Sperm competition and sexual selection in western grey kangaroos

Macropus fuliginosus

Thesis is presented for the Bachelor of Science in Conservation and Wildlife Biology with Honours

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Bachelor of Science (Animal Science) The University of Adelaide
Declaration

I declare that this thesis is my own account of my research and contains, at its main content, work that has not been previously submitted for a degree at any tertiary educational institution.

_____________________________________

Meg L Lane (2014)
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Abstract

Macropods exhibit the second largest sexual dimorphism in body size for any vertebrate, suggesting there is a major role of sexual selection, with the potential for different mating tactics amongst male kangaroos. It is suggested that dominant males invest heavily in large forelimb muscles, which aids in male-male competition, female coercion and increased attractiveness to females, gaining them the majority of matings. A subdominant male may not invest in muscle mass development, but rather rely on other reproductive tactics such as sneaky matings and investment in competitive spermatozoa characteristics; for example more spermatozoa (larger testes) and faster or longer spermatozoa. We examined whether there was a trade-off between investments in forelimb muscle development and sperm competition in the western grey kangaroo (*Macropus fuliginosus*). Sperm traits, body mass, and forelimb muscle masses were measured for 35 male *M. fuliginosus* and an index of ‘muscularity’ was calculated using the residuals of individual muscle masses against body mass. There were no significant relationships between forelimb muscle investment and sperm competition traits. Significant relationships were recorded between relative testes mass and relative epididymis mass ($r^2 = 0.26$, $p = 0.003$), total motility and progressive motility ($r^2 = 0.31$, $p = 0.001$) and spermatozoon velocity and spermatozoon head ($r^2 = 0.47$, $p < 0.001$) and tail lengths ($r^2 = 0.19$, $p = 0.011$) with the longer spermatozoa swimming slower. These results suggest that there is no evidence for a significant relationship between sperm competition and forelimb muscles. There was no evidence for a trade-off between these two measures of sexual selection, suggesting that these traits are independent of each other.
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Chapter 1: General Introduction

1.1 Overview on sexual selection

Sexual selection is an evolutionary process that favours an increase in the frequency of genetic characteristics that result in a reproductive advantage for individuals over conspecifics of the same sex (Birkhead and Pizzari, 2002). The current theory of sexual selection has arisen from Darwin’s first hypothesis to explain intra-specific differences, where males (and less commonly females) demonstrate elaborate morphological and behavioural features including sex-specific displays and secondary sexual characteristics such as plumage and weaponry (Darwin, 1871). These secondary sexual characteristics do not aid in survival of an individual, but rather improve an individual’s reproductive fitness via the three models of sexual selection: intra-specific competition for mates, mate choice and/ or sexual coercion (Clutton-Brock, 2007; Darwin, 1871).

Darwin originally considered that females of all species were monogamous, and therefore only pre-copulatory sexual selection was likely to occur; however, many animal species exhibit polyandry which has increased the selective pressure on the male (Birkhead and Pizzari, 2002). Due to this intense selection pressure, a male may invest in highly pronounced pre- and post-copulatory sexually selected traits to maximise the chance of a successful mating (Birkhead and Møller, 1998; Clinchy et al., 2004; Fisher and Cockburn, 2005). An animals’ reproductive success is defined as the number of surviving offspring produced by an individual (Malo et al., 2005b). In species where male individuals provide limited to no parental care, a male will maximise their reproductive success by siring as many offspring possible.
Phenotypic differences between male and female conspecifics that have evolved due to sexual selection are referred to as sexual dimorphisms (McPherson and Chenoweth, 2012). The degree of sexual dimorphism has been described as the difference between the sum of all the selective pressures acting on the male (natural selection and sexual selection), and the sum of those acting on the female (Ralls and Mesnick, 2009). Sexual dimorphism has been positively correlated with breeding systems in terrestrial mammals. For example, in polygamous breeding systems a male must compete for access to females. Generally, the female is the ‘limiting sex’ due to greater investment into the production and rearing of the offspring (both time and energy) (Bateman, 1948) and this large investment leads to the female selecting a high quality mate, to ensure that the offspring the female produce are healthy and also have the favourable sexually selected traits (Kokko and Johnstone, 2002; Trivers, 1972).

1.1.1 Pre-copulatory sexual selection

Pre-copulatory sexual selection occurs before mating: mainly male-male competition and female choice. Three models of pre-copulatory sexual selection have been described, where the characteristics or behaviours of males influence their chance of gaining successful matings with females (Andersson and Iwasa, 1996). The first is male-male competition (which often involves fighting) where the male is more likely to be successful if he has a greater body size or has larger weapons to assist him in fighting and in self-defense (Andersson, 1994; Glanslosser, 1989; Jarman and Southwell, 1986; Preston and Stockley, 2006; Vanpé et al., 2007). These adaptations are often used to show the male’s dominance over rival males, for example, having bigger horns than the rival’s (Andersson and Iwasa, 1996).
Second, female choice can drive male investment in physical characteristics that females favour as an indication of his genetic fitness (Andersson and Iwasa, 1996). If the mating system is dependent on female choice, the male will invest in the visual traits that the female prefers (e.g. elongated tails, bright colourations or exaggerated physical attributes) (Andersson, 1994). The female may use the size of the physical attribute, such as antler size as an indicator of the male’s genetic quality. Antler or horn sizes are often heritable; therefore the female will mate with the male who will pass on the largest positive trait to the offspring. The brightness of the male plumage is also an indicator of the animal’s health (Vanpé et al., 2007). A study on lemurs (Eulemur fulvus) concluded that females spend significantly more time looking at photographs of the male with a brighter face than the same male that had the colour decreased digitally (Cooper and Hosey, 2003). Therefore the appearance of the male is important when a female is deciding on a mate so she can maximise her offspring’s own fitness.

Often a proportion of these large elaborate characteristics that are selected for may be favourable for survive for the male: i.e., enlarged canines may help to protect the group against predators, however a select few traits may not aid in survival or the mating process, but in-fact hinder the animal’s chances of survival (McPherson and Chenoweth, 2012; Zahavi, 1975). For example, the large male body size of the red-wing blackbird (Agelaius phoeniceus) is advantageous for attracting females and in male-male contests, but leads to the male spending increasingly longer periods of time foraging for food to maintain his large body mass. This therefore leaves less time to defend his territory and attract a female (Searcy, 1979). The long neck of the giraffe is one characteristic that aids in reproductive success and male-male combat, as well as allowing the species to feed on much higher browse that other herbivores (Emlen, 2008).
The third pre-copulatory sexually selected model is sexual coercion, which involves males dictating matings using his strength or often larger size to subdue a female (Bisazza et al., 2000). These three models involve characteristics that the males can, to an extent, control; however there are also selection characteristics that occur after mating, such as sperm competition, which is beyond the control of the male.

