQ8	Mitochondrial inner membrane protein	0.024	0.036	0.03 - 0.04
Q5XJY5	Coatomer subunit delta	0.013	0.036	0.03 - 0.04
Q91ZU6	Bullous pemphigoid antigen 1	0.067	0.036	0.03 - 0.04
Q64727	Vinculin	0.209	0.035	0.03 - 0.04
Q9D2G2	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex, mitochondrial	0.003	0.035	0.03 - 0.04
Q8CGP1	Histone H2B type 1-K	0.022	0.035	0.03 - 0.04

Accession	Protein Description	Mean	Vimpx100	95% CI
P11859	Angiotensinogen	0.006	0.035	0.03 - 0.04
Q9EQP2	EH domain-containing protein 4	0.015	0.035	0.03 - 0.04
Q69Z23	Dynein heavy chain 17, axonemal	0.469	0.034	0.03 - 0.04
Q7TMR0	Lysosomal Pro-X carboxypeptidase	0.007	0.033	0.03 - 0.04
A2AN08	E3 ubiquitin-protein ligase UBR4	0.027	0.031	0.03 - 0.03
O08529	Calpain-2 catalytic subunit	0.002	0.031	0.03 - 0.03
P07759	Serine protease inhibitor A3K	0.925	0.030	0.03 - 0.03
A2AKX3	Probable helicase senataxin	0.017	0.030	0.03 - 0.03
O55234	Proteasome subunit beta type-5	0.006	0.028	0.03 - 0.03
Q80ZN9	Cytochrome c oxidase subunit 6B2	0.023	0.027	0.03 - 0.03
P70412*	CUB and zona pellucida-like domain-containing protein 1	1.943	0.026	0.02 - 0.03
Q9CX56	26S proteasome non-ATPase regulatory subunit 8	0.058	0.026	0.02 - 0.03
P01878	Ig alpha chain C region	0.080	0.025	0.02 - 0.03
P32037	Solute carrier family 2, facilitated glucose transporter member 3	0.060	0.025	0.02 - 0.03
Q8BFZ3	Beta-actin-like protein 2	0.011	0.025	0.02 - 0.03
Q60930	Voltage-dependent anion-selective channel protein 2	0.077	0.024	0.02 - 0.03
Q9EPX2	Papilin	0.374	0.024	0.02 - 0.03
O08716	Fatty acid-binding protein 9	0.169	0.024	0.02 - 0.03
Q06890	Clusterin	0.089	0.024	0.02 - 0.03
Q9CYH2	UPF0765 protein C10orf58 homolog	0.025	0.023	0.02 - 0.03
Q9D023	Brain protein 44	0.015	0.022	0.02 - 0.02
Q9R111	Guanine deaminase	0.003	0.022	0.02 - 0.02
P30416	Peptidyl-prolyl cis-trans isomerase FKBP4	0.004	0.022	0.02 - 0.02
Q7TMY8	E3 ubiquitin-protein ligase HUWE1	0.021	0.021	0.02 - 0.02
Q80YC5	Coagulation factor XII	0.026	0.021	0.02 - 0.02
Q8K0T7	Protein unc-13 homolog	0.024	0.021	0.02 - 0.02
Q9QY48*	Deoxyribonuclease-2-beta	0.083	0.021	0.02 - 0.02

Table 4.4: Proteins significantly important at classifying the samples according to the treatment group when a reduced set of 59 proteins known to be in the ejaculate were modelled using random forest analysis. * indicates significant in the model including all identified proteins.

Accession	Protein description	Ave Prop	Vimp(x100)	95% CI
O08976*	Probasin	0.03	0.802	0.79 - 0.81
P15265*	Sperm mitochondrial-associated cysteine-rich protein	0.16	0.444	0.44 - 0.45
P30933	SVS5	0.06	0.374	0.37 - 0.38
Q9QY48*	Deoxyribonuclease-2-beta	0.16	0.372	0.36 - 0.38
P70412*	CUB and zona pellucida-like domain-containing protein 1	3.83	0.341	0.33 - 0.35
A3KGV1	Outer dense fiber protein 2	0.79	0.333	0.33 - 0.34
P09036	Serine protease inhibitor Kazal-type 3	2.96	0.273	0.27 - 0.28
Q60662	A-kinase anchor protein 4	1.53	0.223	0.22 - 0.23
P00683	Ribonuclease pancreatic	0.06	0.200	0.19 - 0.21
P45700	Mannosyl-oligosaccharide 1,2-alpha-mannosidase IA	0.07	0.150	0.14 - 0.15

Figure 4.1: Plot of the mean proportional abundances of the 10 proteins significantly important for classifying the samples correctly, according to treatment group, within random forest analysis of a reduced set of 59 proteins identified within the ejaculate, known to be male derived. Bars represent mean ± sem.

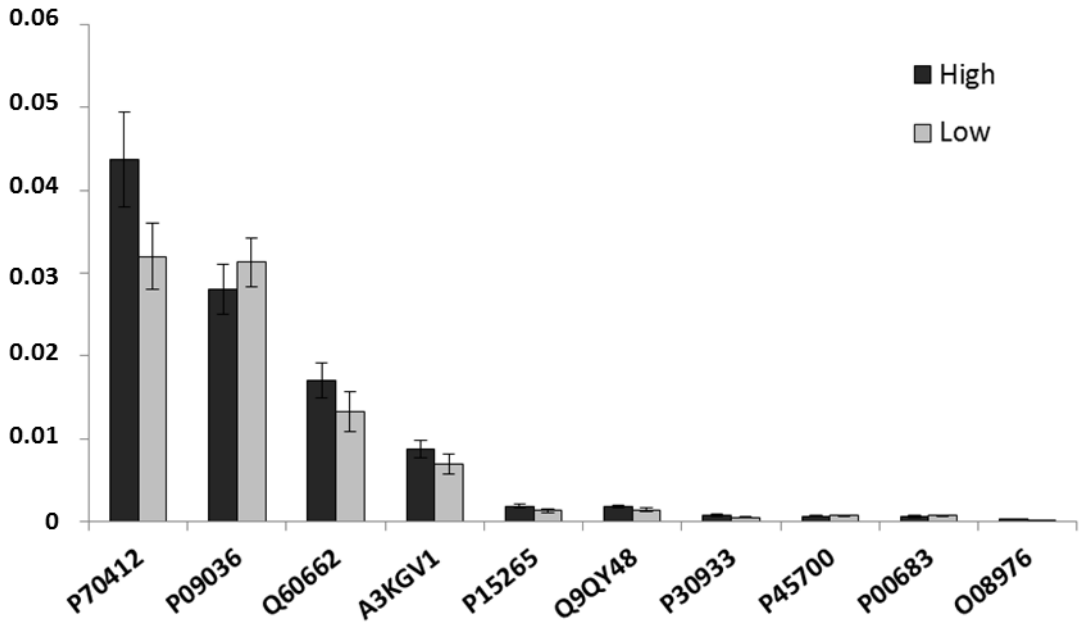


Figure 4.2: Boxplot of the proportion of CUB and zona-pellucida like domain containing protein 1 within ejaculates from male offspring of high competition females and low competition females (df = 28, t = 1.87, *P* = 0.07).

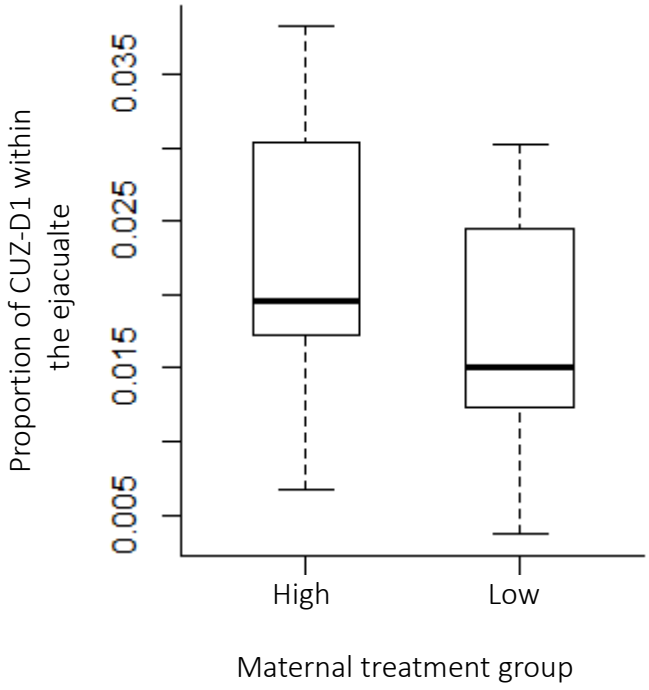


Table 4. 5: Table showing the analysis for maternal treatment group effect on litter size and the mass of the offspring at weaning.

Variable	Model	Group effect t/z	Group effect *P*
Maternal body mass	Maternal body mass ~ Group, random effect = female litter	-1.10	0.33
Number of offspring	Male:Female~Group+Maternal body mass, random effect = female litter, family=binomial)	-1.59	0.11
Offspring body mass at weaning:			
Average male	Average male body mass ~Group, random=female litter	-0.02	0.98
Average female	Average female body mass ~Group, random=female litter	-0.79	0.48
Total litter	Total body mass ~Group, random=female litter	-0.79	0.47
Proportion male	Proportion male (arcsine square root transformed)~Group, random=female litter	-0.11	0.91

Figure 4.3: Boxplot showing the number of offspring produced by high competition group and low competition group mothers.

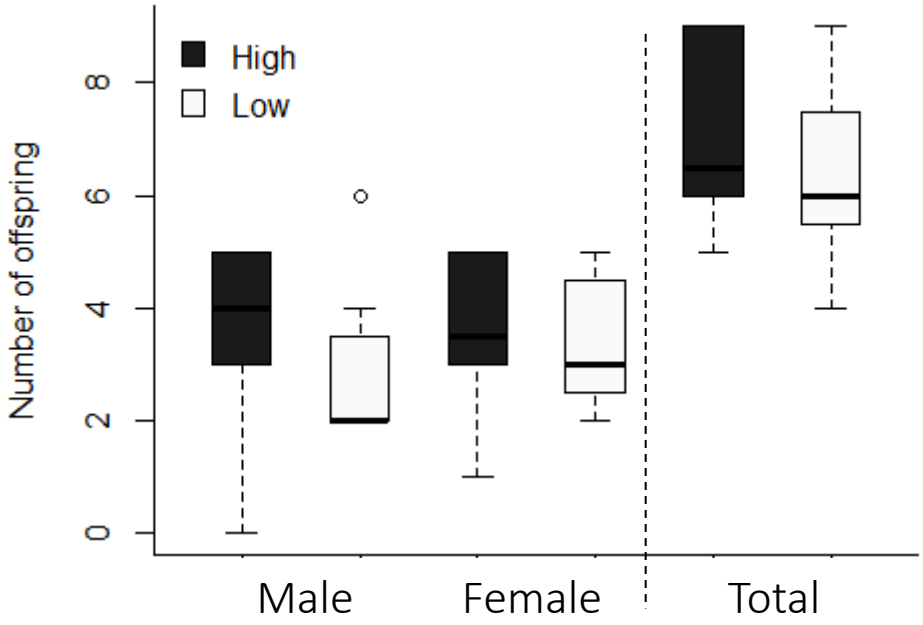


Table 4.6: Results of the analysis for the effect of maternal treatment group on the body mass and reproductive organs of the male offspring.

Group effect from mixed model including: fixed effect = social status (& body mass for sexual organs)
random effect = Maternal litter

	High		Low		Group effect		
	Mean	s.e.	Mean	s.e.	df	t	*P*
Body mass (g)	20.78	0.53	20.09	0.43	18	-0.25	0.802
Preputial glands (mg)	80.48	9.56	66.93	7.16	16	-0.71	0.488
Testes (mg)	191.54	5.01	193.74	9.32	16	-0.22	0.825
Epididymides (mg)	65.09	1.83	60.77	2.09	16	-1.21	0.245
Seminal vesicles (mg)	109.93	9.96	111.85	9.51	16	0.62	0.547
Epididymal sperm count	1.87E+07	2.32E+06	1.91E+07	2.91E+06	17	-0.03	0.974

Table 4.7: Results from the analysis of the effects of maternal environment on mating behaviour. Results presented are the treatment group effects from a linear mixed effects model, including maternal line as a random effect and male social status as a fixed effect.

Group effect from mixed model including: fixed effect = social status
random effect = Maternal litter

	High		Low					
	Mean	s.e.	Mean	s.e.	df	t/z	*P*	Model specifics
Trials to mating	5.50	0.98	4.56	1.04		-0.92	0.36	glmer, family=binomial
Plug (mg)	43.59	3.01	46.19	4.15	18	0.66	0.52	
# sperm in ejaculate	2.09E+07	2.55E+06	1.72E+07	2.01E+06	18	0.10	0.92	
1st Int - ejaculation (s)	3763.47	986.28	2412.63	466.85	20	-1.11	0.28	
Duration of ejaculation (s)	14.90	1.15	14.91	0.81	17	0.04	0.97	
# intromissions	43.50	11.31	31.56	9.24	20	-0.67	0.51	Intromission (log transformed)
Intromissions / minute	0.89	0.13	0.68	0.12	20	-1.55	0.14	Intromission rate (log transformed
Proportion of mating spent intromitting	16.92	5.73	10.47	1.18	20	-1.18	0.25	Proportion of time intromitting (arcsine square root transformed)
# Thrusts	669.38	157.92	537.63	125.22	20	-0.27	0.79	Thrusts (log transformed)
# Thrusts / intromission	18.50067	2.342049	21.16574	2.180587	20	0.83	0.41	Thrusts per intromission (log transformed)

4.4.5 Ejaculate proteins and other mating parameters

As this was a comprehensive analysis of mating behaviour and ejaculate proteins of 30 male house mice, I wanted to consider whether ejaculate composition was associated with other mating traits. I used regression RF analysis for this. When an RF model has a continuous dependant variable it will train the forest to predict the amount of the dependant variable according to the protein composition. You can then take the predictions and perform a linear model of these values against the actual values, to determine how accurate these predictions are. The F values displayed here are the averages of 1000 linear models performed using prediction values from 1000 RF models. For all mating behaviour traits, the protein composition did not predict the continuous traits well.

The number of sperm within the ejaculate correlated well with the predicted values from the RF analysis based on protein composition. This was true when analysing all 649 identified proteins and the shortened set of 59 proteins (649 protein analysis: $F_{1, 28} = 28.97$, $P < 0.001$, 59 protein analysis: $F_{1, 28} = 36.63$, $P < 0.001$). A total of 58 proteins were significantly important from the analysis of 649 proteins, and 26 were important in the analysis of 59 proteins. Of these proteins, 7 were important within both analyses (Table 4.8). This included SVS2 and zona pellucida sperm-binding protein 3 receptor, which are important with respect to fertility. Sperm numbers correlated positively with zona pellucida sperm-binding protein 3 receptor, L-lactate dehydrogenase C chain and Protein-glutamine gamma-glutamyltransferase 4 (TGM4). In contrast, there was a negative correlation with SVS2 (Figures 4.4 – 4.).

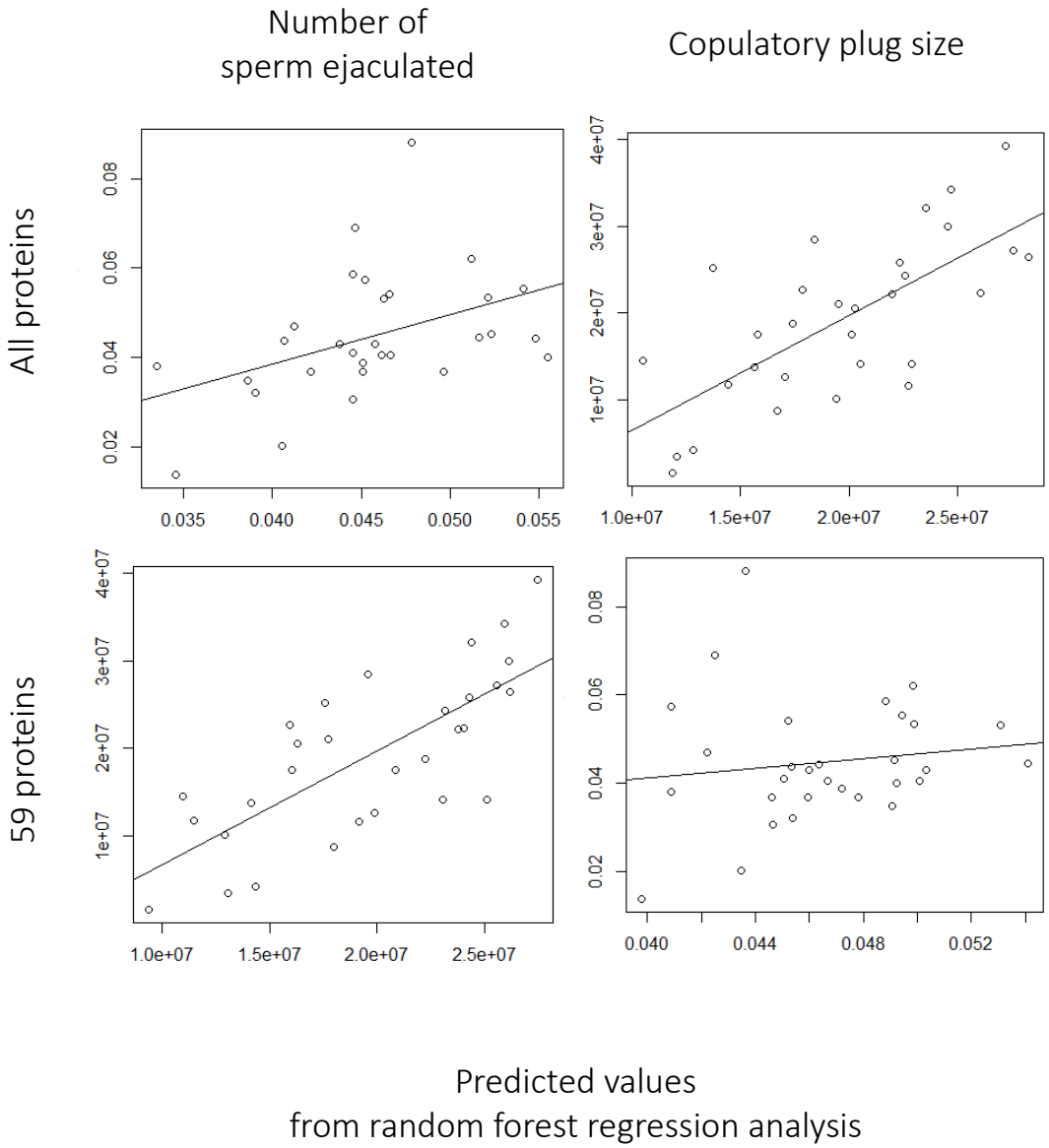
Regression of the large set of 649 proteins also predicted copulatory plug mass ($F_{1, 28} = 6.95$, $P = 0.01$). From this analysis, 59 proteins were significantly important for predicting the plug mass. Most of these were low abundance general proteins, most likely derived from the female. I would be cautious in drawing conclusions from the

plug analysis with regard to differences in the ejaculate proteins as an effect was not found within the smaller set of ejaculate specific proteins.