1.1.2 Post-copulatory sexual selection

Post-copulatory sexual selection occurs after mating and often is out of the direct control of the male, as it takes place within the reproductive tract of the female. Sperm competition is one example of this, where the spermatozoa of multiple males may compete within the female reproductive tract to successfully fertilise the female’s oocyte(s) (Parker, 1970). Sperm competition occurs because of polyandry; the female mating with more than one male (Simmons, 2005). In addition to investing in competitive spermatozoa, males also employ behavioural strategies to ensure they sire the offspring. The male may guard the female after mating to prevent another male accessing her (Sherman, 1983; Sigg and Goldizen, 2006), or he may mate with the female multiple times to increase his spermatozoa deposit (Stockley, 1997). Some species, for example kangaroos (Macropodidae) produce a copulatory plug. This forms when some of the semen coagulates after mating (up to 80 mL of coagulum) (Rodger and White, 1975; Taggart and Temple-Smith, 1994; Tyndale-Biscoe and Renfree, 1987). The role of the copulatory plug is not fully understood; however, it is thought to prevent loss of spermatozoa from the reproductive tract of the female, serve as a reservoir, and act as a barrier to spermatozoa from other males that mate subsequently (Taggart et al., 1998).

Sperm competition has been measured using numerous traits, including relative testes mass, length of the spermatozoon, sperm numbers (ejaculate volume) and sperm velocity. As
sperm competition is largely determined by the number of spermatozoa produced (Gómez Montoto et al., 2011b; Rowe and Pruett-Jones, 2011; Stockley et al., 1997), selection for sperm competition thus favours larger testes relative to body size (Hosken, 1997; Hosken and Ward, 2001; Jennions and Passmore, 1993; Ramm and Stockley, 2010; Wedell et al., 2002). The amount of sperm-producing tissue limits sperm production of the male, which is often correlated to testes size and mass (Garamszegi et al., 2005). Relative investment in sperm-producing tissue will determine the efficiency of sperm production (Peirce and Breed, 2001). Experiments in yellow dung flies (Scathophaga stercoraria) found that testes size increased over 10 generations in polygamous treatment groups, suggesting a rapid evolutionary response in testes size when the risk of sperm competition was increased (Hosken and Ward, 2001).

Sperm length has also been linked to sperm competition. A spermatozoon comprises of three morphologically-distinct components: the head, midpiece and the tail with all three components having an effect on velocity and sperm competition. The degree of elongation of the sperm head affects swimming velocity (Malo et al., 2006) due to a reduction in drag, and therefore reduced energy needed to progress (Gillies et al., 2009). Longer midpieces are generally correlated with increased sperm competition due to more energy reserves (Gomendio and Roldan, 2008), although in red deer (Cervus elaphus hispanicus) spermatozoa with a shorter midpiece have a faster swimming velocity (Malo et al., 2006). The tail length is also important as the longer and faster spermatozoa are more likely to fertilise the ovum, outcompeting other spermatozoa present (Gomendio and Roldan, 2008). The tail of the spermatozoon provides the propulsive force to move the spermatozoon forward and towards the ova. The theory that the longer spermatozoa swim faster definitely doesn’t occur in all species and is a topic of conflicting results as summaries in Table 1.1.
Table 1.1: Summary of previous studies of sperm competition in relation to spermatozoa lengths. (+) positive relationship, (-) negative relationship, NA not researched and (0) no association

<table>
<thead>
<tr>
<th>Taxa</th>
<th>Head</th>
<th>Midpiece</th>
<th>Tail / Total</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mammals</td>
<td>+</td>
<td>+, 0</td>
<td>+, 0</td>
<td>(Anderson et al., 2005; Gage and Freckleton, 2003; Gage et al., 2002; Gomendio and Roldan, 2008; Lemaître et al., 2009; Tourmente et al., 2011)</td>
</tr>
<tr>
<td>Bats</td>
<td>NA</td>
<td>NA</td>
<td>0</td>
<td>(Hosken, 1997; Lemaître et al., 2009)</td>
</tr>
<tr>
<td>Rodents</td>
<td>+</td>
<td>0</td>
<td>+, 0</td>
<td>(Breed and Jason, 2000; Gomendio and Roldan, 1991; Gómez Montoto et al., 2011a; Gómez Montoto et al., 2011b)</td>
</tr>
<tr>
<td>Ungulates</td>
<td>NA, +</td>
<td>NA, -</td>
<td>0, +</td>
<td>(Gomendio et al., 2007; Lemaître et al., 2009; Malo et al., 2006)</td>
</tr>
<tr>
<td>Primates</td>
<td>NA</td>
<td>NA</td>
<td>+, 0</td>
<td>(Anderson and Dixon, 2002; Gomendio and Roldan, 1991; Harcourt et al., 1995; Lemaître et al., 2009)</td>
</tr>
<tr>
<td>Birds</td>
<td>+</td>
<td>0</td>
<td>0, +</td>
<td>(Briskie and Montgomerie, 1992; Garamszegi et al., 2005; Immler et al., 2011; Møller, 1991; Pitcher et al., 2005)</td>
</tr>
<tr>
<td>Shorebirds</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>(Johnson and Briskie, 1999)</td>
</tr>
<tr>
<td>Fishes</td>
<td>NA, 0</td>
<td>NA, 0</td>
<td>-, 0</td>
<td>(Gage et al., 1995; Stockley et al., 1997)</td>
</tr>
<tr>
<td>Invertebrates</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Butterflies</td>
<td>NA</td>
<td>NA</td>
<td>+</td>
<td>(Gage, 1994)</td>
</tr>
<tr>
<td>Bulb Mites</td>
<td>NA</td>
<td>NA</td>
<td>+</td>
<td>(Radwan, 1996)</td>
</tr>
<tr>
<td>Dung Flies</td>
<td>NA</td>
<td>NA</td>
<td>0</td>
<td>(Sharma et al., 2013)</td>
</tr>
</tbody>
</table>

’Sperm number’, either the number of spermatozoa produced in the testes or the number of spermatozoa in each ejaculate, is correlated to the intensity of sperm competition (Byrne, 2004). Typically, sperm number increases as the intensity of sperm competition increases (Gómez Montoto et al., 2011a; Pitnick et al., 2001; Rowe and Pruett-Jones, 2011; Stockley et al., 1997). For polygynous species the ‘Raffle Principle’ predicts that fertilisation probability is positively correlated with the proportion of a given male’s spermatozoa in the female tract,
i.e. the more spermatozoa ejaculated and inseminated by the male, the higher chance he has of fertilising the female (Parker, 1990a). A number of authors have reported a trade-off between spermatozoa numbers (ejaculate investment) and the size of the spermatozoon (Arnaud et al., 2001; Gomendio and Roldan, 2008; Immler et al., 2011). Such a trade-off is an indication that reproduction is a costly investment for males, and they cannot have both larger spermatozoa with high numbers in each ejaculate. Larger animals seem to invest more in a higher number of spermatozoa compared to the size, because the sperm number are diluted in the female’s larger reproductive tract, while smaller species are predicted to be more likely to invest in larger spermatozoa so that they can physically out-compete the other opponents spermatozoa (Immler et al., 2011).

Sperm velocity and motility are two of the most important determinants of male fertility (Gomendio et al., 2007) and are the mediators of the efficiency of inseminated spermatozoa (Froman et al., 2002). The faster the velocity, the higher the fertilisation rate (Gomendio and Roldan, 2008) and the more likely the spermatozoa will overcome physical barriers in the female’s reproductive tract (Gomendio et al., 2007).

Sperm traits are not the only traits that are selected for in sperm competition. The male genital morphology, male accessory glands and their products may also be involved in increasing the male’s reproductive success (Parker, 1970). Some accessory gland products can be beneficial to the male, for example in Drosophila. The accessory gland products from the male Drosophila induce increased egg laying and reduced receptivity to other males (Stockley, 1997), but are harmful to the female (Andersson and Simmons, 2006). The female reproductive tract also affects the male’s sperm competition chance by controlling the fertilisation environment (Snook, 2005).
1.1.3 Relationship between pre- and post-copulatory sexual traits

When males invest in characteristics that aid in their reproduction, there is often a corresponding trade-off due to the energy costs associated with maintenance and growth (Lupold et al., 2014). This trade-off can either be between pre- and post-copulatory characteristics, which can follow the ‘guarder or sneaker’ strategy (Parker, 1990b), or between sperm competition traits (length of the spermatozoon and number of spermatozoa per ejaculate) (Gomendio and Roldan, 1991).

The ‘guarder or sneaker’ strategy relies on the assumption that guarder males will always mate with the female, but are unaware of the sneaker, therefore will expend less on the ejaculate. The sneakers expend more on their ejaculate due to the assumption they will face a high level of sperm competition and have limited matings (Parker, 1990b). This has been observed in salmon Salmo salar, where the sneaker males have larger relative testes mass compared with the guarders (Gage et al., 1995) and in Bluegill sunfish Lepomis macrochirus, where the sneakers have a higher fertilisation success compared to the guard males (Fu et al., 2001). Typically, guard males in most species assert their position by being the larger dominant male, while the sneaker males are the sub-dominant animals in the population and are often smaller and younger (Jackson and Vernes, 2010). However, in some species, such as salmon, the morphology of males is a genetically controlled trait whereby males will stay a sneaker or guarder all their lives.

Both pre- and post-copulatory sexual selection traits interact to ensure the male increases his chance of reproductive success, but there have been limited attempts at understanding these interactions. Female choice and sperm competition have been proven to amplify the pre-copulatory sexual selection in guppies (Andersson and Simmons, 2006), and this may be the case in other animal species.
1.2 Sexual selection in kangaroos

Male kangaroos invest heavily in secondary sexual characteristics, including a larger body mass and larger forelimb muscles compared to the female (Warburton et al., 2013). The sexual characteristics which kangaroos can invest in would appear to be limited due to their highly specialised body form and locomotion. The kangaroo has a highly adapted bipedal bounding style of locomotion that would likely be negatively affected by large horns on their heads, or large elaborate weaponry on their bodies so they have invested in the second largest body dimorphism between the males and the females. The largest macropod, the red kangaroo *Macropus rufus*, has the second largest sexual dimorphism of all mammals. Male red kangaroos average 2.5 times heavier than females, however a male can be up to six times larger than the female at mating (Jarman, 1989, 1991). This sexual dimorphism exceeds the 3:1 male to female body mass ratio for sealions and fur seals (family Otariidae), which had been considered to have the largest sexual dimorphism in body size among mammals (Fisher and Owens, 2000; Weckerly, 1998).

It has also been shown that males invest differentially in muscle development. The forelimb muscle mass in the males are much larger and exaggerated than in the females. The female shows an isometric growth of forelimb muscle, while the males show an allometric growth with forelimb muscle mass, especially in the muscles that are involved in male-male competition (muscles involved in grasping and pulling). These findings suggest that the forelimb muscles in a male kangaroo are sexually selected for to aid in male-male competition, as well as use as a signal of dominance to the females (Jarman, 1989; Warburton et al., 2013).
Kangaroos exhibit hierarchical promiscuity, in that males of higher ranking have a disproportionally higher number of matings than the lower ranked subdominant males (Jarman and Southwell, 1986; Miller et al., 2010). The species within the *Macropus* family are often polygamous (Johnson, 1983) which has lead to a high level of sexual dimorphism. There is a clear size difference between the males, with the larger male having reproductive advantages over a smaller male (Jarman, 1989) as also recorded in tammar wallabies (*Macropus eugenii*) and eastern grey kangaroos (*Macropus giganteus*) (Hynes et al., 2005; Miller et al., 2010).
Miller et al. (2010) found that the larger, alpha male eastern grey kangaroos have higher testosterone concentrations. The alpha males had a significantly higher reproductive success, siring 54% of all the offspring compared with the lower-ranked and smaller males. This level of reproductive success of the alpha males has also been seen in the tammar wallaby, with approximately 50% of the offspring sired by the dominant male and 35% of the second most dominant alpha male, with the smaller lesser ranked males only siring 15% of the offspring (Hynes et al., 2005).

1.2.2 Sexual dimorphism

The selective pressure for larger body masses of the male is driven by limited breeding opportunities and the higher potential for alpha males to mate compared with smaller males, therefore a large sexual size dimorphism is seen in the Macropus family (Miller et al., 2010). Both the male and female can begin to breed before they reach their maximum mature weight, with some females beginning to mate at half their maximum weight, which can make them one-third (swamp wallaby) to one-sixth (red kangaroo) the size of the largest male in the mob (Jarman, 1983), but often males will be very close to their mature body weight before being able to secure a mate.