Table 4.8: The ejaculate proteins which were significantly important within the random forest regression for predicting the number of sperm within the ejaculate when using the 59 proteins known to be male derived. * = proteins also significant within the model including all proteins.

Accession	Protein Description	Ave Prop	Vimp	95% CI
P00342*	L-lactate dehydrogenase C chain	0.031	41.16	41.12 - 41.20
Q60736*	Zona pellucida sperm-binding protein 3 receptor	0.060	27.95	27.91 - 27.98
P07724*	Serum albumin	29.770	27.66	27.63 - 27.69
Q9D9X8*	Sperm acrosome membrane-associated protein 3	0.026	23.49	23.45 - 23.53
Q8BZH1*	Protein-glutamine gamma-glutamyltransferase 4	1.684	23.35	23.31 - 23.40
Q62216*	SVS2	0.019	19.84	19.80 - 19.87
Q6NY15	Testis-specific gene 10 protein	0.028	12.38	12.33 - 12.42
Q64467	Glyceraldehyde-3-phosphate dehydrogenase, testis-specific	0.116	11.84	11.80 - 11.87
Q9Z0J0*	Epididymal secretory protein E1	0.175	10.85	10.80 - 10.89
Q8CFG9	Complement C1r-B subcomponent	0.142	10.71	10.66 - 10.76
C4P6S0	Sperm head and tail associated protein	0.004	10.13	10.08 - 10.17
Q03401	Cysteine-rich secretory protein 1	0.862	10.12	10.08 - 10.15
Q8BND5	Sulfhydryl oxidase 1	1.014	9.57	9.51 - 9.63
A2AJB7	Epididymal-specific lipocalin-5	0.271	7.74	7.69 - 7.79
P09036	Serine protease inhibitor Kazal-type 3	1.525	7.71	7.65 - 7.77
P30933	SVS5	0.033	7.57	7.50 - 7.63
P00683	Ribonuclease pancreatic	1.042	7.23	7.17 - 7.29
Q9DAU7	WAP four-disulfide core domain protein 2	0.002	7.11	7.07 - 7.16
P35700	Peroxiredoxin-1	0.059	6.52	6.47 - 6.57
P45700	Mannosyl-oligosaccharide 1,2-alpha-mannosidase IA	0.034	6.16	6.11 - 6.22
P01027	Complement C3	1.920	6.16	6.11 - 6.22
P70412	CUB and zona pellucida-like domain-containing protein 1	1.943	6.08	6.02 - 6.13
O08976	Probasin	0.013	5.85	5.79 - 5.91
Q3UN54	Secreted seminal-vesicle Ly-6 protein 1	0.031	5.83	5.76 - 5.89
Q64442	Sorbitol dehydrogenase	0.135	5.65	5.61 - 5.70
P21765	Epididymal secretory glutathione peroxidase	0.026	4.69	4.62 - 4.75

Figure 4.4: Plots of the predicted values from random forest regression analysis of ejaculate protein composition with the number of ejaculated sperm or copulatory plug size as the response variable.



4.4.6 Male mating behaviour and ejaculate composition

Males that ejaculated more sperm produced larger copulatory plugs ($F_{1,28}$ = 4.03, *P* = 0.05). Larger males produced a larger copulatory plug and ejaculated a greater number of sperm (plug mass: df = 14, t = 3.03, *P* = 0.005; number of sperm: df = 14, t = 2.58, *P* = 0.022). Larger males also needed to be trialled with a smaller number of females before successfully mating (df = 16, t = 4.84, *P* < 0.001). As body size correlated with these traits, it was included as a covariate in further analysis. A greater number of females trialled also correlated with a lager copulatory plug (df = 13, t = 3.70, *P* = 0.003), but not with the number of sperm ejaculated (df = 13, t = 1.65, *P* = 0.12).

There were also correlations between specific behaviours within the matings and the size of the ejaculates. Males that performed more thrusts and intromissions also ejaculated more sperm (thrusts: df = 17, t = 2.82, *P* = 0.012; intromissions: df = 17, t = 32.19, *P* = 0.043). There was no correlation between copulatory plug size and these mating behaviours (thrusts: df = 17, t = -0.17, *P* = 0.87; intromissions: df = 17, t = -0.08, *P* = 0.94). There was a strong correlation between the number of thrusts and intromissions performed ($F_{1,30}$ = 155.5, *P* < 0.001). A longer mating duration strongly correlated with a greater number of thrusts and intromissions performed (thrusts: df = 19, t = 8.19, *P* < 0.001; intromissions: df = 19, t = 8.52, *P* < 0.001), and the number of sperm ejaculated ($F_{1,22}$ = 7.53, *P* = 0.01), but not copulatory plug size ($F_{1,22}$ = 0.001, *P* = 0.97). Mating duration did not correlate with other mating behaviour traits; mean intromission duration, number of thrusts per intromission or the proportion of the mating spent within an intromission. See Figures 4.5 and 4.6.

Figure 4.5: Plots showing the relationship between the number of ejaculated sperm, the copulatory plug mass and body mass. High and Low represent the maternal treatment group, either high or low likelihood of multiple mating.

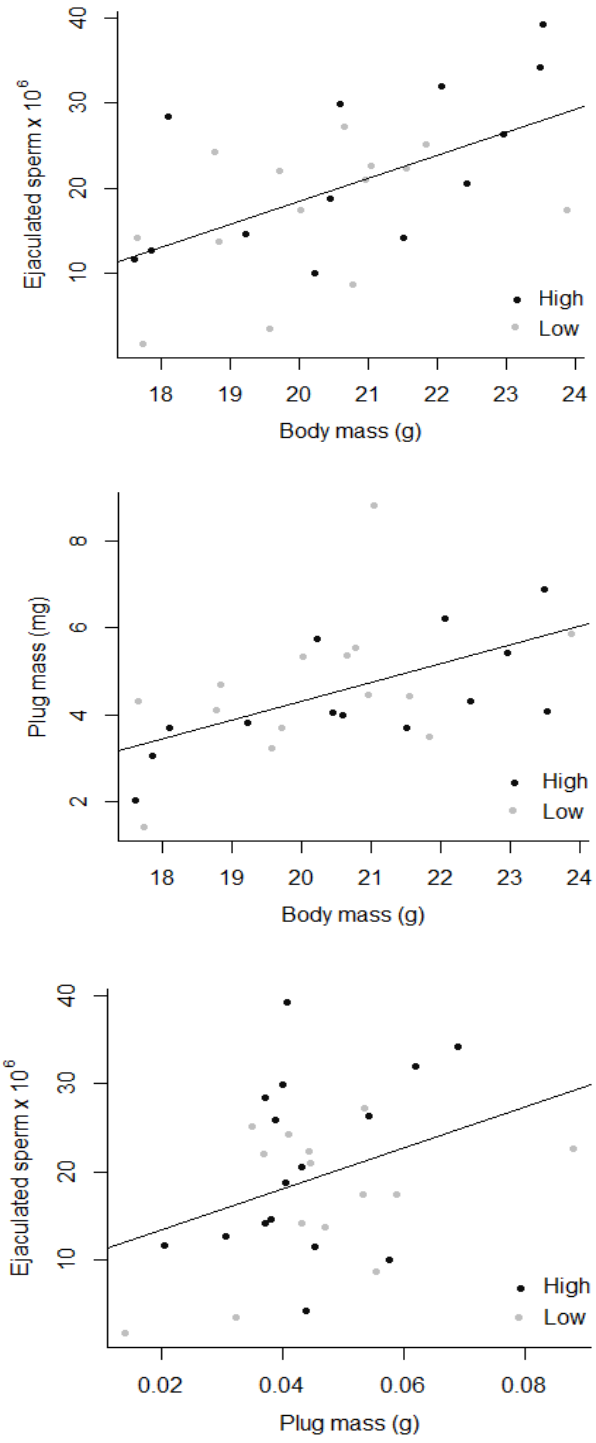
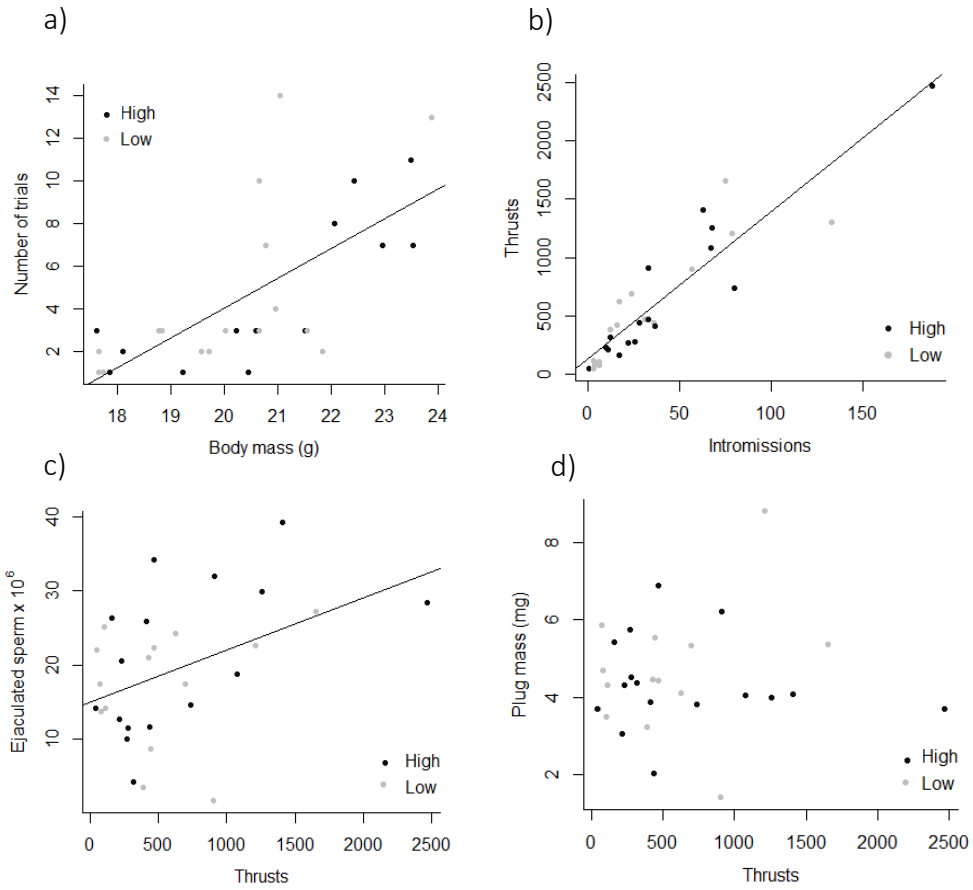


Figure 4.6: Plots showing the relationship between mating traits and ejaculate composition: a) the number of trials before a male mated and body mass, b) the number of intromissions and the number of thrusts, c) the number of ejaculated sperm and the number of thrusts, and d) the size of the copulatory plug and the number of thrusts. High and low represents the maternal treatment group, either a high or low likelihood of multiple mating.



4.5 Discussion

4.5.1 Maternal effects on ejaculate proteins

RF analysis of the ejaculated proteins was effective at classifying the samples correctly, beyond what would be expected by chance. The classification accuracy of the RF models reveals that the overall protein composition of the ejaculates differs according to the maternal treatment group. RF analysis identified proteins that were significantly important for classifying the samples correctly. CUB and zona pellucida-like domain-containing protein 1 (CUZD1) is a relatively abundant ejaculated protein, produced by the epididymis (Yamazaki, Adachi *et al.* 2006), which tended to be found in higher proportions within the high maternal group males. Further, sperm specific A-Kinase anchor protein 4 (AKAP4) tended to be found in higher proportions, and Serine protein inhibitor kazel type-3 (Spikl) in lower proportions, within the high maternal group males. I would be tentative to suggest that these trends correspond to significant differences in these proteins that were not seen with these sample sizes. Additionally, it is worth noting that the RF model is a supervised approach and so guided in its classification. Nonetheless, the overall composition of the ejaculates appears to be influenced by maternal group.

The potential mechanism of this is uncertain. Recent studies of maternal effects are increasingly highlighting epigenetic changes within the female, which can be passed on to her offspring (Weaver, Cervoni *et al.* 2004). Furthermore, trans-generational effects on offspring ageing and mortality due to maternal sexual activity have been shown within *Drosophila melanogaster* (Dowling, Williams *et al.* 2014). It is possible that increased interactions with males cause minor differences in the female genome, which then lead to differences in the ejaculates of male offspring.

Alternatively, the effect seen here may be due to a hormonal mechanism. Endocrine mechanisms of maternal effects have been documented within mammals. For example changes in maternal glucocorticoid levels, in response to population density or experimental manipulation, alter offspring growth rate in the red squirrel (*Sciurus*

vulgaris) (Dantzer, Newman *et al.* 2013). Additionally, prenatal social stress results in masculinised behaviour of female offspring in guinea pigs (*Cavia porcellus*), via a neuro-endocrine mechanism (Kaiser, Kruijver *et al*. 2003).

The effect found here is subtle and the mechanism cannot be concluded from this study. However, it is the first hint that the seminal fluid proteome may be influenced by maternal environment which is an interesting result worthy of further study.

4.5.2 Maternal effects on litters and male offspring

Offspring sex ratio, litter size and pup mass were not influenced by maternal environment prior to conception. Neither was the mass of male offspring or their reproductive organs. This study therefore highlights the importance of considering the molecular environment, as phenotypic differences in offspring may be more subtle than morphological adaptations. Anogenital distance was not measured within this study. In hindsight this may have been a useful measure as there is evidence for plasticity in this trait due to maternal influences (Allen and Haggett 1977; Zielinski, Vandenbergh *et al.* 1991).

Mating behaviour, the number of sperm ejaculated and the size of the copulatory plug were also not influenced by maternal environment prior to conception. Male offspring of females that have been selected through polyandry show increased competitive ability (Firman 2011), however, this is likely to be due to genetic selection rather than maternal effects on their offspring. If females were able to influence the reproductive phenotype of male offspring, in response to the level of local competition within their population, this could give a clear advantage over competitor males. As male house mice respond plastically to their local level of sperm competition by increasing sperm production (Ramm and Stockley 2009a) and altering mating behaviour and ejaculate allocation (Preston and Stockley 2006; Ramm and Stockley 2007) the adaptive benefit of a maternal effect on these traits may be counteracted by mechanisms of phenotypic plasticity. Particularly as mating

behaviour and ejaculate allocation are influenced by the local environment at the time of mating (Preston and Stockley 2006; Ramm and Stockley 2007).

Correlations of mating behaviour and ejaculates

As there were no differences found in the mating behaviour of males according to treatment group, data was combined to analyse the relationship between behavioural traits and ejaculate composition.

4.5.3 Trends in ejaculate protein composition

Predictably, the number of sperm within the ejaculate strongly correlated with the ejaculate protein composition. Specifically, proteins that were deemed significantly important in relation to the number of sperm tended to be sperm specific proteins. SVS2, a major component of the seminal vesicle secretion, was found to have a negative correlation with the number of sperm within the ejaculate. As this protein is primarily found within the copulatory plug, and males that ejaculated a greater number of sperm also produced a larger plug, it is possible that this difference was due to more of SVS2 being utilised within the plug in males that ejaculate more sperm.

The protein composition of the ejaculate washed from the uterus only correlated with the size of the copulatory plug when all protein within this solution were analysed. This would include a large amount of tissue and female derived proteins. Further, the significance value was only marginally below the 0.05 threshold. I would therefore remain cautious with the interpretation of these results. Most of the proteins deemed significant predictors of plug size within the ejaculate were very low abundance, non-specific proteins. Two male derived serine protease inhibitor proteins (Serpin-1 and Spikl) were significant at predicting copulatory plug size. It could have been predicted that a greater plug size would correlate with a greater quantity of protease inhibitors, as these proteins act to reduce protein degradation and therefore may protect the plug. However, the relationship of each was contradictory and so no conclusions can be drawn from this.

4.5.4 Trends in male mating behaviour

Smaller male house mice required more trials with females before a successful mating would occur. An increased number of trials lead to a larger copulatory plug, regardless of body size. The proteins that compose the copulatory plug have a particularly rapid turnover rate (Claydon, Ramm *et al.* 2012). It is feasible that the smaller males wait until they have produced additional seminal vesicle proteins before they will mate, allowing the production of an equivalent plug. The same relationship is not found with the number of sperm within the ejaculate, however sperm production in the house mouse takes around 30 days (Amann 1970; Claydon, Ramm *et al.* 2012).

Males that performed a great number of thrusts and intromissions also ejaculated a greater number of sperm. From this study it cannot be determined if males invest a greater amount in mating behaviour if they have a larger store of sperm available, or whether males are stimulated to ejaculate a greater number of sperm if they perform more mating behaviours. It is also unknown whether it is the male or female that determines the duration of the mating and the frequency of the intromissions and thrusts. Regardless of these unknowns, these results show that males which invest more energy into their mating behaviour also invest more in their ejaculate, by increasing their sperm output.

4.6 Conclusions

The use of proteomics has shown that, within the house mouse, the protein composition of the ejaculate may be influenced by the environment their mother experienced prior to conception. The mechanism for this is uncertain. Litter size and broader measures of reproductive physiology were not influenced by the maternal environment. This result highlights the potential for subtle maternal effects that may affect future fertility.

Chapter 5
Sperm competition and sperm production in mammals

5.1 Chapter overview

Sperm competition has led to selection on males to evolve larger testes. Here, I find that males primarily increase sperm output by increasing the rate of sperm production.

Sperm competition leads to an increased relative testis mass across a diversity of species. It is expected that this leads to greater sperm production, therefore giving males an advantage under competition. There is increasing evidence that functional adaptations within the testes also increase the rate of spermatogenesis within promiscuous species, and so differences in testes mass may be underestimating the

increased sperm production capacity. Here, an analysis of data collected from the literature finds that relatively larger mammalian testes do produce more sperm. Further, relatively larger testes are more efficient at sperm production, producing more sperm per gram of testicular tissue. Combining data from the literature with new data collected from histological analyses, I show that increased sperm production is primarily due to a faster rate of spermatogenesis, or shorter seminiferous epithelium cycle length (SECL). The effects of sperm competition on other testicular traits are also analysed. Finally, broad morphological differences in the sperm collected from species within two distinct orders, rodents and ungulates, are presented.

5.2 Introduction

Sperm competition occurs when sperm from more than one male compete within the female reproductive tract to fertilise an ovum (Parker 1970). Under the theory of a fair raffle, whereby all sperm have an equal chance of being successful (Parker 1990), males that can produce and ejaculate more sperm will gain an advantage. Males under greater selection from sperm competition have evolved relatively larger testes (Harcourt, Harvey *et al.* 1981). As larger testes produce more sperm (Amann 1970; Moller 1989), an increased relative testis mass can improve a male's chance of gaining fertilisations. Increasingly, there is evidence that selection via sperm competition may also be acting on traits that alter the efficiency and rate of spermatogenesis. Therefore differences in relative testis mass could be underestimating differences in sperm production (Lüpold, Linz *et al.* 2009; Ramm and Schärer 2014).

Spermatogenesis is relatively conserved across mammals (Roosen-Runge 1977). The mitotic spermatogonia are recruited to mature within the semiferous epithlium. These spermatocytes, supported by the Sertoli cells (Jégou 1992), continue developing within the seminiferous tubules, moving towards the tubule lumen with each stage of development. After completing approximately four and a half cycles of

the seminiferous epithelium (Clermont 1972), the immature sperm (spermatids) are released from the seminiferous epithelium and travel to the epididymides for storage. Spermatogenesis is a continuous process and each cell transitions through histologically recognisable stages. Groups of maturing spermatocytes are associated with other groups of cells in defined stages, such that spermatagonia at a set developmental stage are always associated with the same type of spermatocytes and spermatids. The cycles of the seminiferous epithelium are based on these typical cellular associations. Each group of cellular associations follows in a continuous sequence and a full series of these associations is considered one cycle (Clermont 1972).

There are three primary mechanisms by which sperm production could be increased:-

1) Having a greater number of supportive Sertoli cells, or increasing their capacity to support a greater number of spermatogonia.
2) Increasing the proportion of sperm producing tissue within the testes.
3) Increasing the rate of production, by reducing the time each cycle of the seminiferous epithelium takes.

Sertoli cells support a limited number of germ cells (Johnson, Carter *et al.* 1994) and after puberty the quantity of these is reasonably stable (Kluin, Kramer *et al.* 1984; Herrera-Alarcón, Villagómez-Amezcua *et al.* 2007). Selection may therefore favour more Sertoli cells, or for them to have a greater supportive capacity. Sertoli cells are more efficient and able to support more developing germ cells under a high level of sperm competition, in both birds (Lüpold, Wistuba *et al.* 2011) and mammals (delBarco-Trillo, Tourmente *et al.* 2013).

The proportion of spermatogenic tissue within testes is highly variable. Within a study of testes from 12 mammalian species, the proportion of seminiferous tissue ranged from 33% to 93% (Russell, Ren *et al.* 1990). This wide range may in part be due to selection via sperm competition, as there is evidence in both avian (Lüpold,

Linz *et al.* 2009; Rowe and Pruett-Jones 2011) and mammalian (Montoto, Arregui *et al.* 2012) species that larger relative testes mass correlates with increased proportion of seminiferous tissue.

The Seminiferous Epithelium Cycle Length (SECL), and therefore rate of sperm production, varies across species. In mammals there is increasing evidence that the SECL correlates negatively with relative testis mass, indicating sperm competition increases the rate of sperm production (Peirce and Breed 2001; Parapanov, Nusslé *et al.* 2008; Ramm and Stockley 2010; delBarco-Trillo, Tourmente *et al.* 2013). Taken together, these studies suggest sperm production should be more efficient in species with high levels of sperm competition, creating a greater sperm output per unit of testis. This study aims to test this in mammals, to determine whether functional adaptations of testes result in differences in sperm production efficiency, and which of these factors are most effective at increasing sperm output.

As well as maximising sperm production, the testicular architecture may be optimised to the morphology of the sperm it is producing. The subject of sperm competition and sperm length is a contentious one. Various studies have found no evidence of a relationship (Briskie and Montgomerie 1992; Gage and Freckleton 2003; Ramm and Stockley 2010), however there is an opposing argument that sperm competition promotes longer sperm (Briskie, Montgomerie *et al.* 1997; LaMunyon and Ward 1999; Tourmente, Gomendio *et al.* 2011a). Within mammals, it is possible that metabolic constraints limit the effects of sperm competition on sperm size, and accounting for this within the analysis reveals selection for larger sperm (Gomendio, Tourmente *et al.* 2011; Tourmente, Gomendio *et al.* 2011b; delBarco-Trillo, Tourmente *et al.* 2013). Within this study, broad morphological measurements of sperm are made in order to incorporate this in the analysis of testicular architecture, and to compare the general trends across the species with samples available. There is not scope to delve into the discussion of sperm length in relation to sperm competition with the limited data set here.

This study uses comparative analysis techniques to account for the non-independence of data points due to shared ancestry (Freckleton, Harvey *et al.* 2002; Nunn 2011; Orme, Freckleton *et al.* 2012). Phylogenetic analysis methods have become more sophisticated, and more commonly used, in recent years. This is perhaps due to the increased availability, and accessibility, of these methods within open platforms, particularly R (R-Development-Core-Team 2011). Details of the analysis can be found within the methods section, but the importance of performing comparative analysis correctly, accounting for phylogenetic similarities, is worth noting here.

5.3 Methods

5.3.1 Literature data collection

Data on testicular traits and daily sperm production were sourced from the literature. Information was available for 35 mammalian species across six orders. Where possible, data were collected from the same source for each species. Data is presented within the Appendix (Table A1).

5.3.2 Testes sample collection

Samples of testicular tissue from captive bred large mammals were collected from local zoological collections, Chester Zoo and Knowsley safari park (n=11 species, 15 samples). Samples from domestic mammals were collected from EM&T Jackson abattoir, Knutsford, and Ness Heath Farm, which is attached to the Leahurst campus, University of Liverpool (n=4 species, 6 samples). Red squirrels were collected from carcasses received by the National Trust, Formby (n=2). All other rodent samples were collected from wild caught or laboratory bred animals within the MBE group's collection (n=5 species, 13 samples).

Large mammal testicular and epidiymal samples were collected following castration or death. Males were castrated due to either already having produced sufficient numbers of offspring previously or, at becoming sexually mature, to reduce the likelihood of aggressive behaviour. Death was either due to illness or, in the case of the domestic mammals, being killed for food. Immediately on removal, samples were chilled prior to collection. The longest delay between removal and processing was three days, but most were collected on the same day.

After collection, scrotal tissue and the tunica vaginalis were removed from both testes within the laboratory. Histological samples were collected from only one testis, allowing the other to remain whole for volumetric measurements to be taken at a later date. It was also expected that testes from each side would have the same architecture and epidiymal sperm. The left or right testis was stored at -20°C at

random. The second testis was washed twice in ddH2O and then once in 70% ethanol to remove residual dirt and blood. The epididymis was cleaved from the testis and approximately 1cm^3 sections of testicular tissue were taken from three evenly distributed segments of each testis. These were fixed in Bouin's fixative (Saturated picric acid 15: Formaldehyde 5: Glacial acetic acid 1) for two days, prior to storage in 70% ethanol.

Sperm were collected from the caudal section of the epididymis. An approximately 1cm^3 section was dissected and placed in a Petri dish. This tissue was scored repeatedly and 400µl PBS added. This was left for 5 minutes before collecting the sperm suspension. The Petri dish was then washed with a further 400µl of PBS. The sperm suspension was frozen at -80°C prior to proteomic analysis. These results are presented within Chapter 6. Sperm remaining on the cauda epididymis were rinsed and collected with a further 50µl of PBS. Sperm within 10µl of this suspension were viewed under Leica DM 1000 light microscope. A camera and associated software (FlyCap; Point Grey) attached to the microscope were used to take images of multiple sperm, which could be clearly seen at 400x magnification for further measurements to be taken later.

All rodent samples collected from within MBE were dissected immediately after death. Red squirrels were collected and dissected within a day of arrival at Formby. These carcasses had been found by members of the public or staff at Formby National Trust Centre and so I cannot be certain how fresh these were, however, from the quality of the samples I would assume dissection occurred within two days post mortem.

One testis was placed whole, or sliced in half if larger than 2cm in length, into Bouin's fixative for 2 days before being stored in 70% ethanol. The other testis was stored in the freezer. Both epididymides were dissected and the cauda section isolated. The paired cauda epididymides were placed onto a Petri dish with 200µl of PBS, before being macerated with a scalpel. After leaving for 5 minutes the sperm

suspension was collected to be stored at -80°C. The Petri dish was rinsed with PBS to collect remaining sperm to photograph, as described above.

5.3.3 Testis histology and measurements

Histological processing of the fixed testicular tissue was performed by the histology services department at the University of Liverpool. Briefly, each section of fixed tissue was trimmed to an appropriate size before being dehydrated in alcohol and embedded in paraffin wax. A microtome was used to create 5µm thick sections, which were then stained with Haematoxylin and Eosin (H&E).

Testicular sections were viewed using a Leica DM 1000 light microscope. A digital camera, attached to the microscope, and associated software (FlyCap; Point Grey) were used to take multiple images of each slide at 200x magnification (Figure 5.1). Measurements of testicular architecture were taken from the digital images using ImageJ (Rasband 1997-2012). For each male, measurements were taken from images of three histological slides. Multiple tubules were measured from each slide, so that each male had a minimum of 23 measures of tubule diameter (mean±s.e.m. = 67±4.5) taken and the tubule area calculated from these. For each male, a minimum of 52 measurements of the epithelium height were made (mean±s.e.m. = 128±9.3). From these measurements, the relative proportions of seminiferous epithelium, tubule lumen and interstitial tissue could be calculated.

5.3.4 Sperm measurements

Measurements of sperm morphology were made using ImageJ software (Rasband 1997-2012). For each individual, measurements of tail length, head length and head width were taken for a minimum of 5 sperm that could be clearly seen. The head width measurements were taken at the widest point of the sperm head. For the rodent species, the head width measurement included the hooked section (see Figure 5.2).

5.3.5 Data analysis

Data analysis was performed in R (R-Development-Core-Team 2011). Phylogenetic Generalised Least Squares (PGLS) analysis was used to control for the effects of phylogeny, and shared inheritance of traits (Felsenstein 1985; Freckleton, Harvey *et al.* 2002; Gage and Freckleton 2003). The supertree of Bininda-Emonds et al (2007) was used to construct the phylogeny (Figure A1). The R packages APE (Paradis, Claude *et al.* 2004), CAPER (Orme, Freckleton *et al.* 2012) and Geiger (Harmon, Weir *et al.* 2008) were then used to perform the PGLS analysis. Partial residual plots of the data were constructed using the package Faraway (Faraway 2014). All data were transformed as appropriate.

PGLS analysis accounts for phylogenetic non-independence of data points by calculating a variance-covariance matrix, for how similar traits should be if they are following Brownian model of evolution, and applying this to a linear model (Freckleton, Harvey *et al.* 2002; Orme, Freckleton *et al.* 2012). Not all traits will directly follow a Brownian model. To account for this, the internal branch lengths are multiplied by a constant that improves the fit of the model. The PGLS method also incorporates a lambda (λ) transformation, and calculates a maximum likelihood value (Orme, Freckleton *et al.* 2012). A larger value of λ indicates a greater amount of covariance between closely related species, with λ = 1 corresponding to a Brownian model of inheritance (Freckleton, Harvey *et al.* 2002). When λ = 0, there is no evidence for shared inheritance of the traits. In this instance, assuming an ultimetric phylogeny, the model will give the same result as a standard linear model. Within the analysis presented here, when λ = 0 the results for the linear model are shown for simplicity, and to avoid accusations of statistical machismo (McGill 2012).

Here, PGLS is used to determine the effect of sperm competition on the evolution of testicular traits. The level of sperm competition within a species is not a specific continuous trait that can be utilised within a regression analysis. Commonly, comparative studies use the effect of testes mass, when controlled within the model for body mass, as an indicator of the level of sperm competition (e.g. Gage and

Freckleton 2003; Ramm and Stockley 2009, but see Briskie and Montgomerie 1992) . Sperm competition leads to the evolution of larger testes in mammals (Moller 1989; Harcourt, Purvis *et al.* 1995; Ramm, Parker *et al.* 2005). Within this analysis, testes mass was strongly predicted by body mass ($\lambda = 0$, n = 50, $F_{1, 48} = 373$, $P < 0.001$). The species here were also classified according to mating system, either single or multi-male. Species with single male mating systems tend to only mate with one male per reproductive bout, and those classified as multi-male commonly mate multiply each oestrus. Mating system, either single or multi-male, was a significant predictor of testes mass when body mass was included in the model ($\lambda = 0$, n = 50, $F_{2, 48} = 9.36$, $P = 0.004$, Figure 5.3). Or, a multi-male mating system does lead to larger relative testes mass within the species here. Therefore here a significant testes mass effect, when body mass is included as a covariate, is considered to be a significant effect of selection via sperm competition.

Figure 5.1: Cross section of a seminiferous tubule from a histological section of a *Mus spiceligus* testis stained with hematoxylin and eosin (H&E) (200x magnification). Black double arrow indicates seminiferous epithelium height.

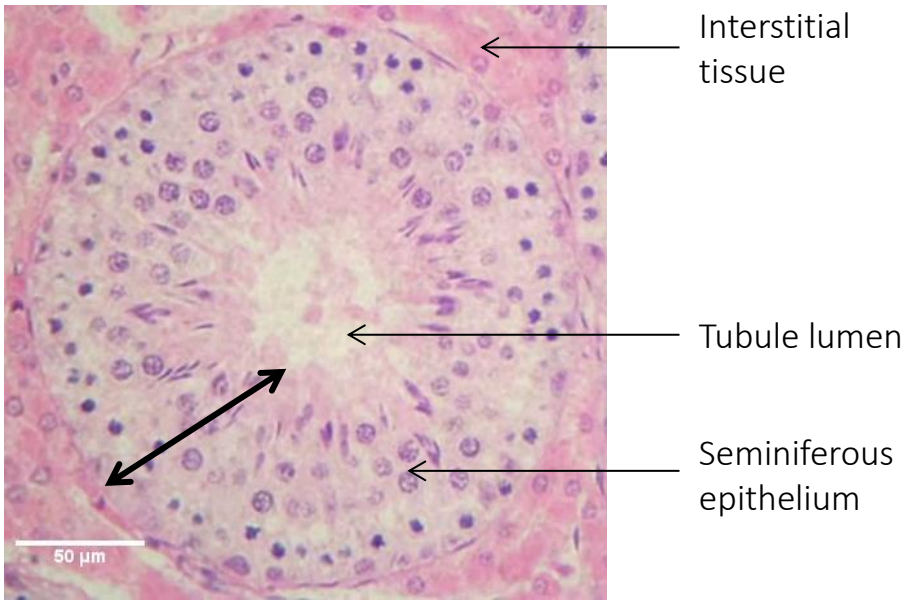


Figure 5.2: Images of sperm from a woodmouse and a ram taken at 400x magnification. Dotted lines indicate measurement taken for sperm head height, solid lines sperm head width. For rodents, sperm head height measurements included the hook. Tail length was measured by following the length of the tail using a freehand line on ImageJ.