The characteristic bauplan of kangaroos and wallabies, with large powerful hindlimbs and tail, together with relatively small and slightly muscled forelimbs has been correlated with adaptation for bipedal bounding locomotion (Hopwood, 1974). The smaller forelimbs help to support the body mass together with the tail during slow pentapedal movement (Harvey and Warburton, 2010) see also (Windsor and Dagg, 1971), and are also involved in male-male fighting (Warburton et al., 2013). Male kangaroos have much more strongly development muscles of the forelimb, particularly those performing grasping and pulling function (Croft and Snaith, 1990; Glanslosser, 1989), but the forelimbs also have very large, thick claws
(Glanslosser, 1989), which would seem to act as weapons, much as antlers or horns might be used in quadrupedal animals in these aggressive interactions (Jarman, 1983). The degrees to which the forelimb muscles are developed, seems to vary between kangaroo species. Hopwood (1981) investigated the muscle masses of the forearm and the hindlimb of red kangaroos and grey kangaroos, comparing males and females. The forelimb muscles (proximal and distal) were significantly greater in males of both species compared with the females. When comparing animals of the same body mass between the species, the grey kangaroos have a relatively greater proportion of muscles in the hindquarters, while the red kangaroos have a relatively greater proportion of muscle in the forequarters and the tail. Not only is the muscle mass larger in the males, the length of the forearm (wrist to elbow) relative to the length of the hind foot is also greater in males (Jarman, 1983).

Few other sexually dimorphic traits are exhibited among macropodids. The red kangaroo and the wallaroo exhibit different colourings and markings between males and females, while in other species, males and females are generally fairly similar in appearance (Jarman, 1983). Some of the smaller macropodoids have insignificant differences between body masses of the sexes, but they do invest in weaponry. In the bettongs, potoroos and rat-kangaroos the canine teeth are larger in the males compared with the female (Jarman, 1983).
1.2.3 Sperm competition

There has been limited research into sperm competition in marsupials. Taggart et al. (1998) and Rose et al. (1997) studied sperm competition and reproductive mating strategies in a range of marsupial and monotreme species and found a positive allometric relationship between the body mass and testes mass. *Macropus fuliginosus* and red-neck wallaby (*Macropus rufogriseus*) have high numbers of epididymal spermatozoa for their body mass compared with the swamp wallaby (*Wallabia bicolor*) and wombat (*Vombatus ursinus*). A negative relationship between spermatozoa tail length and log body mass was found. Species under 1kg (e.g., the honey possum *Tarsipes rostratus* - 356 µm; and kowaei *Dasyuroideae byrne* - 243 µm), had the longest sperm tails, as well as the highest relative length for their total body mass. The eastern grey kangaroo (112 µm) and the swamp wallaby (102µm) had relatively long sperm tails for large marsupials compared with the koalas (*Phascolarctos cinereus*) and wombats that ranged between 72-82 µm. These data presented for the macropodoids, especially the kangaroos indicated a multi male mating
system reflected by the average to large relative testes mass, long sperm tails and high numbers of epididymal spermatozoa (Taggart et al., 1998).

Marsupial spermatozoa have an unusual maturation and capacitation process throughout the testes, to epididymis to the site of fertilisation after ejaculation. The capacitation process occurs in the female reproductive tract (usually in the lower segment of the oviduct) to allow the spermatozoa to go through the acrosome reaction (an exocytotic reaction required for the spermatozoa to penetrate the oocyte and fuse to the oocyte membrane) and be able to fertilise (Mate and Rodger, 1996). These aspects have been studied thoroughly in several species including Bennett’s wallaby, Macropus rufogriseus rufogriseus, tammar wallaby, Macropus eugenii, and the common brush tail possum, Trichosurus vulpecular (Boere et al., 2011; Clulow et al., 1992; Cummins, 1976). Cryopreservation has also been studied in numerous species to assist in captive breeding programs, but with minimal success. The most successful study was by Holt et al. (1999) using tammar wallaby spermatozoa and the result was 50% motility pre-warming to 5-10°C, and when warmed further to 20-25°C the motility decreased to less than 10%. This freezing process used 20% glycerol; this is a very high glycerol concentration, but was proved to be the most successful (McClean et al., 2007; McClean et al., 2008).

Holt et al. (1999) observed some unusual patterns in the eastern grey and red-necked wallaby epididymal spermatozoan motility while cooling the spermatozoa for cryopreservation. While cooling the spermatozoa from 20°C to 15°C the progressive motility declined due to spermatozoa tail bending, and this was also seen again at below 5°C. This motility decline will obviously affect the visual assessment of the spermatozoa during cooling. Boere et al. (2011) determined that 15 min at 36°C was the minimum incubation time needed in their experiment with Bennett’s wallaby; however, the minimum incubation time has not been determined for the Macropus fuliginosus.
Behavioural observations have also been conducted on the eastern grey kangaroo. Eastern greys have been confirmed to have a stable dominance hierarchy with the largest male defending the females while she is in oestrus (Lee and Cockburn, 1985). Majority of the females will mate with the dominant male (Rubenstein and Wrangham, 1986), while multiple mating have been observed, but it is unsure if these occur when the female is in oestrus, or past her oestrus period (Paplinska et al., 2010). The relative testes mass of the eastern grey kangaroo is relatively low; however, that for Macropus fuliginosus is much higher, suggesting that there is more sperm competition occurring in this species. This may indicate that the dominant male is not as good at guarding the mated females and multiple sneaker matings do occur (Paplinska et al., 2010).