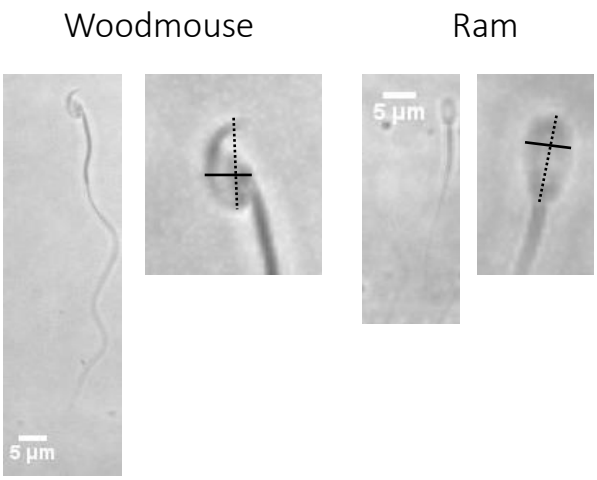
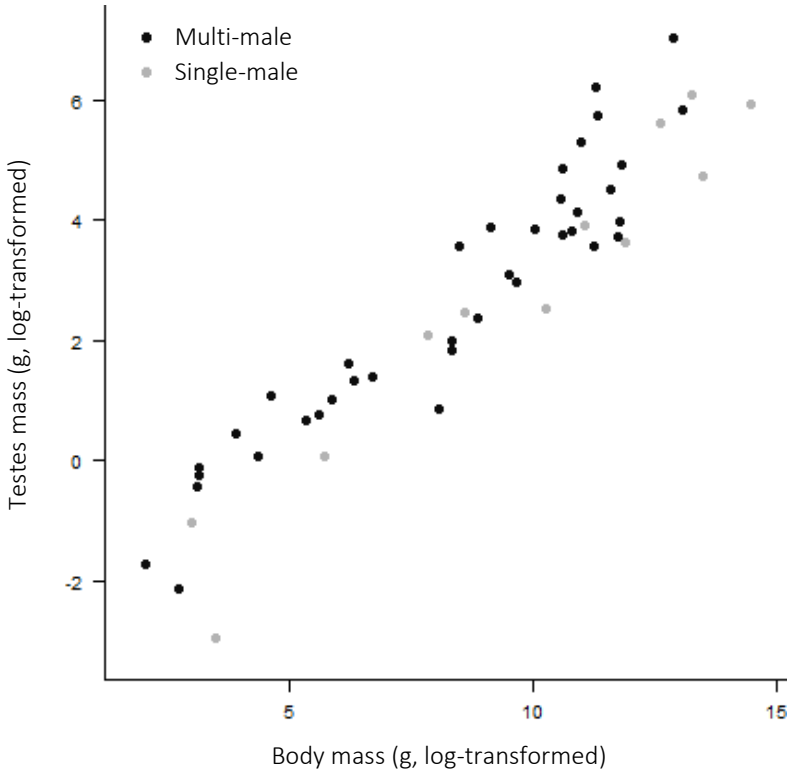


Figure 5.3: Scatterplot of the relationship between body mass and testes mass within the mammalian species sampled. Dark circles indicate a multi-male mating system, whereby females commonly mate with more than one male each oestrus cycle, grey circles represent a single-male mating system, within which females tend to only mate with one male per reproductive bout.



5.4 Results

5.4.1 Sperm competition and sperm production

Testes are known to be relatively larger in species under selection via sperm competition (Kenagy and Trombulak 1986; Harcourt, Purvis *et al.* 1995; Stockley, Gage *et al.* 1997). Here, a significant testes mass effect when controlling for body mass is considered to indicate an adaptation to sperm competition, as has been suggested previously (Gage and Freckleton 2003; Ramm and Stockley 2010).

Testes mass predicted daily sperm production (DSP) when accounting for body mass ($\lambda = 0$, n = 30, $F_{2, 28} = 81.15$, $P < 0.001$). DSP per gram of testicular tissue was also predicted by testes mass, when controlled for body mass ($\lambda = 0$, n = 31, $F_{2, 29} = 4.12$, $P = 0.05$). Therefore sperm competition, as indicated by larger relative testes mass, is associated with greater sperm production efficiency.

5.4.2 Testicular architecture and sperm production

To understand the likely mechanism of increased sperm production efficiency, the relationship between sperm production and testicular traits was analysed. The traits for which data was available from the literature were the seminiferous epithelium cycle length (SECL), the proportion of seminiferous tissue, and the number of Sertoli cells. These factors, along with body mass and testes mass, were incorporated in a PGLS model that had DSP per gram of testes as the dependent variable. In this full model, only SECL was a significant predictor of DSP per gram ($\lambda = 0$, n = 27, t = -3.18, $P = 0.011$). As body mass is included as a covariate of the model, the effects of overall size are accounted for and so a model with total DSP was performed with the same predictor variables. Here, again SECL was a significant predictor, as was testes mass ($\lambda = 0$, n = 27 SECL: t = -2.39, $P = 0.04$, testes mass: t = 2.74, $P = 0.023$; see Table 5.1). This effect became more pronounced in a reduced model. Although there was no effect of the proportion of seminiferous tissue on sperm production, it is worth noting that, without accounting for body mass, this is a strong predictor of

DSP (λ = 0.1, n = 40, t = 2.91, *P* = 0.008). However, although in theory this trait may not be linked to body mass, in reality males with a larger body mass have a smaller proportion of seminiferous tissue (λ = 0.55, n = 40, t = -2.74, *P* = 0.009). See Figure 5.4 for the relationship between testicular architecture and sperm production.

5.4.3 Sperm competition and testicular traits

This analysis found that sperm competition appears to select for a faster rate of sperm production, as there was a significant effect of testes mass controlled for body mass, on the SECL (λ = 0, n = 31, TM effect: t = - 3.33, *P* = 0.024). The number of Sertoli cells was also significantly predicted by testes mass (λ = 0.74, n = 20, TM effect: t = 4.55, *P* < 0.001). The proportion of sperm producing seminiferous tissue was not predicted by testes mass, when controlled for body mass (λ = 0.49, n = 40, TM effect: t = 1.20, *P* = 0.24). Further details can be found in Table 5.2 and Figure 5.5.

5.4.4 Testis histology

None of the histological measures of testicular architecture were predicted by sperm competition. Unfortunately, due to the species available, there were only a few species for which the data could be included in the analysis of sperm production efficiency.

5.4.5 Sperm length

Sperm competition, as indicated by a significant effect of testes mass when controlled for body mass, did not predict sperm length. There was also no relationship between sperm morphology and sperm production rates. Males with a smaller body mass did have a longer sperm length (λ = 0, n = 30, t = -4.67, *P* < 0.001). In accordance with this, ungulates had shorter sperm than rodents (n = 24, W = 125, *P* < 0.001). The broad differences in sperm length between rodents and ungulates were primarily due to differences in the length of the tail (Tail: λ = 0.0, n = 14 Family effect: t = -3.16, *P* = 0.008; Head: λ = 0.47, n = 14 Family effect: t = -2.29, *P* = 0.04).

When accounting for these broad differences by including body mass in the phylogenetically controlled model, mating behaviour, either multi or single male mating system, did predict sperm length (λ = 0.85, n = 30 mating effect: t = -2.62, *P* = 0.014). There was very little variance in the length of sperm found within ungulate species. In contrast, the sperm of rodents were far more variable and those from species with multi-male mating systems were considerably longer (see Figure 5.6).

The interesting hook morphology found within the rodent species here (see Figure 5.2) is well documented in murine rodents and exaggerated due to sperm competition (Breed 2005; Immler, Moore *et al.* 2007). The pronounced hooks made the head width measurements not directly comparable. Here, rodents were found to have wider sperm heads, when measured including the hook, than ungulates (λ = 0, n = 14, t = -2.43, *P* = 0.032). It is clear that the sperm of large mammals is morphologically very different from rodents.

Figure 5.4: Partial residual plots to show the relationship between daily sperm production and body mass, testes mass, seminiferous epithelium cycle length, and proportion of seminiferous tissue. Partial residual plots show the relationship between the designated independent variable and daily sperm production, when accounting for the rest of the variables in the model.

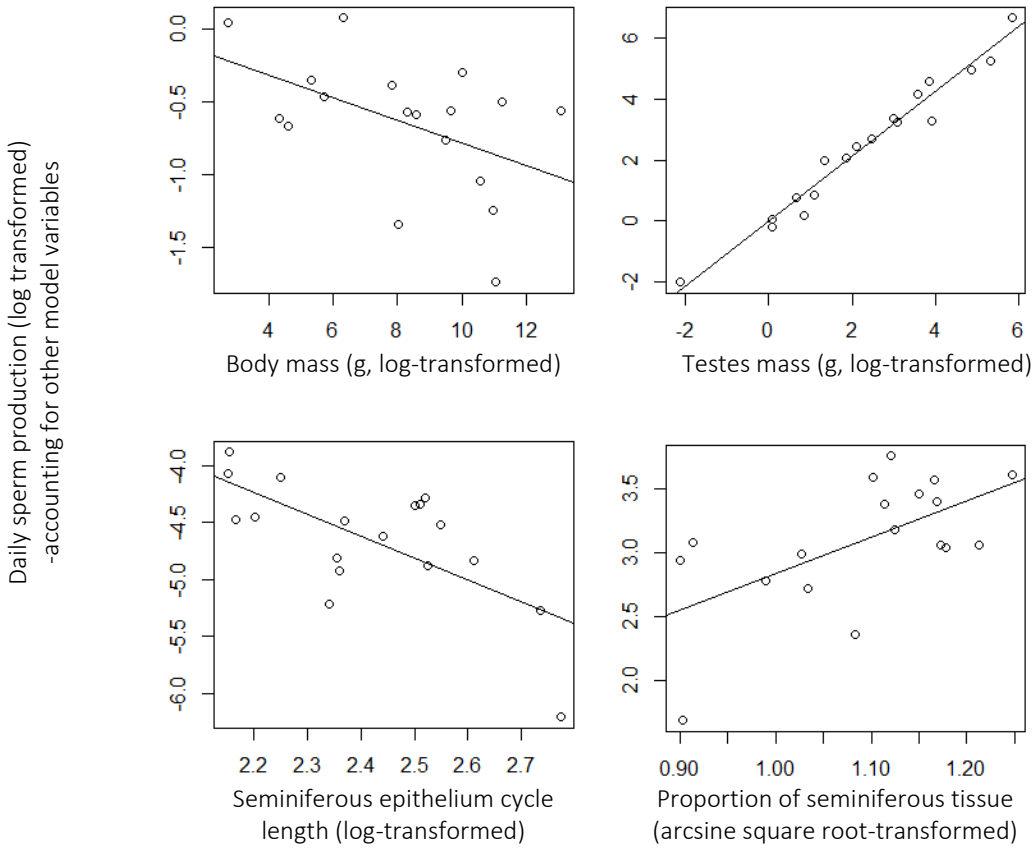


Table 5.1: Table of values following analysis of the effect of testicular traits on daily sperm production. DSP/g = Daily sperm production / gram testicular tissue, BM= Body mass, TM = Testes mass, SECL = Seminiferous epithelium cycle length, %SE = proportion of seminiferous tissue, #Sertoli = the total number of Sertoli cells within the testes. When λ=0 there is no indication of phylogenetic signal and so the model is equivalent to a linear model.

Term	Estimate ± s.e.	t	*P*-value	r^2_{adj}
Model: DSP/g ~ BM + TM			**n=31, λ=0**	
Intercept	4.57 ± 0.49	9.398	<0.001	0.23
Body mass	-0.24 ± 0.09	-2.81	0.009	
Testes mass	0.23 ± 0.11	2.03	0.05	
Model: DSP ~ BM + TM+SECL+%SE+#Sertoli			**n=27, λ=0**	
Intercept	7.74 ± 3.96	1.96	0.082	0.93
Body mass	-0.23 ± 0.20	-1.15	0.28	
Testes mass	1.27 ± 0.46	2.74	0.023	
SECL	-1.97 ± 0.83	-2.39	0.04	
%SE	1.84 ± 1.67	1.10	0.3	
#Sertoli	-0.07 ± 0.44	-0.17	0.87	
Model: DSP ~ BM + TM+SECL+%SE			**n=30, λ=0**	
Intercept	5.32 ± 2.74	1.944	0.0739	0.94
Body mass	-0.08 ± 0.15	-0.51	0.6181	
Testes mass	1.06 ± 0.19	5.659	<0.001	
SECL	-1.92 ± 0.74	-2.61	0.0214	
%SE	2.84 ± 1.40	2.022	0.0643	

Figure 5.5: Partial residual plots from models investigating the relationship between testes mass, when accounting for body mass indicating an effect of sperm competition, on testicular traits. These plots show the relationship between the designated response variables and testes mass, given that body mass was a co-variable in the models.

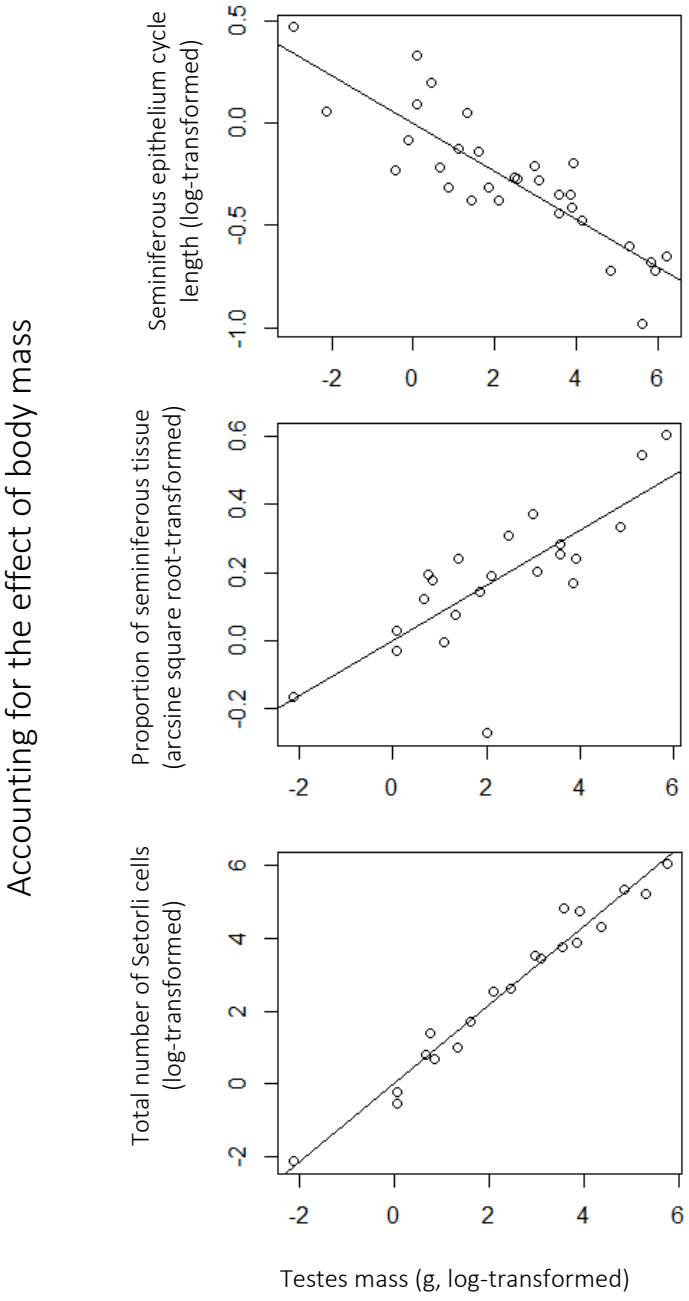
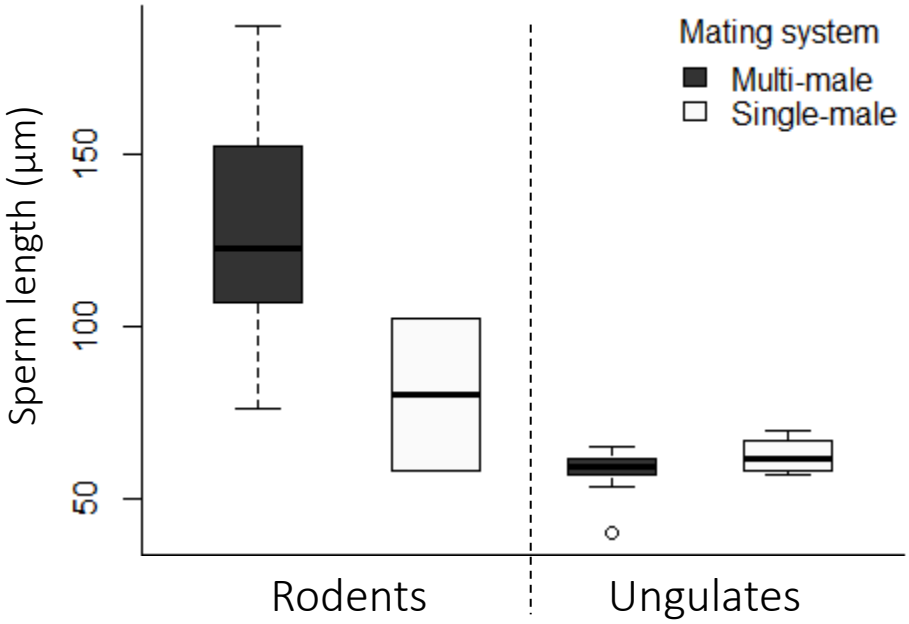


Table 5.2: The effect of testes mass, when controlling for body mass, on testicular traits. Values of λ are a maximum likelihood estimate, with a potential range between 0 – 1: larger values indicate a greater phylogenetic signal. SECL = Seminiferous epithelium cycle length, BM = Body mass, TM = Testes mass, %SE = Proportion seminiferous tissue, #Sertoli = the number of Sertoli cells within the testes.

Term	Estimate ± s.e.	t	*P*-value	r^2_{adj}
Model: SECL ~ BM + TM			**n=31, λ=0**	
Intercept	1.81 ± 0.14	13.42	<0.001	0.37
Body mass	0.11 ± 0.03	4.145	<0.001	
Testes mass	-0.12 ± 0.04	-3.33	0.002	
Model: %SE ~ BM + TM			**n=40, λ=0.49**	
Intercept	1.37 ± 0.11	12.3	<0.001	0.15
Body mass	-0.04 ± 0.02	-2.44	0.019	
Testes mass	0.03 ± 0.02	1.2	0.238	
Model: #Sertoli ~ BM + TM			**n=20, λ=0.74**	
Intercept	4.65 ± 0.67	6.96	<0.001	0.94
Body mass	-0.17 ± 0.12	-1.41	0.18	
Testes mass	1.13 ± 0.14	7.87	<0.001	

Figure 5.6: Boxplot of sperm length, according to mating system, within rodents and ungulates. A multi-male mating system refers to one whereby females commonly mate with more than one male each oestrus cycle. In contrast, in a single-male mating system females tend to only mate with one male per reproductive bout.



5.5 Discussion

5.5.1 Sperm competition selects for more efficient sperm production

This analysis shows that selection through sperm competition appears to increase sperm production efficiency, as males with relatively large testes for their body size also produce more sperm per gram of testicular tissue each day. This increased efficiency is primarily due to an increased rate of sperm production, through the shortening of the SECL. That sperm competition selects for a shortened SECL has been shown previously (Parapanov, Nusslé *et al.* 2008; Ramm and Stockley 2010; delBarco-Trillo, Tourmente *et al.* 2013). The association with SECL and daily sperm production (DSP) may be due, in part, to these two factors being autocorrelated. DSP is generally calculated by counting the number of spermatids within a testicular homogenate before dividing this figure by the SECL, as this is the number of days each sperm will remain in this stage of development (e.g. Amann 1970). Regardless, this analysis provides further evidence that sperm competition selects for a faster rate of sperm production, and that this leads to increased sperm production efficiency.

There was no association between the number of Sertoli cells and DSP. Males with relatively larger testes had more Sertoli cells overall, but these were not found at a greater density. This is in agreement with previous work in mammals (delBarco-Trillo, Tourmente *et al.* 2013). It is likely that instead of gaining a greater density of Sertoli cells, sperm competition selects for Sertoli cells to support a greater number of developing germ cells (Lüpold, Wistuba *et al.* 2011; delBarco-Trillo, Tourmente *et al.* 2013).

There was no significant relationship between the proportion of sperm producing seminiferous tissue within the testes and DSP, when accounting for body mass. Similarly, there was no evidence within this analysis that sperm competition selects for a greater proportion of sperm producing tissue. This is in contrast to previous analysis in avian species (Lüpold, Linz *et al.* 2009; Rowe and Pruett-Jones 2011),

however the range of values within the analysis here was far greater than within the earlier studies (32.7% – 92.7% here, compared to Lüpold et at: 87.7% – 95.7%; Rowe and Pruett-Jones: 99.74% - 98.99%).

Studies of mammalian species have also found an effect of sperm competition on the proportion of seminiferous tissue (Montoto, Arregui *et al.* 2012; delBarco-Trillo, Tourmente *et al.* 2013). The different result found here may be due to this study having a smaller proportion of small mammals (including Rodentia, Lagomorpha and Eulipotyphla). The earlier of the two previous studies was an analysis of four *Mus* species (Montoto, Arregui *et al.* 2012). The more recent analysis contained 56% small mammal species (delBarco-Trillo, Tourmente *et al.* 2013), compared to 42% within this study. As the proportion of seminiferous epithelium tissue is negatively correlated with body mass, it is possible that the large mammals within this analysis are masking the effect of sperm competition within the smaller mammals. Analysing only rodent species within the data available here does not give a significant result, however this is on a sample size of 12. The limited data here is due to the primary analysis focussing on the effects of testicular traits on DSP, and so only species with values for DSP are included.

When considering the proportion of sperm producing tissue within the testes it is also worth bearing in mind the function of the interstitial tissue. Much of the space between the tubules is filled with androgen producing Leydig cells (Stocco and McPhaul 2006). In capybaras (*Hydrochaeris hydrochaeris*), a species with testes containing 72% interstitial tissue (Moreira, Clarke *et al.* 1997), increases in relative testes mass are more closely associated with larger androgen-dependant scent glands than greater sperm production (Moreira, Macdonald *et al.* 1997). In large ungulate species, high levels of testosterone often correlate to larger secondary sexual characteristics (i.e. horns or antlers) (Suttie, Lincoln *et al.* 1984; Martin, Presseault-Gauvin *et al.* 2013). These weapons are under strong sexual selection (Bro-Jørgensen 2007a), and so this may result in a trade-off between the proportion of testicular tissue devoted to sperm production, as opposed to androgen

production. Within smaller species of mammals, it may be harder for males to compete in pre-copulatory sexual selection. In this instance there may be a greater emphasis on increasing sperm production in order to remain competitive.

5.5.2 Testicular histology

This analysis found no evidence for sperm competition selection acting on any of the histological traits measured here. This is in agreement with a larger study of mammals, analysing data collected from literature sources (delBarco-Trillo, Tourmente *et al.* 2013). It is possible that functional constraints of spermatogenesis limit adaptations of the gross architecture of the testes. Instead, adaptations to the relative size of the testes, the rate of sperm production, supportive capacity of Sertoli cells and proportion of sperm producing tissue appear to be enough to sufficiently increase sperm production. Further, no relationship was found between histological measures and sperm length, suggesting testis architecture is not, at least in part, determined by sperm morphology.

5.5.3 Sperm morphology

Gross differences in sperm morphology may further highlight the differing priorities of males from these distinct mammalian orders. Sperm from murine rodents is well characterised by the impressive hooked head morphology (Breed 2005; Šandera, Andrlíková *et al.* 2011). This adaptation allows sperm to aggregate into trains, which move faster through the reproductive tract (Moore, Dvorakova *et al.* 2002; Immler, Moore *et al.* 2007). The analysis here found the sperm tails of rodents to be far longer than in larger mammals. When considering the huge difference in the size of the tracts the sperm will navigate, this represents a considerable difference in the relative investment in the sperm morphology of these species.

The relationship between sperm length and sperm competition is somewhat contentious. There is evidence that sperm competition leads to longer sperm (Briskie, Montgomerie *et al.* 1997; LaMunyon and Ward 1999; Tourmente, Gomendio *et al.* 2011a; Tourmente, Gomendio *et al.* 2011b). However, many

studies, as within this analysis, find no relationship between competition and sperm size (Briskie and Montgomerie 1992; Gage and Freckleton 2003; Ramm and Stockley 2010). It has been suggested that, within mammals at least, the differences in selection on sperm length are due to metabolic constraints (Gomendio, Tourmente *et al.* 2011; Tourmente, Gomendio *et al.* 2011b; delBarco-Trillo, Tourmente *et al.* 2013).

The intricacies of the relationship between sperm competition and selection on sperm morphology may be beyond the scope of this chapter; however it is of note how distinct the morphologies of rodent, compared to ungulate, sperm are (see Figure 5.2). This may be due to different investment strategies, with larger males instead requiring greater effort to secure mates.

5.6 Conclusions

Here, I show that sperm competition leads to an increase in the efficiency of sperm production within mammals. This is primarily driven by a decreased SECL, increasing the rate of spermatogenesis. In contrast to previous studies, I find no evidence for sperm competition selecting for an increased proportion of seminiferous tissue. This may be due to this study including a greater proportion of larger species, for which the androgen producing interstitial tissue of the testes may have greater importance. Differences in investment strategies, according to family groups, are further highlighted when considering gross sperm morphology. Rodents tend to have highly evolved sperm, adapted to a high level of post-copulatory sexual selection. In contrast, males from larger species may instead invest in being more successful at pre-copulatory sexual selection.

Chapter 6
Proteomic investigation of mammalian sperm

6.1 Chapter overview

Proteins involved in reproduction are known to evolve rapidly. Here, proteomic analysis is performed on epididymal sperm samples from a broad range of mammalian species.

Sexual conflict and post-copulatory sexual selection can lead to rapid evolution of the proteins involved in fertilisation processes. The reproductive proteomes of relatively closely related species may therefore be very different from each other. Here, I use proteomic analysis of cauda epididymal samples collected from a diverse range of mammals to study variation in the sperm proteome. I find that these samples are broadly similar, however proteins that are involved in sperm – egg

interactions show lower levels of sequence homology. This is in accordance with previous evidence for the rapid evolution of those proteins necessary for sperm – egg interactions. Further, this study provides the first analysis of proteins within the cauda epidiymal samples of many of the species sampled here.

6.2 Introduction

Proteins involved in reproduction evolve rapidly, despite being required for the crucial process of fertilisation (Swanson and Vacquier 2002; Clark, Aagaard *et al.* 2006; Turner and Hoekstra 2008). The rapid evolution of reproductive proteins is of particular interest to evolutionary biologists, as it may lead to reproductive isolation and speciation (Gavrilets 2000). There is increasing evidence that post-copulatory sexual selection and sexual conflict drive both rapid evolution, and co-evolution, of male and female mammalian reproductive proteins (e.g. Swanson, Yang *et al.* 2001; Dorus, Evans *et al.* 2004; Turner and Hoekstra 2006; Ramm, Oliver *et al.* 2008; Dorus, Wasbrough *et al.* 2010). These studies utilise genomic techniques to compare the rate of genetic divergence, of genes specific to reproduction, within closely related species. More recently, proteomic techniques have been used to compare mammalian ejaculate proteins (Druart, Rickard *et al.* 2013), finding functional adaptations that have evolved according to the level of sperm competition (Ramm, McDonald *et al.* 2009). For wide scale analyses of rates of protein evolution genomic approaches offer the best results. Here I wanted to identify the major components of the epididymal secretion from a diverse set of mammals, as well as making inferences as to the evolution of these proteins, and so a proteomics approach was taken.

In order to identify proteins using proteomic techniques, knowledge of the protein sequence is required. Databases, such as UniProtKB, offer a vast resource of protein sequence information that can be used to identify peptides (Consortium 2014). As 98% of the protein sequences within UniProtKB have come from translating the coding sequences of known genes, most of the protein sequence information

available is reliant on the level of genomic information for that species. When analysing species which have no protein sequence information available, database searches will instead find proteins that share identical peptides. These are often homologous proteins from closely related species. *De novo* sequencing techniques enable the sequencing of peptides without protein sequence information (e.g. Dancik, Addona *et al.* 1999). Recent advances in proteomic analysis software utilise *de novo* sequencing to increase the accuracy of database searching when studying species for which protein sequence information is unavailable (Ma, Zhang *et al.* 2003; Zhang, Xin *et al.* 2012).This provides a useful tool for comparing the proteomes of various, non-sequenced species. Here, I use proteomics techniques to compare the proteins present within samples obtained from the cauda epididymides of 19 mammalian species belonging to two broad groups: rodents and ungulates.

When considering rapidly evolving proteins, such as those within the reproductive tract, techniques relying on the presence of orthologous proteins may appear unorthodox. However, although many reproductive proteins are evolving rapidly, there is evidence that a majority of functional proteins are relatively conserved (Dean, Clark *et al.* 2009; Carnahan-Craig and Jensen-Seaman 2014). Comparing the proportion of the protein also identified within other species, and therefore conserved, can imply rates of amino acid sequence divergence. Furthermore, proteomics methods provide an accurate depiction of the proteins present within the sample. Methods relying on mRNA transcripts are not representative of protein expression (Gygi, Rochon *et al.* 1999), which could be of particular importance to the reproductive proteins that limit mRNA expression to the testes, although the proteins are present within sperm (e.g. Zonadhesin, Gupta 2005).

As well as a broad overview of those proteins found within epididymal samples obtained from a range of mammalian species, I consider sperm specific proteins known to either be structurally important or involved in sperm – egg interactions (see Gupta 2005 for a detailed introduction to proteins of spermatogenesis). For fertilisation to successfully occur, the sperm sheds its acrosomal layer, penetrates

the zona-pellucida, and aligns horizontally to the oolemma, allowing it to bind at the equatorial segment. The sperm membrane and oolemma are then able to fuse, before the oocyte engulfs the sperm in a phagocytic manner (Primakoff and Myles 2002; Rubinstein, Ziyyat *et al.* 2006). Although the molecular interactions surrounding this process are yet to be entirely understood, various key proteins have been identified at each level of this process. Zonadhesin, sperm surface protein 17, spermadhesin and zona pellucida binding proteins are all known to assist with sperm binding to and penetration of the zona pellucida (Hardy and Garbers 1995; Kong, Richardson *et al.* 1995; Gao and Garbers 1998; Burkin and Miller 2000; Töpfer-Petersen and Calvete 2005). Sperm equatorial segment protein 1 and izumo sperm – egg fusion protein allow the sperm to fuse to the oolemma, with the latter protein being required for this process to occur and so essential to fertilisation (Inoue, Ikawa *et al.* 2005; Fujihara, Murakami *et al.* 2010). There are various proteins essential to the structural integrity of sperm. Here, for simplicity, I present data on the outer dense fibre proteins (ODF), and a-kinase anchor proteins (AKAP), which make up much of the sperm fibrous sheath (Vera, Brito *et al.* 1984; Oko 1988; Brown, Miki *et al.* 2003). An overview of the chosen proteins and their functions can be found in Table 6.4.

6.3 Methods

6.3.1 Samples

Epididymal sperm samples were collected as described in Chapter 5.

6.3.2 Proteomic analysis

In order to identify the proteins present within the epididymal sperm of each species, samples collected from the cauda epididymis were analysed using proteomic methods similar to those described in Chapter 2. In total, 30 samples from 19 species were analysed (see Table 6.1). Briefly, each sample was defrosted and vortexed for 1 minute. The homogenous solution was assayed to determine the protein concentration using the Coomassie plus protein assay before a trypsin digest was performed on 50 mg protein in a final volume of 200 µl. Tryptic digests were diluted 1:1 with 97:3:0.1 HPLC grade water:MeOH:TFA and then resolved over a 90 minute linear organic gradient using ultra performance liquid chromatography (Waters nanoAcquity) coupled with tandem mass spectrometry using the LTQ Orbitrap Velos (Thermo).

6.3.3 Protein identification

In order to maximise the number of peptides that were successfully matched to protein sequences, PEAKS (Bioinformatics solutions inc.) software was used to perform protein identification. PEAKS is a preferred method for improving the accuracy of protein identifications for species that do not have protein sequence data available (Ma, Zhang *et al.* 2003; Zhang, Xin *et al.* 2012). This software performs *de novo* sequencing on all peptide hits from within the raw data files. Here, I then specified for PEAKS to compare the *de novo* peptide sequences with those within a Uniprot validated database for all mammalian species. Combining *de novo* sequencing with database searching, PEAKS analysis is more mutation tolerant and improves the accuracy of each protein identification (Ma, Zhang *et al.* 2003; Zhang, Xin *et al.* 2012).

6.3.4 Data analysis

Due to the exploratory nature of this study, statistical analysis would be of limited value. Instead, information regarding the proteins identified within a cauda epididymal sample from each species available is presented here. As sequence data is not available for most of the species tested, the proteins are identified based on peptides that are present within sequenced species. Therefore, well characterised and evolutionary conserved proteins are more likely to be identified than those proteins that are highly species specific. As reproductive proteins are generally rapidly evolving (Swanson, Yang *et al.* 2001; Swanson and Vacquier 2002), many of the proteins identified may only have been matched to a few conserved peptides. Conversely, evolutionarily stable proteins will be identified with a greater number of peptides and are likely to have many species matches.

To discover the most confidently identified proteins within the data a reduced set was produced, containing only proteins identified within the following parameters; coverage ≥ 20%, number of unique peptides ≥ 2, -10lgP ≥ 50. Coverage is the proportion of the protein sequence for which peptides are identified. Proteins do not digest into only easily identifiable peptides; instead many peptides are undiscoverable due to extremes of size or charge. Therefore, using HPLC-MSMS techniques, coverage values of 100% are uncommon. In contrast, coverage values below 20% suggest a less confident identification. Similarly, in order to increase confidence in the protein identification, a minimum of two unique peptides was specified. Peptides are considered unique in relation to that specific protein. Discovery of multiple unique peptides suggests it is likely the protein is present. The third measure used to increase the confidence of protein matches within the reduced data set is the -10lgP score. This value is calculated by PEAKS to represent the likelihood of the peptide being an accurate, as opposed to random match. It is equivalent to -10*log(P-value), whereby the P-value is the probability the peptide was matched to the database by chance, calculated according to the quality of the peptide spectrum match. As the -10lgP score is on a log scale, a cut off of 50 is equivalent to a 0.001% probability that the spectrum was matched by chance. PEAKS

recommends a threshold of 20 (equivalent to a 1% chance of a random match), and so within the full list of proteins each is identified with at least this level of confidence.

Data is presented for both the reduced list and the entire complement of proteins. Information on the number of identified proteins, the most common species match, the number of species matches and correlated traits are investigated. Further, the proteins that were identified within all species are described. Specific proteins known to be present within the ejaculate were considered more closely. These were either structural sperm proteins, or those known to be involved in sperm egg interactions (see Section 6.2). For each of these proteins, for each species analysed, the coverage, number of unique peptides and most common species match is presented.

Table 6.1: Epididymal sperm samples collected for proteomic analysis.

Common name	Latin name	#samples	Rodent / Ungulate	Mating system
Alfreds Spotted Deer	*Cervus alfredi*	1	U	Multi-male
Ankole	*Bos indicus*	2	U	Single-male
Bank Vole	*Clethrionomys glareolus*	2	R	Multi-male
Blackbuck	*Antilope cervicapra*	1	U	Multi-male
Brown Norway Rat	*Rattus norvegicus*	2	R	Multi-male
Boar	*Sus domesticus*	2	U	Multi-male
Buffalo	*Syncerus caffer*	1	U	Single-male
Bull	*Bos taurus*	1	U	Single-male
Field Vole	*Microtus agrestis*	2	R	Multi-male
Gemsbok Oryx	*Oryx gazella*	1	U	Single-male
Lechwe	*Kobus leche*	1	U	Multi-male
Ram	*Ovis aries*	3	U	Multi-male
Red Squirrel	*Scuirus vulgaris*	1	R	Multi-male
Scimitar Oryx	*Oryx dammah*	1	U	Multi-male
Warthog	*Phacochoerus africanus*	1	U	Multi-male
WildBoar cross	*Sus scrofa*	1	U	Multi-male
Wildebeast	*Connochaetes gnou*	1	U	Multi-male
Wistar rat	*Rattus norvegicus*	2	R	Multi-male
Woodmouse	*Apodemus sylvaticus*	3	R	Multi-male
Zebra	*Equus grevyi*	1	U	Multi-male

6.4 Results

6.4.1 General protein composition of mammalian sperm

Following proteomic analysis, an average of 873 proteins were identified within each cauda epididymal sample (range: 549 – 1103). The mean coverage values and most commonly matched species for each sample are presented within Table 6.2. Within each sample, proteins were matched to the sequence data available within a database containing all available mammalian sequence data. Identified proteins matched to an average of 98 species within each sample (range: 55 - 140). A mitochondrial protein, Cytochrome b, was the protein most commonly identified with the most species matches (mean ± s.e. 36.4 ± 2.2 species, n=11).