1.3 Study species: western grey kangaroo (Macropus fuliginosus)

Macropus fuliginosus is subdivided into subspecies according to the distribution over Australia; M. f. fuliginosus is the subspecies found only on Kangaroo Island, M. f. ocydromus is found in Western Australia, and M. f. melanops from the east of Australia covering South Australia, Victoria, New South Wales and up into Queensland (Dawson, 2012; Poole et al., 1982). The eastern grey kangaroo overlaps this home range throughout western Victoria and south-western New South Wales, and through similar in morphology, the two species rarely interbreed in the wild (Poole, 1975; Poole and Catling, 1974) and the two species can be separated via reproductive, morphological and serological differences (Kirsch and Poole, 1967, 1972).
Figure 1.3: Distribution of *Macropus fuliginosus* shaded in grey sourced from Mayberry *et al.* (2010)

The *Macropus fuliginosus* does not only have a large dimorphism between the male and female body mass, but also differences in the timing, duration and rate of growth. Females sexually mature by 14 months, and typically begin producing offspring at 16kg (2 years old). They continue to grow after sexual maturity and may reach up to 34kg by 7-8 years old. Males sexually mature at 31 months, around 25kg, although they are not considered an adult until around 55-60kg, which is at approximately 6-7 years of age. The male can reach a body mass of up to 85kg at his peak (Dawson, 2012). There is a sex difference in the head-body length (range 950-2225 mm in males and 950-1750 mm in females) and tail length (average of 1000 mm in males and 815 mm in females) (Richardson, 2012). Interestingly, unlike all the other species of kangaroos, wallabies and kangaroo rats, *M. fuliginosus* lacks the ability to initiate embryonic diapauses (Tyndale-Biscoe, 2005). Male *M. fuliginosus* have a unique musty odour, which is believed to be due to a secretion from their paracloacal glands (Richardson, 2012).
1.4 General Aims

Large sexual dimorphism in body size and muscle development in kangaroos suggests that sexual selection is likely to be a significant driving force in the development and biology of these animals. Inherent variation in the size and muscle mass of the male leads to the hypothesis that males may use different strategies to ensure matings, and that sperm competition may also take place. The traits of sperm competition and characteristics of sexual selection have been studied comprehensively in many animal species, however there have been few studies published in these fields to date on the kangaroos. There are also few studies that have studied both selection pressures of sexual selection and sperm competition in the one animal species. As such, knowledge of this trade-off and the relationship between these two selection characteristics is limited.

The present study investigated the relationship between sexually selected forelimb muscle mass and traits associated with sperm competition in the *Macropus fuliginosus*. Traits associated with sperm competition are sexually selected for in kangaroos along with sexually dimorphic traits such as body mass and muscle investment. Theory predicts a trade-off between the pre- and post-copulatory sexual traits (Lupold *et al*., 2014).

The aims of the present study was as follows:

1. To determine the optimal processing methods for *Macropus fuliginosus* sperm

Before we could determine a relationship between forelimb muscle mass and sperm competition, a comprehensive review of methods was undertaken to determine the appropriate techniques for sperm processing for *Macropus fuliginosus*. In chapter 2, I have investigated the different incubation times and concentrations to establish the best methods to maintain kangaroo sperm motility and viability. Two methods of assessing spermatozoa
velocity were evaluated to determine if they are equivalent. This is the first study on sperm competition in the *Macropus fuliginosus*; therefore the initial experimental methods will determine the optimal methods of processing.

(2) A brief description of the *Macropus fuliginosus* spermatozoa;

Marsupial spermatozoa have a different maturation and capacitation process throughout the testes and epididymis compared with eutherian mammal spermatozoa. This process has been thoroughly studied in several marsupial species, however not *Macropus fuliginosus*. In chapter 3, I have investigated the main measures of the *Macropus fuliginosus* including lengths, average velocities and the common morphological abnormalities seen.

(3) Determine whether there is any correlation between investment in forelimb muscle and investment in sperm competition in *Macropus fuliginosus*.

All sexually mature males have the ability to mate with a female; however there is a bias towards the bigger, more muscular dominant males mating with all the females, rather than the smaller males (Jackson and Vernes, 2010). Therefore we hypothesise that the males that have lower muscle mass in their forelimbs relative to their body mass will have more competition reproductive/spermatozoa traits due to their limited access to matings (A phenotypic trade-off). In chapter 3, I investigated the sperm traits that are being used as a measure of sperm competition. These are RTM (relative testes mass), the lengths of spermatozoa (head, midpiece and tail), forward velocity, proportion of morphologically normal spermatozoa and motility (both total motility and progressive motility). The results of this study will further aid in determining the links between sexual selection and sperm competition in general and give a general summary of sperm competition in macropods as this has not been explicitly explored. The kangaroo is an interesting animal to study in this area because of its restricted body structure (due to locomotion. Complication
Chapter 2: Establishing protocols for the collection and examination of live western grey kangaroo (*Macropus fuliginosus*) spermatozoa

2.1 Introduction

The evaluation of sperm motility is considered to be a fundamental laboratory assessment of the viability and fertilisation capacity of spermatozoa (Varner, 2008). Extraction of spermatozoa from the epididymis for assessment is a common technique used in numerous species. As the spermatozoa from the caudal epididymis have adequate maturity to fertilise (Turner, 1995), these techniques can be used to extract sperm and genetic material from animals that have been harvested, or unexpectedly passed away.

Assessments of raw ejaculated semen samples are an indication of sperm performance in their natural fluid; however, they are in such high concentrations that it is difficult to assess the individual motility patterns (Varner, 2008). The same situation occurs when spermatozoa are flushed from the epididymis; the concentration is extremely high, and rather than the other accessory gland fluids, the spermatozoa are in the flushing buffer. When the sperm concentration is too high, they appear clustered together and the maximum velocity decreases (WHO, 2010). The appropriate diluted sample for stallion spermatozoa is 25 x10^6 sperm/mL using a high quality extender (Varner, 2008) so this was used as a starting point for the dilution of the kangaroo.

Marsupial spermatozoa have previously been recorded as immotile when first flushed from the epididymis of Bennett’s wallaby (Boere *et al.*, 2011) and tammar wallaby (Clulow *et al.*, 1992). This immotile state of the spermatozoa turned to vigorous motility after 15 min in a diluted culture medium (Boere *et al.*, 2011). This study further investigates this prediction...
and determine the appropriate incubation time after sperm extraction. Spermatozoa are temperature sensitive (thermotaxis) (Bahat and Eisenbach, 2006) and this temperature dependence alters the motility and the tail movement. This is why it is important to bring the sample up to the appropriate temperature to accurately assess the maximum motility. The kangaroo testes are kept at 2-5°C lower than body temperature (Dawson, 2012), therefore the optimal temperature would be assumed to be approximately 32-34°C due to the kangaroos low body temperature, which is on average approximately 36.2°C (Maloney et al., 2011).

The second question posed in this thesis is whether there is a difference in sperm quality between processing within a day of collection (12 hours) to two days (36 hours). This is an important question to assess due to the constraints that may be faced by the distances of some kangaroo collection site (distance from laboratory) and the impact this may have on measures of spermatozoan quality/health. The importance of assessing the spermatozoa as soon as possible is known; however, it is unknown if there will be a significant decrease in motility from 12 versus 36 hours post death.