Considering only the reduced data set, an average of 226 proteins were identified per sample (range: 131 – 341). Within each sample, protein sequences were matched to an average of 38 species (range: 26 – 60). The most commonly identified species remained the same for most samples. For the swine (domestic boar, wild boar, and warthog) the most common species identified changed from *Homo sapiens* within the full data set, to *Sus scrofa* within the reduced set. Information for each sample can be found in Table 6.3.

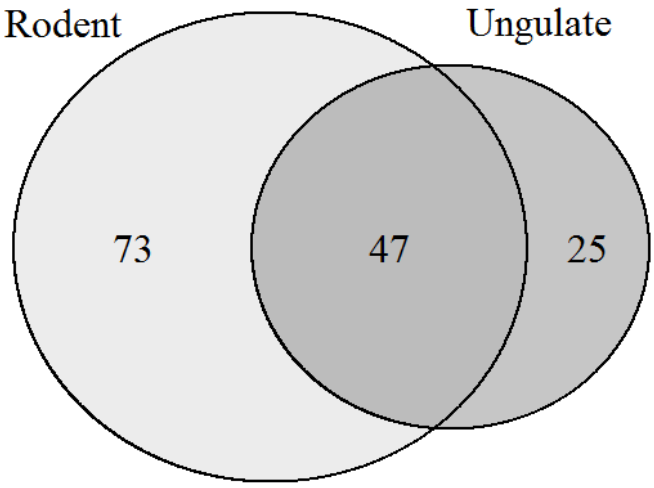
In total, 120 proteins were identified within all rodent samples, and 72 within all ungulate samples. Of these, 47 were identified within samples from all species. Within the reduced set of proteins, 7 proteins were identified in all rodent, and 10 within all ungulate samples, see Figure 6.1. Only four were identified within all samples from the reduced data set, 14-3-3 protein epsilon, 14-3-3 protein zelta/delta, Calmodulin and Histone H4. Many proteins were identified in most, but not all, samples. Within the rodent species, 191 proteins were identified in 10 out of 12 samples. A total of 188 proteins were identified in 15 out of 18 ungulate samples.

6.4.2 Data on proteins of particular interest

For simplicity, I choose 9 proteins to consider in more detail, see Table 6.4. Three of these, AKAP3, ODF1, and ODF2 are known structural components of sperm. The other six proteins were chosen due to their known involvement in sperm – egg binding. These results are presented within Table 6.5, Table 6.6 and Figure 6.2. Overall, the structural proteins were identified with a greater coverage than those involved with sperm – egg interactions ($t = -9.53$, $df = 124.8$, $P < 0.001$). Examples of protein sequence coverage and identified peptides can be found in Figure 6.3 and Figure 6.4. Dotplots comparing homologous protein sequences of ODF2 and zonadhesdin from two model species can be found within Figure 6.5. These were produced within R (v3.1.0) using the seqinr package (Charif, Lobry *et al.* 2012).

Figure 6.1: Number of proteins identified within cauda epididymal samples from all species of rodent, ungulate and those within all species a) using the total data set, and b) using only the reduced set. The reduced set was selected by using more stringent criteria to identify the most confident matches (coverage ≥ 20%, number of unique peptides ≥ 2, -10logP ≥ 50).

(a)



(b)

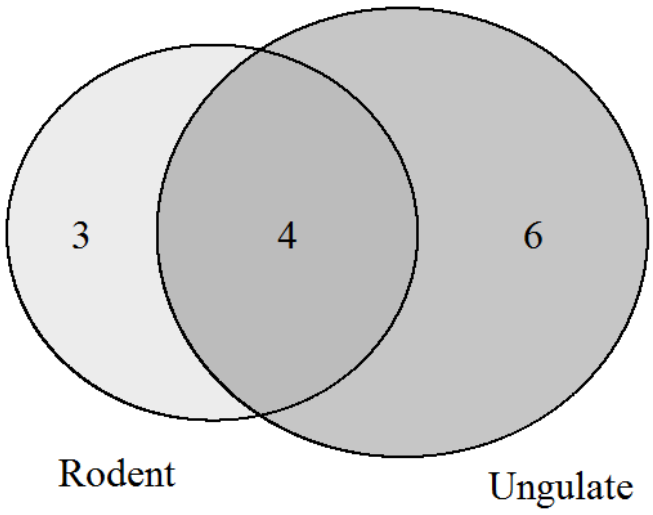


Table 6.2: Overview of the proteins identified within mammalian cauda epididymal sperm samples.
**Bos = Bos taurus, Homo = Homo sapiens, Mus = Mus musculus, Rattus = Rattus norvegicus, Sus = Sus scrofa*

Sample	#Proteins	Ave cov (%)	#Species ID'd	Most common species*	Protein with most species hits	#species
Alfreds Spotted Deer	1054	18.63	124	*Bos*	Cytochrome b	33
Ankole1	983	17.28	102	*Bos*	Cytochrome b, Actin cytoplasmic 1	18
Ankole2	549	16.76	98	*Bos*	Haemoglobin subunit beta	30
BankVole1	875	16.27	140	*Mus*	Cytochrome b	42
BankVole2	832	15.81	121	*Mus*	Cytochrome b	44
Blackbuck	897	14.16	88	*Bos*	Caveolin-1	30
BNRat1	914	17.27	123	*Rattus*	Cytochrome b	36
BNRat2	880	17.37	101	*Rattus*	Cytochrome b	37
Boar1	740	16.74	55	*Homo*	Actin cytoplasmic 1	17
Boar2	812	19.09	61	*Homo*	Actin cytoplasmic 1	18
Buffalo	898	15.6	102	*Bos*	F-actin-capping protein subunit alpha-2	25
Bull	910	20.15	84	*Bos*	Haemoglobin subunit beta	28
FieldVole1	979	16.48	80	*Mus*	Caveolin-1	30
FieldVole2	1026	16.96	89	*Mus*	Actin cytoplasmic 1, Haemoglobin subunit alpha	17
GemsbokOryx	1103	19.28	116	*Bos*	Cytochrome b	32
Lechwe	1044	17.38	100	*Bos*	Caveolin-1	30
Ram1	818	18.16	131	*Bos*	Cytochrome b	41

Sample	#Proteins	Ave cov (%)	#Species ID'd	Most common species*	Protein with most species hits	#species
Ram2	905	17.37	106	*Bos*	Cytochrome b	39
Ram3	990	20.1	79	*Bos*	Actin cytoplasmic 1	19
RedSquirrel	838	16.36	101	*Homo*	Testin	31
ScimitarOryx	718	15.81	86	*Bos*	Actin cytoplasmic 1, Cytochrome c oxidase subunit 2	17
Warthog	770	17.58	73	*Homo*	Actin cytoplasmic 1	18
WildBoarX	855	16.46	65	*Homo*	Actin cytoplasmic 1	17
Wildebeast	1004	14.91	96	*Bos*	F-actin-capping protein subunit alpha-2	25
Wistar1	840	17.18	64	*Rattus*	Actin cytoplasmic 1	17
Wistar2	791	16.37	93	*Rattus*	Cytochrome b	37
Woodmouse1	661	16.46	93	*Mus*	Actin cytoplasmic 1	19
Woodmouse2	643	16.27	107	*Mus*	F-actin-capping protein subunit alpha-2	30
Woodmouse3	1076	17.82	133	*Mus*	F-actin-capping protein subunit alpha-2	31
Zebra	791	16.04	125	*Homo*	Cytochrome b	41

Table 6.3: Overview of the protein identifications within cauda epididymal samples using more stringent selection (coverage ≥ 20%, unique peptides ≥ 2, -10lgP ≥ 50).

**Bos = Bos taurus, Homo = Homo sapiens, Mus = Mus musculus, Rattus = Rattus norvegicus, Sus = Sus scrofa*

Sample	# Proteins	Ave cov (%)	#Species ID'd	Most common species*
Alfreds Spotted Deer	324	37.03	43	*Bos*
Ankole1	254	36.83	40	*Bos*
Ankole2	171	35.69	26	*Bos*
BankVole1	188	37	41	*Mus*
BankVole2	188	35.4	38	*Mus*
Blackbuck2	161	35.75	32	*Bos*
BNRat1	288	37.01	37	*Rattus*
BNRat2	270	38.19	39	*Rattus*
Boar1	189	34.05	34	*Sus*
Boar2	218	39.23	32	*Sus*
Buffalo	207	35.19	35	*Bos*
Bull	320	41.4	41	*Bos*
FieldVole1	204	36.12	51	*Mus*
FieldVole2	233	36.53	42	*Mus*
GemsbokOryx	341	39.38	43	*Bos*
Lechwe	275	36.71	34	*Bos*
Ram1	254	36.86	40	*Bos*
Ram2	258	37.37	47	*Bos*
Ram3	321	40.5	41	*Bos*
RedSquirrel	142	34.62	60	*Homo*
ScimitarOryx	156	35.81	41	*Bos*
Warthog	194	36.75	33	*Sus*
WildBoarX	211	36.63	34	*Sus*
Wildebeast	208	31.45	41	*Bos*
Wistar1	257	36.58	32	*Rattus*
Wistar2	253	34.2	31	*Rattus*
Woodmouse1	149	36.27	40	*Mus*
Woodmouse2	131	36.56	32	*Mus*
Woodmouse3	229	36.97	36	*Mus*
Zebra	146	38.82	35	*Homo*

Table 6.4: The known functions of proteins which were selected for further investigation within this study.

Protein name	Short name	Function	Reference
Outer-dense fiber protein 2	ODF2	Sperm fibrous sheath; sperm motility	Vera *et al.* 1984; Oko 1987
Outer-dense fiber protein 1	ODF1	Sperm fibrous sheath; sperm motility	Vera *et al.* 1984; Oko 1988
A-Kinase Anchor Protein 3	AKAP3	Sperm fibrous sheath; binds interacting proteins	Brown 2003
Sperm surface protein Sp17	SpS17	Binds zona pellucida	O'Rand 1995
Zona pellucida-binding protein 1	ZP-binding protein	Binds zona pellucida	Burkin and Miller 2000
Spermadhesin-1	Spermadhesin	Ungulate specific, peripheral sperm proteins, initial sperm - ZP interactions	Hasse *et al.* 2005;
Izumo sperm-egg fusion protein 4	Izumo	Required for sperm - egg fusion	Inoue *et al.* 2005
Sperm equatorial segment protein 1	Sp Equatorial	Sperm - egg fusion	Fujihara *et al.* 2010
Zonadhesin	Zonadhesin	Multiple adhesion domains, bind to ZP and oocyte ECM	Hardy and Garbers 1995

Figure 6.2: Protein sequence coverage of peptides identified within cauda epididymal sperm samples from 19 mammal species. Proteins were structural components of sperm (black circles) or involved in sperm-egg interactions (grey circles) * $P < 0.001$. Circles represent mean ± sem.

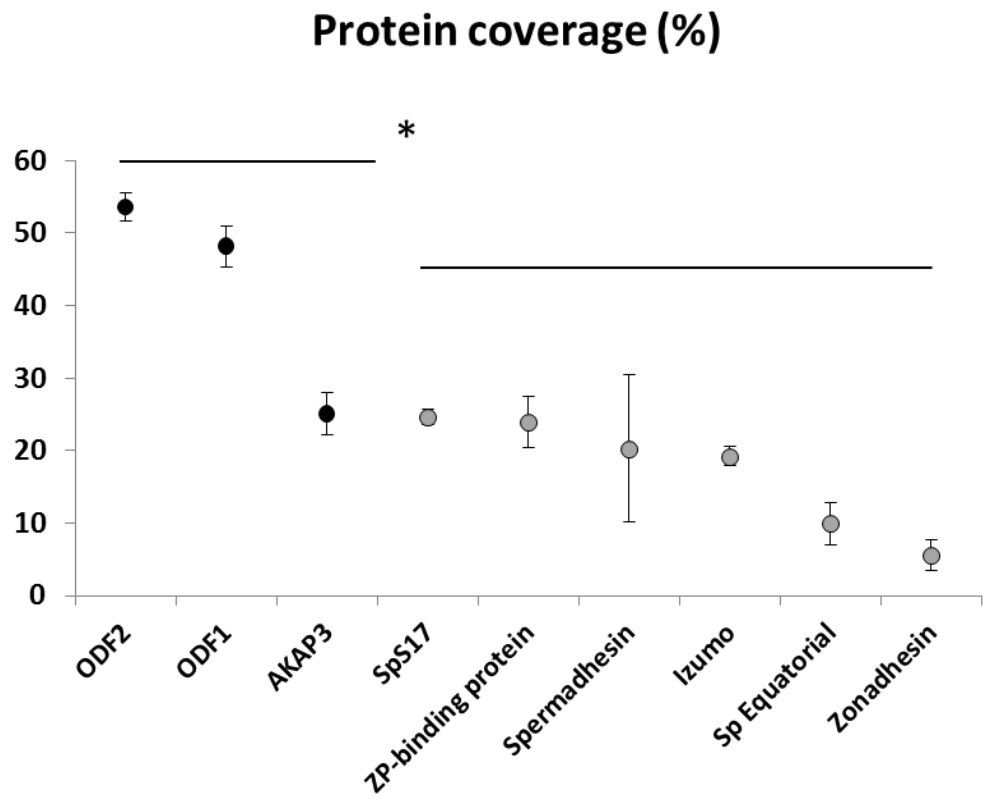


Table 6.5: Sequence coverage, the number of unique peptides and the best species match for proteins which are structural components of sperm identified within cauda epididymal samples.

	AKAP3			ODF1			ODF2		
Species	Cov (%)	#Unique	Sp match	Cov (%)	#Unique	Sp match	Cov (%)	#Unique	Sp match
Alfreds Spotted Deer	34	27	*Bos*	64	32	*Bos*	63	51	*Bos*
Ankole	23	17	*Bos*	47	11	*Bos*	55	40	*Bos*
Bank Vole	22	9	*Mus*	41	10	*Mus / Ratt*	55	0	*Maca*
Boar	18	5	*Bos*	67	68	*Sus*	57	0	*Maca*
Buffalo	24	18	*Bos*	21	6	*Bos*	42	29	*Bos*
Bull	59	60	*Bos*	66	51	*Bos*	63	69	*Bos*
Field Vole	17	4	*Mus*	42	12	*Mus / Ratt*	52	0	*Maca*
Gemsbok Oryx Lechwe	38	37	*Bos*	51	33	*Bos*	60	73	*Bos*
Ram	42	28	*Bos*	50	14	*Bos*	64	59	*Bos*
Rat (Brown Norway)	22	18	*Mus*	48	12	*Mus / Ratt*	54	0	*Maca*
Rat (Wistar)	23	18	*Mus*	48	12	*Mus / Ratt*	47	1	*Bos*
RedSquirrel	8	7	*Mus*	45	10	*Homo*	35	0	*Bos*
Scimitar Oryx	34	27	*Bos*	47	12	*Bos*	59	44	*Bos*
Warthog	13	3	*Bos*	68	41	*Sus*	51	0	*Maca*
Wild BoarX	14	3	*Bos*	43	11	*Sus*	50	0	*Maca*
Wildebeast	24	11	*Bos*	34	7	*Sus / Bos*	47	5	*Bos*
Woodmouse	24	20	*Mus*	44	10	*Mus / Ratt*	55	0	*Maca*
Zebra	13	6	*Bos*	41	12	*Sus / Bos*	56	0	*Maca*

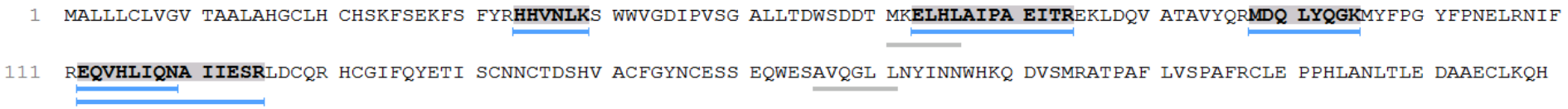
Table 6.6: Sequence coverage, the number of unique peptides and the best species match for proteins involved in sperm – egg interactions identified within cauda epididymal samples.

	Zonadhesin			ZP-binding protein 1			Izumo sperm – egg fusion protein 4			Sperm surface protein 17			Sperm equatorial segment protein 1			Spermadhesin		
Species	Cov (%)	#Unique	Sp match	Cov (%)	#Unique	Sp match	Cov (%)	#Unique	Sp match	Cov (%)	#Unique	Sp match	Cov (%)	#Unique	Sp match	Cov (%)	#Unique	Sp match
Alfreds Spotted Deer	1	2	*Sus*	28	3	*Sus*	23	6	*Maca*	30	1	*Mus*	5	3	*Bos*	7	1	*Bos*
Ankole	0	1	*Sus*	19	3	*Sus*	26	6	*Maca*	23	2	*Mus / Ory*	5	3	*Bos*	22	2	*Bos*
Bank Vole	0	2	*Mus*	11	1	*Homo*	23	6	*Mus*	22	3	*Maca*						
Boar	33	81	*Sus*	54	35	*Sus*	26	6	*Maca*	23	3	*Mus / Ory*	3	1	*Homo / Maca / Mus / Ratt*			
Buffalo				3	1	*Sus*	9	2	*Maca / Mus*	23	2	*Mus / Ory*	4	2	*Bos*			
Bull	3	5	*Sus*	33	11	*Sus*	21	6	*Maca*	23	3	*Mus / Ory*	35	14	*Bos*	76	8	*Bos*
Field Vole	1	5	*Mus*	7	1	*Homo*	21	5	*Mus*	22	3	*Macr*	3	1	*Mus*			
GemsbokOryx	1	2	*Sus*	21	4	*Sus*	18	5	*Maca*	30	4	*Mus*	20	8	*Bos*	10	2	*Bos*
Lechwe																10	1	*Bos*
Ram	1	3	*Sus*	23	3	*Sus*	21	1	*Maca*	23	3	*Mus / Ory*	25	10	*Bos*			
Rat (Brown Norway)	0	2	*Mus*	30	9	*Mus*	12	3	*Mus*	30	2	*Mus*						

	Zonadhesin			ZP-binding protein 1			Izumo sperm – egg fusion protein 4			Sperm surface protein 17			Sperm equatorial segment protein 1			Spermadhesin		
Species	Cov (%)	#Unique	Sp match	Cov (%)	#Unique	Sp match	Cov (%)	#Unique	Sp match	Cov (%)	#Unique	Sp match	Cov (%)	#Unique	Sp match	Cov (%)	#Unique	Sp match
Rat (Wistar)	1	3	*Mus*	35	11	*Mus*	15	4	*Mus*	35	3	*Mus*						
RedSquirrel				7	1	*Mus*	20	5	*Maca*	17	2	*Oryc*						
ScimitarOryx	0	1	*Sus*	22	4	*Sus*	21	1	*Maca*	30	3	*Mus*	17	7	*Bos*	10	1	*Bos*
Warthog	24	57	*Sus*	50	25	*Sus*	23	5	*Maca*	23	2	*Mus / Ory*	3	1	*Homo / Maca / Mus / Ratt*			
WildBoarX	23	46	*Sus*	45	15	*Sus*	26	6	*Maca*	23	2	*Mus / Ory*	3	1	*Homo / Maca / Mus / Ratt*			
Wildebeast	0	1	*Sus*	11	3	*Mus*	12	2	*Maca*	14	1	*Ory*	9	3	*Bos*	7	1	*Bos*
Woodmouse	0	2	*Mus*	17	4	*Mus*				34	3	*Mus*	2	1	*Mus*			
Zebra	1	1	*Homo*	14	2	*Sus*	10	2	*Mus*	18	1	*Ory*	5	2	*Maca*			

Figure 6.3: Protein sequence of Izumo sperm – egg fusion protein 4 with matched peptides which were identified within the a) Gemsbok Oryx and b) Field Vole cauda epididymal samples. Blue line indicates matched peptide, grey line indicates *de novo* sequenced peptides with a high likelihood of being a mutated version of the peptide within the known sequence.

a) Gemsbok Oryx



b) Field vole

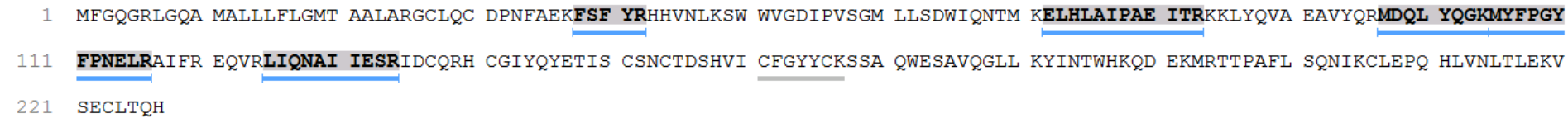


Figure 6.4: Protein sequence of Outer Dense Fibre Protein 2 with matched peptides which were identified within the a) Gemsbok Oryx and b) Field Vole cauda epididymal samples. Blue line indicates matched peptide, grey line indicates *de novo* sequenced peptides with a high likelihood of being a mutated version of the peptide within the known sequence.

a) Gemsbok Oryx

b) Field vole

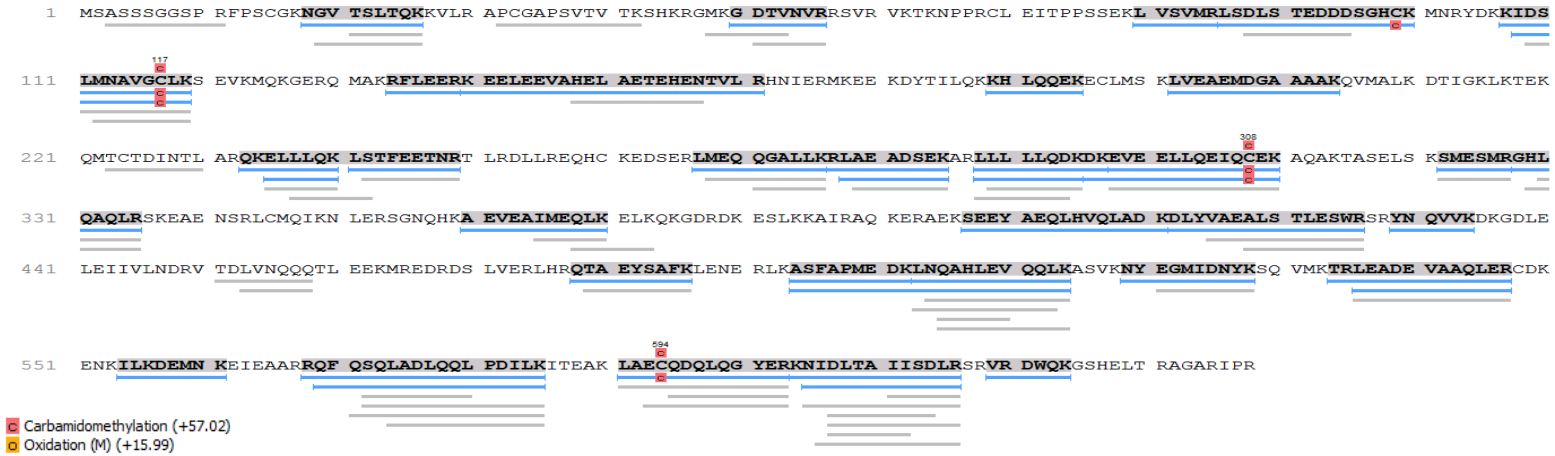
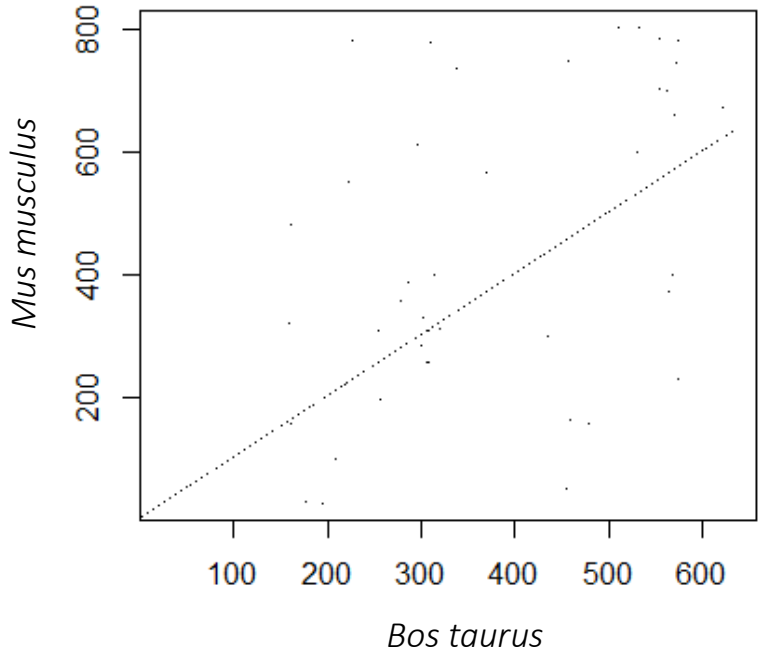
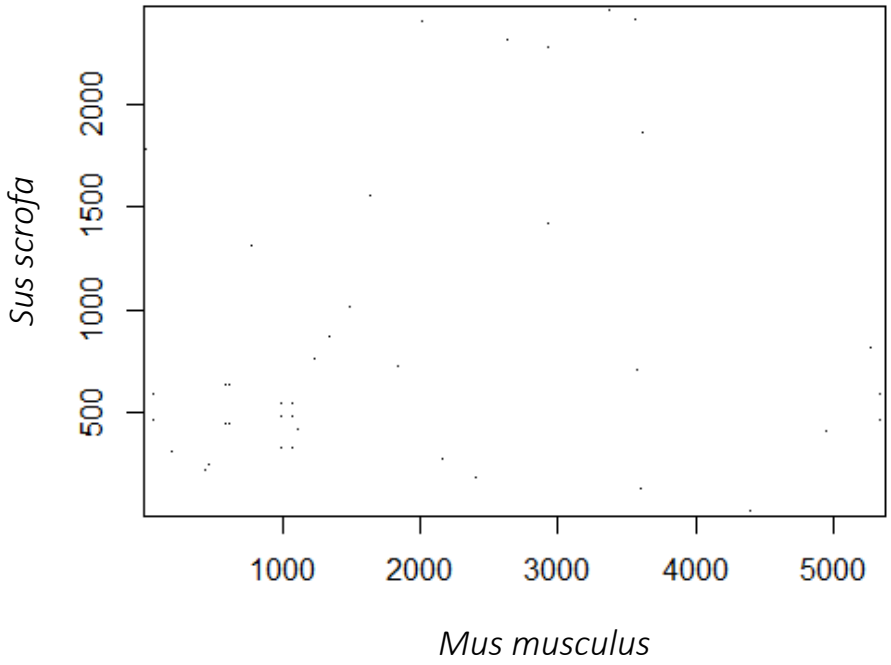


Figure 6.5: Dotplots of the protein sequences of a) outer dense fiber protein 2, and b) zonadhesin, from the house mouse compared against the homologous protein sequence within a) the bull and b) the pig. A minimum match length of 3 amino acids was specified. The broken diagonal line within plot (a) demonstrations a large amount of sequence homology.

(a) Outer dense fiber protein 2



(b) Zonadhesin



6.5 Discussion

Proteomic analysis of cauda epididymal sperm identified a large number of proteins within a broad range of mammalian species. Neither this, nor the level of protein coverage, was related to the sequence availability of the species, suggesting that a large proportion of mammalian epididymal sperm proteins contain homologous peptides. This agrees with more recent studies showing that many of the proteins within the ejaculate are not evolving at an elevated rate (Dean, Clark *et al.* 2009; Carnahan-Craig and Jensen-Seaman 2014). The protein most commonly observed with the highest amount of species matches was cytochrome-b. This mitochondrial protein is commonly used to assess phylogenetic relationships (e.g. Farias, Ortí *et al.* 2001) and so a vast amount of species will have sequence information available. As much of this protein is conserved, many species will share peptides from this protein.

Limitations of sequence availability were apparent when considering the results for members of the suidae family. Analysing the entire list of proteins, the most commonly identified species match was *Homo sapiens.* After reducing the data set using more stringent selection criteria this altered to *Sus scrofa*. The higher rate of matching to human protein sequences within the full data set may reflect the greater availability of protein sequence information of humans, particularly for highly conserved cellular proteins. The two other species for which *Homo sapiens* was the most common match were *Sciurus vulgaris* (red squirrel) and *Equus grevyi* (Grévy's zebra). Both pigs and these species are known to be polyandrous (Ginsberg and Rubenstein 1990; Wauters, Dhondt *et al.* 1990; Delgado-Acevedo, Zamorano *et al.* 2010). This may result in a greater rate of protein evolution for these species, and so less sequence homology with the more closely related model species.

Broad differences observed in protein identifications are likely to be due to disparities in initial sample quality and technical variation. Regardless, the proteins known to be present in sperm were identified within most, if not all, species. Only four proteins were found within all samples after the stringent selection criteria

(coverage ≥ 20%, number of unique peptides ≥ 2, -10IgP ≥ 50) were applied. These were two 14-3-3 proteins, regulatory molecules (Fu, Subramanian *et al.* 2000), histone H4, present in the chromatin (Bhasin, Reinherz *et al.* 2006), and calmodulin, a calcium binding messenger protein (Cheung 1980). All four proteins are known to be present within all eukaryotic cells. Calcium signalling, which is regulated by calmodulin, impacts fertility in both males and females. It is required for normal sperm motility, hyperactivation, capacitation and sperm – egg interactions (Primakoff and Myles 2002; Schuh, Cartwright *et al.* 2004; Suarez 2008a; Lasko, Schlingmann *et al.* 2012; Xiang, Cui *et al.* 2012), as well as follicular development and ovulation in females (Wu, Gonzalez-Robayna *et al.* 2000).

Known sperm proteins (Table 6.4) were identified in all species bar the Lechwe. This sample was obtained from a very young male following castration. No sperm were found during microscopic examination of the sample. It is therefore likely that sperm were yet to be produced and stored in the epididymis. In all other species sampled, AKAP and ODF proteins were well identified (three are described in Table 6.4). These are structural proteins that make up the sperm tail (Vera, Brito *et al.* 1984; Brown, Miki *et al.* 2003). Compared to the proteins analysed that are involved in sperm – egg binding, more peptides from the structural proteins were identified. Proteins within the zona-pellucida are known to have an elevated rate of evolution (Swanson, Yang *et al.* 2001; Turner and Hoekstra 2006), therefore sperm proteins that bind to the zona-pellucida are likely to coevolve to maintain fertilisation ability. Reduced cross-species protein sequence homology is to be expected in rapidly evolving proteins. There is previous evidence that 22% of genes encoding for sperm membrane proteins exhibit accelerated evolution (Dorus, Wasbrough *et al.* 2010). Here, I find species differences in specific proteins that are involved in sperm – egg interactions. Some caution should be taken when considering this result as the structural proteins tend to be classified in a greater number of species. In four of the proteins involved in sperm-egg interactions there was no *Bos taurus* sequence available, which commonly give the best results for many of the ungulate species. However, the two proteins for which bovine sequences are available show

comparably low sequence coverage values, showing the pattern is likely to hold even if a greater number of sequences were available. Additionally, the identified ungulate proteins which have matches with the *Sus scrofa* ODF1 sequence give a mean coverage of 49% (± 7), which is in line with the values given when matching again the *Bos taurus* sequence.

Spermadhesins are a set of ungulate-specific proteins that modulate the initial binding of sperm with the zona-pellucida (Tedeschi, Oungre *et al.* 2000; Caballero, Vazquez *et al.* 2004; Töpfer-Petersen and Calvete 2005). Within this study there was no evidence for similar proteins being present within rodent sperm, as there was no homologous peptide matching to the spermadhesin sequences. This protein was identified within most ungulates tested, including the lechwe sample that contained no sperm. This protein is therefore likely to be found within diverse ungulate species and is not specific to the domestic species.

Further study of genomic or transcriptomic data for the genes encoding the most diverse proteins identified within this study would complement the data presented here. Analysing the evolutionary rate of specific genes, and whether this is related to sexual selection, can improve our understanding of the evolution of reproductive proteins and speciation. Coupling this data with information on which sections of the protein sequence are less conserved can give clues to the binding patterns of these proteins that are essential to fertility.

6.6 Conclusions

Here, I show that the proteins present within cauda epididymal samples of male mammals contain many homologous proteins, and are broadly similar across species. I describe additional evidence that proteins which interact with the egg are adapting the most rapidly. This study also provides the first proteomic analysis of many of the species sampled here, and could assist further investigation into the

evolution of reproductive proteins within ungulates, which has thus far received little attention.

Chapter 7

General discussion

7.1 Chapter overview

In this chapter I discuss the results of the previous chapters within a broader context. I begin by considering a key novel finding from this thesis: the use of proteomics to reveal evidence of ejaculate plasticity in house mice. I then move onto discuss the diversity in mammalian testicular architecture and ejaculate proteins investigated in Chapters 5 and 6, and the mating behaviour of male house mice from Chapters 2 and 4. Finally, I consider the wider implications of this work and the potential for further research.

7.2 Plasticity in the production of seminal fluid proteins

The results presented in Chapters 2 and 4 show that male house mice can plastically alter their ejaculate protein production. This is the first example of plasticity in the production of specific seminal fluid proteins in a mammalian system. These studies utilised recent advances in proteomic methodologies, HPLC-MSMS followed by label-free quantitative analysis using Progenesis LC-MS, to provide new insights into the variation within mammalian ejaculates.

Previous evidence of plasticity in seminal fluid proteins comes from studies of *Drosophila*. The seminal fluid proteins, and their functions, have been well characterised within *Drosophila* (e.g. Chapman 2001). Plasticity in the expression of selected genes has been found in response to the intensity of sperm competition (Fedorka, Winterhalter *et al.* 2011), although it is worth noting that transcription and protein expression are not entirely correlated (Ghazalpour, Bennett *et al.* 2011; Vogel and Marcotte 2012). Expression of specific proteins has been shown to vary in ejaculates according to the mating status of the female (Sirot, Wolfner *et al.* 2011), and as a result of experimental evolution within lines selected to have either large or small accessory sex glands (Wigby, Sirot *et al.* 2009). These studies used an ELISA method to determine the expression of specific proteins that are relevant in *Drosophila*: sex peptide and ovulin (Wigby, Sirot *et al.* 2009; Sirot, Wolfner *et al.* 2011). In contrast, the proteomics analysis utilised in the studies here allow high throughput analysis of entire proteomes from specific accessory glands and the ejaculate. This allows subtle differences in the expression of all proteins to be explored, in addition to considering those known to be functionally important.

In rodents, the seminal vesicles are relatively larger within polyandrous species (Ramm, Parker *et al.* 2005). These prominent accessory sex glands are larger in bank voles that have experienced a high risk of sperm competition during development (Lemaitre, Ramm *et al.* 2010) and are also larger in dominant male bank voles (Lemaitre, Ramm *et al.* 2012). In the house mouse, previous studies have not identified a difference in the size of the seminal vesicles due to sperm competition

risk (Ramm and Stockley 2009a). Here, I found that dominant males have larger seminal vesicles (Chapter 2). Using proteomics techniques allowed me to also explore more subtle differences that cannot be tested by considering morphology alone. I found that subordinate males produced a seminal vesicle secretion which had a higher protein concentration than dominant males, and contained proportionally more of the plug forming protein SVS2.

Theory predicts that males should invest more in components of the seminal fluid that will influence the level of bias, by increasing the likelihood of success when mating competitively (Cameron, Day *et al.* 2007). Or, males should produce more of the ejaculate components that will give them a greater chance of gaining successful fertilisations when their ejaculates are likely to face competition. In the house mouse, the copulatory plug is substantial and can drastically improve fertility (Dean 2013). As subordinate male house mice invest considerably less in ejaculate production, particularly of sperm, it is possible that they are instead opting to invest in copulatory plug formation by increasing the proportion of SVS2 produced. Although this is not tested directly here, subordinate males produced a copulatory plug that was of equivalent size to the dominant males, despite having significantly smaller seminal vesicle glands and so less total protein available for plug formation. As the seminal vesicle glands were essentially empty following copulation (personal observation), it is unlikely that this is due to differential allocation of seminal vesicle products by dominant males.