There are currently no clear protocols for the extraction and assessment of the spermatozoa of *Macropus fuliginosus*. Most of the research into marsupial spermatozoa has focused on the maturation process through the testes and epididymis, the interactions of the spermatozoa with the female reproductive tract, and cryopreservation methods. This chapter aims to determine these protocols including the optimum concentrations and incubation times for the diluted spermatozoa sample, velocity methods and the most favourable time from death to processing (12 vs 36 hours).
2.2 Methods

2.2.1 Animal collection

Male *Macropus fuliginosus* of varying body size were sourced by professional kangaroo shooters between March 2014 and August 2014 as part of commercial harvesting. They were collected from six sites in Western Australia (11 from Lake Carmody, 3 from East Jurien Bay, 4 from North Pinjarra, 3 from Fairbridge Village, 5 from Lakes Road and 8 South east Pinjarra) (Table 2.1). Each individual was weighed using a mass balance before anything was removed from the carcass. The whole scrotum with contents was excised from each animal immediately after death, placed in a plastic bag in a styrofoam box. They were refrigerated at 4°C and transported back to Murdoch University for analysis. Only one epididymis was used from the first 11 kangaroos from Lake Carmody, which was examined the day after collection. For all other kangaroos one epididymis was removed and assessed within 12 h of death, and the scrotum with its remaining organs was stored in the styrofoam box in refrigerator (4°C) for another 24 hours. The second epididymis was then removed and analysed at approximately 36 h post death. The project was conducted under the approval of Murdoch University Animal Ethics Committee (Cadaver Notification) and a Regulation 17 license SF009843.
Table 2.1: Each kangaroo collected with location of collection, date and body mass. Number 19** and 32** were removed from the analysis because of the lack of sexual maturity and no spermatozoa present.

<table>
<thead>
<tr>
<th>Number</th>
<th>Location</th>
<th>Date of Collection</th>
<th>Total Body Mass (kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Lake Carmody</td>
<td>24/03/2014</td>
<td>42</td>
</tr>
<tr>
<td>2</td>
<td>Lake Carmody</td>
<td>24/03/2014</td>
<td>33</td>
</tr>
<tr>
<td>3</td>
<td>Lake Carmody</td>
<td>24/03/2014</td>
<td>68</td>
</tr>
<tr>
<td>4</td>
<td>Lake Carmody</td>
<td>24/03/2014</td>
<td>38</td>
</tr>
<tr>
<td>5</td>
<td>Lake Carmody</td>
<td>24/03/2014</td>
<td>28</td>
</tr>
<tr>
<td>6</td>
<td>Lake Carmody</td>
<td>24/03/2014</td>
<td>23</td>
</tr>
<tr>
<td>7</td>
<td>Lake Carmody</td>
<td>24/03/2014</td>
<td>57</td>
</tr>
<tr>
<td>8</td>
<td>Lake Carmody</td>
<td>24/03/2014</td>
<td>55</td>
</tr>
<tr>
<td>9</td>
<td>Lake Carmody</td>
<td>24/03/2014</td>
<td>51</td>
</tr>
<tr>
<td>10</td>
<td>Lake Carmody</td>
<td>24/03/2014</td>
<td>24</td>
</tr>
<tr>
<td>11</td>
<td>Lake Carmody</td>
<td>24/03/2014</td>
<td>70</td>
</tr>
<tr>
<td>12</td>
<td>East Jurien Bay</td>
<td>2/06/2014</td>
<td>23</td>
</tr>
<tr>
<td>13</td>
<td>East Jurien Bay</td>
<td>2/06/2014</td>
<td>32</td>
</tr>
<tr>
<td>14</td>
<td>East Jurien Bay</td>
<td>2/06/2014</td>
<td>23</td>
</tr>
<tr>
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<td>North Pinjarra</td>
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</tr>
<tr>
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<td>North Pinjarra</td>
<td>12/06/2014</td>
<td>71</td>
</tr>
<tr>
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<td>12/06/2014</td>
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</tr>
<tr>
<td>18</td>
<td>North Pinjarra</td>
<td>12/06/2014</td>
<td>69</td>
</tr>
<tr>
<td>19**</td>
<td>North Pinjarra</td>
<td>12/06/2014</td>
<td>10</td>
</tr>
<tr>
<td>20</td>
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<td>15/07/2014</td>
<td>83</td>
</tr>
<tr>
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<td>Fairbridge Village</td>
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</tr>
<tr>
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</tr>
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<tr>
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<td>Lakes Road</td>
<td>29/07/2014</td>
<td>84</td>
</tr>
<tr>
<td>26</td>
<td>Lakes Road</td>
<td>29/07/2014</td>
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<tr>
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<td>68</td>
</tr>
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<td>South East Pinjarra</td>
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</tr>
<tr>
<td>29</td>
<td>South East Pinjarra</td>
<td>25/08/2014</td>
<td>31</td>
</tr>
<tr>
<td>30</td>
<td>South East Pinjarra</td>
<td>25/08/2014</td>
<td>33</td>
</tr>
<tr>
<td>31</td>
<td>South East Pinjarra</td>
<td>25/08/2014</td>
<td>29</td>
</tr>
<tr>
<td>32**</td>
<td>South East Pinjarra</td>
<td>25/08/2014</td>
<td>29</td>
</tr>
<tr>
<td>33</td>
<td>South East Pinjarra</td>
<td>25/08/2014</td>
<td>41</td>
</tr>
<tr>
<td>34</td>
<td>South East Pinjarra</td>
<td>25/08/2014</td>
<td>40</td>
</tr>
<tr>
<td>35</td>
<td>South East Pinjarra</td>
<td>25/08/2014</td>
<td>41</td>
</tr>
</tbody>
</table>
2.2.2 Spermatozoa recovery

For each animal, one testis and associated structures was removed from the scrotum via a long incision in the side of the scrotum, and the epididymis was dissected free of all other tissue to leave approximately 50mm of the vas deferens and the cauda epididymis for sperm collection, in a modification of the method used in other species (Cary et al., 2004; Martinez-Pastor et al., 2006; Papa et al., 2008). A dissecting microscope was used to visualise the open end of the cut vas deferens so that a blunt 25G needle could be inserted into the lumen and secured with forceps to create a tight seal. A 5mL syringe containing room temperature (22°C) EquiPro (Equine semen extender, Minitube Australia Pty Ltd, Smythesdale, Victoria) was attached to the needle and using a pulsing technique with the syringe, the spermatozoa were flushed through the epididymis into a sterile 10 mL centrifuge tube. When necessary, i.e. when no liquid was flowing out, extra incisions were made to the cauda epididymis to allow the liquid to flow more freely. Between 2 mL and 6 mL of EquiPro was used to flush, depending on the ease of flushing, but the volume used was noted so accurate concentrations could be calculated.

2.2.3 Spermatozoa analysis

One drop of the flushed liquid from the epididymis was assessed under a phase-contrast microscope (Olympus CX41) with a warming stage attached at 37°C (Tokai Hit Olympus) to ensure there were spermatozoa present in the solution and make a subjective assessment of how concentrated the initial solution was. An aliquot of the flushed solution was then diluted to a standard concentration of approximately 25 million spermatozoa per mL for further assessment. Visual subjective assessments of total motility (i.e. percentage of spermatozoa moving) and progressive motility (i.e. percentage of spermatozoa moving forward) were made after the standard concentration had incubated at 37°C. The total motility assessment was based on a 0-100% scale with 0% (no movement), and 100% (all
spermatozoa showing movement). The progressive motility assessment then focused on the forward moving spermatozoa from 0% (none of the moving spermatozoa progressive), to 100% (all the moving spermatozoa swimming progressive). All subjective measures were performed by the same individual to minimize variation.

A buffered formalin saline composition (BFS) was used to make a standard 1:20 dilution of the spermatozoa (5µ spermatozoa sample added to 95µ BFS) and spermatozoa were counted using a haemocytometer visualised under 10x power on the phase contrast microscope, to calculate the total numbers of spermatozoa flushed from the epididymis.

Photographs were taken of individual spermatozoa using Moticam 2300 (3.0MP Live resolution, Digital Microscopy) from the air-dried slides and the lengths were measured by tracing the spermatozoa on the photograph using Motic Image Plus (Version 2.0). The heads of linear spermatozoa were measured tip to midpiece using a straight line, and those that were T-shaped were measured tip to tip. Both the measure of and midpiece and midpiece+tail were measured using the free hand setting. The measure of midpiece+tail was made instead of tail only as the midpiece could not be well differentiated for all spermatozoa.

One sample slide per kangaroo was prepared for morphological assessment of 100 spermatozoa. 15 µm of the initial flushed spermatozoa was spread on a microscope slide and air-dried, and then stained using SpermBlue following methods from van der Horst and Maree (2009). SpermBlue fixative and stain were supplied by Microptic SL (Barcelona, Spain). Morphological defects were recorded.

These slides were also used to count the proportion of straight head orientation compared to T-shaped head orientation. Lin and Rodger (1999) studied the transit throughout the
epididymis of the tammar wallaby and brushtail possum and concluded that light microscopy could be a definitive indicator of sperm maturation.

2.2.4 Varying incubation times

The incubation time before the spermatozoan motility assessment was examined to determine when motility appeared best. Different lengths of times that the sample spent in the incubator were tested, as well as two methods either room temperature (22 – 25°C) EquiPro compared with 37°C EquiPro.

a. Kangaroos 12-22- Group 1

Samples were flushed with room temperature EquiPro and then diluted samples were prepared with 37°C EquiPro. The samples were assessed subjectively for total motility, progressive motility and velocities after 10 min and 15 min.

b. Kangaroos 23-35- Group 2

Samples were flushed with room temperature EquiPro, and then the diluted samples were prepared using room temperature EquiPro. These samples were assessed subjectively for total motility, progressive motility and velocity after 15, 20, 25 and 30 min.

2.2.5 Varying spermatozoa concentrations

Three sperm concentrations were assessed to see if the concentration of the sample affected the total motility, progressive motility and velocity i.e. if a high concentration of spermatozoa restricted each spermatozoon from swimming forward. A low concentration (~15million/mL) recommended concentration (~25million/mL) and high concentration (>60million/mL) were compared.
2.2.6 Two different velocity methods

Video recordings were made of each sample using the Motic Image Plus Version 2.0 computer program for subsequent analysis of motility and velocity of five spermatozoa. Sperm velocity was calculated by timing a spermatozoons’s path across the computer screen. In addition to using Motic to assess the sperm velocity, the program Sperm Class Analyzer, SCA Motility and concentration (Microptic, Barcelona, Spain) was used. A sample of the diluted flushed sample was analysed using a negative phase microscope (Nikon Eclipse e200). The sperm Class Analyzer (SCA) did not have a setting for kangaroos, so the chicken setting was used as this was the most similar in size and speed to the kangaroo spermatozoa (Froman and Feltmann, 2000). This program recorded a video of the sperm sample for one second, then traces the path of the moving spermatozoan, categorising the velocities into rapid progressive >100 µ/s, medium progressive between 100 µ/s and 50 µ/s, slow <50 µ/s and static, not moving. The velocity assessments on the SCA were performed at either 15 min (kangaroos 12-22) or 30 min (kangaroos 23-35).

2.2.7 Time to processing (12 versus 36 hours)

To determine if there was a significant difference between flushing the spermatozoa and analysing at 12 hours post death compared to 36 hours post death, epididymis 1 was analysed at 12 hours, and epididymis 2 at 36 hours. The 36 hours post death analysis was the same as at 12 hours, with varying incubation times, varying concentration and the two different velocity methods being tested.

2.2.8 Statistical Analyses

Statistical analysis was performed using Statistica 7.1 for Windows and Excel 2007 for Windows. Statistica was used to calculate p values for analysis (an alpha level of p= 0.05 was used to determine significance). All variables were assessed for normality, and if
variables were not normally distributed statistical transformations were performed to meet the necessary parametric criteria.

To analyse the effect of time after death (comparing data from epididymis 1 and epididymis 2) for motility, concentration and velocity, the data was analysed using either a repeated measure ANOVA or a General Linear Mixed Model. The difference between the velocity methods was calculated by determining the relationship between the average velocity measured manually and the fastest velocity using the SCA computer program using a paired t-test.

### 2.3 Results

#### 2.3.1 Sperm Morphology

Of the 33 kangaroos sampled, the sperm morphology is summarised in Table 2.2. Not all midpieces could be visualised in all the spermatozoa measured, and some kangaroos were more visible than others, e.g. Kangaroo 4 only had 1/20 midpieces measured. Randomly selected samples of spermatozoa were re-measured to calculate the coefficient of variation for each measure.