In Chapter 4, the effect of maternal social experience prior to pregnancy (either many or few interactions with three males or one male) on the ejaculate composition of male offspring was tested in the house mouse. Random forest analysis found that overall the protein composition of the ejaculates differed according to the maternal environment. Although significant differences in the expression of major seminal proteins were not observed, CUZD1, a protein that is important for fertility (Yamazaki, Adachi *et al.* 2006), tended to be more abundant within the ejaculates of male offspring of the high competition maternal group.

There were no morphological or behavioural differences observed in these males. This study highlights the effectiveness of using proteomic analysis to search for more subtle differences in reproductive investment. To my knowledge, this is the first experiment testing the effect maternal social experience has on male offspring investment in reproduction.

Female plasticity in the protein composition of the oviduct, in response to the likelihood of multiple mating, was tested in Chapter 3. Although this specific question has not been addressed previously, a recent study in the house mouse used a similar approach to investigate female plasticity, comparing the ova defensiveness of females according to the level of prior male interaction (Firman and Simmons 2013). The study presented in Chapter 3 found no evidence for differential protein expression in the oviduct according to the prior level of interaction with males. Instead, the protein composition was more closely linked to genetic similarities, as sibling females had more similar results despite being balanced across treatment groups. On these results alone I would not rule out the possibility of subtle changes in the oviduct secretion that were not seen due to analysing the entire oviduct proteome, which may have swamped the secreted proteins. In pigs, the oviduct secretion is known to alter according to the stage of the oestrus cycle (Seytanoglu, Georgiou *et al.* 2008) and in response to the presence of gametes (Georgiou, Sostaric *et al.* 2005). It is possible that more selective analysis of just the oviduct secretion may reveal subtle differences, following a different dissection and sample collection methodology.

Overall, these studies are important as they reveal phenotypic adaptations that may not have otherwise been revealed without the use of proteomics techniques. Many ejaculate proteins of the house mouse, which are important for fertility and influencing the outcome of sperm competition, have a particularly high rate of production (Claydon, Ramm *et al.* 2012). Plastic investment in these proteins could therefore occur relatively easily in response to variation in the risk of sperm

competition, and could be a far faster way of improving fertility than increasing sperm output, due to the duration of spermatogenesis (Amann 1970).

7.3 Evolution of mammalian ejaculates in response to sperm competition

There is a wealth of literature regarding the evolution of ejaculates in response to sperm competition. The most well documented adaptation is increased relative testes mass in males from promiscuous species (Kenagy and Trombulak 1986; Harcourt, Purvis *et al.* 1995; Stockley, Gage *et al.* 1997). Relatively larger testes also exhibit greater sperm production efficiency (Chapter 5). It has been established that males with relatively larger testes also have a shorter SECL, leading to faster sperm production (Pierce and Breed 2001; Parapanov, Nusslé *et al.* 2008; Ramm and Stockley 2010; delBarco-Trillo, Tourmente *et al.* 2013 and Chapter 5). Of the measures tested here, a shortened SECL was most significantly correlated with an increased sperm production efficiency (Chapter 5). The mechanism for an increased rate of production is unknown. It could relate to a greater number of Sertoli cells or improved Sertoli cell efficiency, as is seen in blackbird species (Lüpold, Wistuba *et al.* 2011). An investigation of cell activity levels within testicular tissue of a few species with contrasting mating systems could offer insights greater insights into this.

Contrary to a previous study (Ramm and Stockley 2010), I found no correlation between sperm length and the rate of sperm production. I also found relative testes mass, indicating sperm competition, did not predict sperm length. The link between sperm length and sperm competition has been under much debate (e.g. Gage and Freckleton 2003; Tourmente, Gomendio *et al.* 2011; Tourmente, Gomendio *et al.* 2011). The analysis in Chapter 5 compared two distinct mammalian groups: rodents and ungulates. The hooked sperm heads found within murine rodents have been well documented (Breed 2005). In contrast, sperm from ungulate species have a far simpler, spatula like morphology. I found that, despite their far larger size, ungulates have shorter sperm than rodents. Analysis taking into account the distinct groups

revealed a link between mating system and sperm length. Although it may be argued that phylogenetic control should be sufficient to account for species differences, perhaps such divergent sperm types proved too disparate for the model to clarify more subtle differences in closely related species.

In Chapter 6 I go on to consider the evolution of reproductive proteins using a proteomic approach. Proteins that are structurally important in sperm were relatively homologous within the species tested here. In contrast, those known to be involved in sperm – egg interactions had far fewer matching peptides. This is in agreement with studies investigating the rates of molecular evolution of the reproductive genome (Turner and Hoekstra 2006; Vicens, Lüke *et al.* 2014). Discussion of potential for further work related to this is included in Section 7.6.

7.5 Wider implications and impact

Understanding the plasticity of reproductive proteomes could have important implications within the field. Proteomics analysis is being used increasingly in the field of ecology (Karr 2007; Diz, Martinez-Fernandez *et al.* 2012; Valcu and Kempenaers 2014). The studies presented here use a high throughput method of obtaining label-free relative quantitative data for complex biological samples. Previous studies of ejaculate protein plasticity have instead compared mRNA transcription levels or protein abundances using ELIZAs (Wigby, Sirot *et al.* 2009; Fedorka, Winterhalter *et al.* 2011; Sirot, Wolfner *et al.* 2011). All of these approaches are targeted to analyse specific proteins. Using high throughput techniques will increase our knowledge of ejaculate composition, as well as improving our understanding of subtle differences in protein abundance. Combined, the studies presented here highlight which samples these techniques work best with, for example secretions or ejaculates were more successfully analysed than whole tissues such as the oviduct. The more that these methods are utilised for reproductive tissue samples, the more useful these methods will become.

Finding evidence for ejaculate plasticity according to male status and maternal social experience could have important implications for the animal production industry. Seminal proteins can protect sperm during freezing and improve fertilisation rates in large mammals (Hernandez, Roca *et al.* 2007; Novak, Ruiz-Sanchez *et al.* 2010). Decreasing fertility has been particularly problematic within the dairy industry, as years of selection for increased milk yield has led to lower fertility in cows (Lucy 2001). If housing can improve the fertility of bulls this could have important implications to animal husbandry.

7.6 Further studies

Following from this work, it would be useful to perform proteomic analysis of the copulatory plug of males of different social status. This would reveal if different proportions of SVS2 within the seminal vesicles resulted in differing plug composition. Due to the unusual chemical composition of the copulatory plug, attempts to analyse this were unsuccessful. Breaking the plug into its protein components is akin to un-baking a cake. Using methods similar to previous analyses that yielded protein identifications (Dean, Findlay *et al.* 2011), I was able to identify proteins; however the abundances observed were so far from what would be reasonably expected that they could not be trusted. More specifically, SVS2 was only identified in very low quantities. As this protein accounts for a major proportion of seminal vesicle fluid (20%-30%) and known to be a primary component of the copulatory plug (Williams-Ashman 1984), it is highly unlikely it would only be present in trace amounts. Instead, it is likely the bonds catalysed by trans-glutaminases when forming the plug are not broken when trying to dissolve the plug.

To better understand the implications of plasticity in seminal fluid production, it would be interesting to analyse the outcomes of sperm competition for males with different seminal fluid protein profiles. To test this, you would require the following: two males mated to one female, the seminal fluid protein composition of each males ejaculate at the point they mated, and the paternity share of each male from the

litter produced. This may be particularly challenging due to the rapid turnover of seminal fluid proteins (Claydon, Ramm *et al.* 2012) as we do not know if mating would alter the composition of an ejaculate collected at different time point. It would be worthy of further investigation, however, and understanding how ejaculates alter as males age and in response to sexual experience would be worthwhile.

It would also be useful to support the comparative proteomic approach used in Chapter 6 with some genomic and molecular evolution analyses. It would be particularly interesting to compare the rates of evolution of proteins involved in sperm – egg interactions from ungulate samples compared to rodent samples. Pre-copulatory intrasexual mechanisms of sexual selection are common in large mammals, resulting in the vast array of weaponry seen in ungulates (Clutton-Brock and McAuliffe 2009). Males typically have to fight for access to females, and dominant males may restrict mating access during periods of female receptivity. It is therefore likely that sperm competition is less intense in many ungulates compared to some of the highly promiscuous rodent species. The proteins involved in sperm-egg interactions are known to evolve rapidly (Turner and Hoekstra 2006; Vicens, Lüke *et al.* 2014). This is further driven by sexual conflict, which is accelerated due to sperm competition. This rapid evolution of fertilisation proteins driven by sexual conflict may be an important factor in speciation (Gavrilets 2000). Comparing the evolutionary rates of genes from such diverse mammalian groups may help improve our understanding of reproductive isolation in highly speciose rodents compared to less speciose ungulates. .

Finally, I would be particularly interested in further proteomic study of female reproductive investment. Studies of sperm competition and sexual conflict tend to focus on male traits. This is most likely for some obvious reasons, such as they are easier to measure and theoretical predictions generally consider ejaculate investment. However, further understanding of the molecular environment within the female tract, in response to experimental evolution or variation in mating rate

for example, could provide important insights into mechanisms of cryptic female choice, coevolution of males and females, and general fertilisation processes. Furthermore, mating is often costly to females, either due to toxic components of the seminal fluid or more generally due to mating increasing contact with pathogens. I would be particularly interested in picking apart the drivers of adaptive change, for example the cost of being pursued, mating rate or seminal fluid, in the female reproductive tract.

7.7 Concluding remarks

The work presented within this thesis provides a broad study of sperm competition in mammals using proteomics. The novel approach used here provides exciting insights into the plasticity of reproduction at a molecular level. Increasingly, proteomics techniques are being used in the field of behavioural ecology. At the 2014 ISBE meeting in New York, Marlene Zuk argued that it is important not to get bogged down in technical details when using genomics techniques, warning against "ooh shiny" syndrome. However, I think it is important not to consider molecular techniques as a black box, where samples go in and data comes out. With proteomics analysis it is particularly important to have at least a reasonable understanding of the validity of the method you are using. There are often various approaches that could be taken and thorough experimental design can make the use of proteomics incredibly valuable. As the use of proteomics increases within the field of ecology, we will gain a greater understanding of reproductive phenotypes at a molecular level.

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Appendix
Comparative data

The data collected from literature sources for the comparative analysis presented within Chapter 5 is given here, with sources. Table A1 provides the data collected for the analysis of daily sperm production rates.

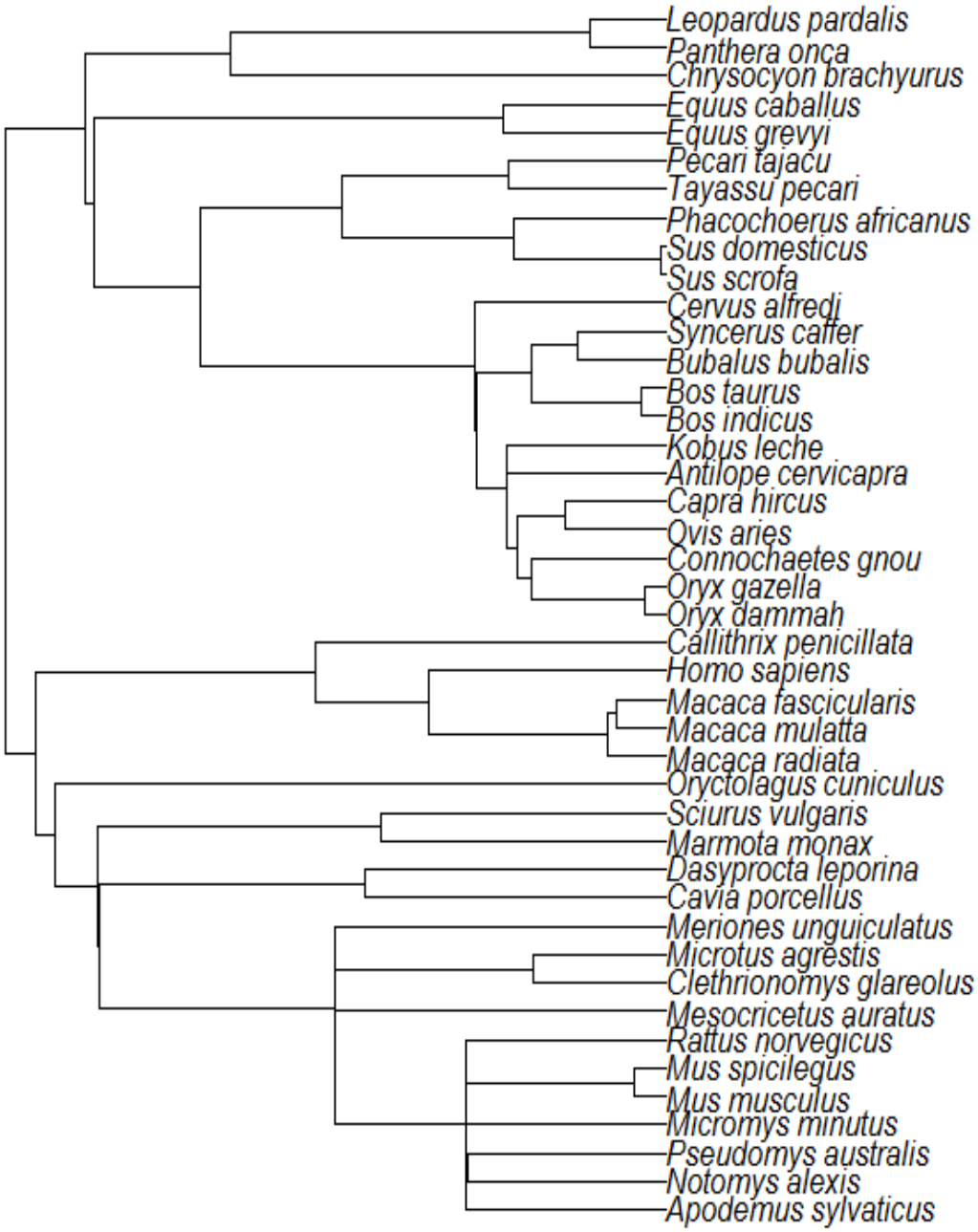
A phylogeny for all species is also presented (Figure A1).

Table A1: Data collected from literature sources for the analysis of sperm production efficiency presented in Chapter 5. BM = body mass, TM = paired testes mass, DSPG = daily sperm production per gram of testicular tissue, SertG = number of Sertoli cells per gram of testicular tissue, %SE = the proportion of seminiferous tissue within the testes, Sperm = length of sperm (Sperm measures here are those taken from Gage et al. 1998[1], the analysis also included those measured by myself and Emily Logie).

Species name	**BM (g)**	**TM (g)**	**DSPG**	**SertG**	**%SE**	**SECL**	**Sperm**	**Sources**
Agouti paca	5400	11.8	39	43	84.7	11.5		2
Bos indicus	554000	447.2	12.2		76.55		64.1	3: 4
Bos taurus	1866000	384	12		67.11	13.5		5-7
Bubalus bubalis	302000	277	14.4			8.6	57	8-10
Callithrix penicillata	300	1.075	18.1	34.7	81.4	15.4		11
Canis lupus	15400	19.86	14.5	43.4	83.3	13.6	60.7	12-15
Capra hircus	59000	202	30.3	21.4	87.7	10.6	59.4	16: 17
Cavia porcellus	813.3	4.1		55.1	90.2	8.45	114.1	14: 18: 19
Chinchilla lanigera	500	5	61	44		10.2		2: 20
Chrysocyon brachyurus	28000	12.68	28.7			13.6		21: 22
Clethrionomys glareolus	21.8	0.646		18: 23		6.7		18: 44
Dasyprocta leporina	2500	8.2	52	57	80.5	9.5		2
Equus caballus	473000	340	16	61.3	79.6	12.2	60.6	6: 17: 24
Felis silvestris	3100	2.34	15.7	32	78	10.4	59.5	25
Homo sapiens	63540	50.2	4.1	48.8	61.6	16	57.4	6: 14: 26
Hydrochaeris hydrochaeris	54100	63.4	10	19		11.9		2
Leopardus pardalis	13200	22	18.3	46	69.8	12.5		27
Macaca fascicularis	4787	35.7		101.9	83.6	10.5	77.1	14: 18: 26
Macaca mulatta	9200	49	23.3			10.5	81.9	26: 28
Macaca radiata	7000	10.6	34.7					29
Marmota monax	4165	7.4		47.1	82.7			14: 18

Species name	BM (g)	TM (g)	DSPG	SertG	%SE	SECL	Sperm	Sources
Meriones unguiculatus	77	1.08	33	28	85	10.55		30
Mesocricetus auratus	100	3	24	34.8	85.4	8.74	186.7	6: 14: 31
Microtus agrestis	46.4	0.804		18: 23		7.8		18: 44
Mus musculus	15.3	0.119	46.5	36.2	84.5	8.63	123.6	2: 14: 18: 32
Notomys alexis	32	0.052	9.8			14	102.5	33
Octodon degus	268	2.16	15.63	80.3	92.7			14: 34
Oryctolagus cuniculus	4100	6.4	25	24.9	73.2	10.7	56.5	6: 14: 35: 36
Ovis aries	80000	500	21		69.9	10.4		6: 7
Panthera onca	77000	35.4	16.9	29	61.3	12.8		37
Pecari tajacu	22600	47.4	23.2	28	62.6	12.3	40	38
Proechimys oris	207	1.94	82	53	89.9	8.6		39
Pseudomys australis	48.8	1.57	25.9			11.2	122.5	33
Rattus norvegicus	560	3.8	23	28.5	81.1	12.4		6: 14: 40
Sus domesticus	81723	318	23.7	27.8	60.5			41: 42
Sus scrofa	39700	128.2	28.6	42.4	73.8	9.05		43
Tayassu pecari	39000	78.4		23				44

Figure A.1: Phylogeny used for the comparative analysis of data collected from literature sources presented within **Chapter 5**. Adapted from the phylogeny of Bininda-Emonds et al. 2007[45].



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