<table>
<thead>
<tr>
<th>Sperm component</th>
<th>Average length (µm)</th>
<th>Number measured</th>
<th>Range length (µm)</th>
<th>Error in measure (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Head</td>
<td>6.4 ± 1.0</td>
<td>660</td>
<td>4.0 - 12.2</td>
<td>4.1</td>
</tr>
<tr>
<td>Midpiece</td>
<td>10.8 ± 1.5</td>
<td>528</td>
<td>6.4 - 16.2</td>
<td>3.2</td>
</tr>
<tr>
<td>Midpiece + Tail</td>
<td>109.5 ± 6.7</td>
<td>660</td>
<td>60.0 - 125.0</td>
<td>0.5</td>
</tr>
<tr>
<td>Total</td>
<td>115.9 ± 3.3</td>
<td>660</td>
<td>110.1 - 123.2</td>
<td>7.7</td>
</tr>
</tbody>
</table>

Table 2.2: Average spermatozoa lengths recorded. These lengths were measured on the computer program Motic and error in measure calculated by re-measuring range samples.
Very few morphological defects were seen in the sperm samples (Table 2.3). The T-shaped head orientation was seen in approximately 14% of the spermatozoa assessed (1,813 of 13,200 counted in T-shape orientation) (Figure 2.1).

Table 2.3: Morphological defects recorded. These parameters were defined for this study as they were the most common defects from the first observations

<table>
<thead>
<tr>
<th>Defect</th>
<th>Description</th>
<th>Occurrence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Head defect</td>
<td>Head abnormal shape or other defect</td>
<td>4.0</td>
</tr>
<tr>
<td>Midpiece defect</td>
<td>Midpiece bent or other defect</td>
<td>1.7</td>
</tr>
<tr>
<td>Tail A (top 3rd of tail) defect</td>
<td>Top 3rd of tail coiled or bent</td>
<td>0.5</td>
</tr>
<tr>
<td>Tail B (middle 3rd of tail) defect</td>
<td>Middle 3rd of tail coiled or bent</td>
<td>2.1</td>
</tr>
<tr>
<td>Tail C (end 3rd of tail) defect</td>
<td>End 3rd of tail coiled or bent</td>
<td>1.5</td>
</tr>
<tr>
<td>Coiled end of tail</td>
<td>Very end of the tail coiled</td>
<td>1.1</td>
</tr>
<tr>
<td>Detached head</td>
<td>Head not attached to midpiece</td>
<td>6.1</td>
</tr>
<tr>
<td>Completely coiled</td>
<td>Entire tail coiled</td>
<td>0.9</td>
</tr>
</tbody>
</table>

Figure 2.1: Phase contrast microscope taken under 40 x magnifications from an air-dried sperm smear. These two photos compare the different head orientations of *Macropus fuliginosus*. (A) illustrates the normal sperm head orientation (K21-1) while (B) show the T-shaped sperm head orientation (K18-3).

2.3.2 Does the length of time since cull influence spermatozoa morphology?

There was a significant effect of time since cull (12 hours to 36 hours) on the sperm head morphology (paired $t_{21} = 2.20$, $p= 0.039$). The spermatozoa assessed 36 hours post cull had significantly more T-shaped heads compared to the sample assessed at 12 hours post cull (Figure 2.2).
Figure 2.2: Average percent of T-shaped spermatozoa at testes 1 (12 hours) and testes 2 (36 hours). Effect on time after cull seen between the two indicating testes 2 has more T-shaped spermatozoa than testes 1 with standard deviation bars.

2.3.3 Is there an effect of incubation time on spermatozoa traits (motility and velocity)?

A significant effect of time after cull (12 compared with 36 hours) was found for both group 1 ($F_{1,9} = 7.00$, $p=0.027$) and group 2 ($F_{1,11} = 13.78$, $p=0.003$) on sperm velocity (Figure 2.3) showing that the velocity was significantly lower at 36 hours compared with 12 hours.

Group 1 also had a significant effect of three factors of time post cull (12 vs 36 hours) x incubation time x kangaroo ID, ($F_{3,136} = 2.97$, $p=0.034$) on sperm velocity.

Group 2 showed a significant effect of incubation on velocity ($F_{3,19} = 10.81$, $p<0.001$) as well as a effect of the interaction between testes assessment time (12 vs 36 hours) and animal ID ($F_{11,18} = 2.48$, $p=0.041$) and the interaction of all three factors; testes x incubation time x animal ID ($F_{16,282} = 1.88$, $p=0.022$).
Figure 2.3: (A) Showing that velocity increased as incubation time increased. The velocity was also significantly lower when assessed at 36 hours compared to 12 hours. (B) Shows the same relationship however 15 min and 20 min there was a slight decrease at both time points. Standard deviation bars on both graphs.

Group 1 showed an effect of time after cull on total motility ($F_{1,9} = 24.9$, $p= 0.001$) where the total motility significantly decreased from 12 hours to 36 hours (Figure 2.4), and there was no effect of incubation time ($F_{1,9} = 0.04$, $p= 0.848$). Progressive motility was unaffected by either time after cull ($F_{1,9} = 1.63$, $p= 0.234$) or incubation times ($F_{1,9} = 0.04$, $p= 0.848$).
Figure 2.4: Effect of testes (assessing at 12 and 36 hours post death) on total motility of the group 1 kangaroos (standard deviation bars). There was a significant decline in total motility from 12 hours to 36 hours.

Group 2 showed an effect of time after cull ($F_{1,6} = 17.22$, $p = 0.006$), incubation time ($F_{3,18} = 16.10$, $p < 0.001$) and a time post cull x incubation time interaction ($F_{3,18} = 11.43$, $p < 0.001$) on total motility (Figure 2.5A). Progressive motility was affected by incubation time ($F_{3,18} = 3.49$, $p = 0.037$) but not by the time post cull ($F_{1,6} = 1.98$, $p = 0.209$) (Figure 2.5B).

2.3.4 Does sperm concentration influence the spermatozoa traits (motility and velocity)?
Velocity was not affected by diluent concentration ($F_{2,14}= 3.58, p= 0.056$). Total motility was also not affected by diluent concentration ($F_{2,36}= 1.26, p= 0.295$). Total motility decreased from 12 to 36 hours, however each diluent concentration decreased differently as seen in Figure 2.6A ($F_{2,36}= 5.56, p= 0.009$).

There was a significant effect of diluent concentration ($F_{2,36}= 4.23, p= 0.022$) on progressive motility where the high concentration had the highest progressive motility, while the recommended concentration had the lowest progressive motility (Figure 2.6B).

![Figure 2.6: (A) Effect of concentration and time post cull on total motility. (B) Effect of concentration on progressive motility showing the high concentration had the highest progressive motility, and the recommended concentration with the lowest progressive motility. Both graphs indicating the standard deviation.](image)

### 2.3.5 Is there a correlation between measures of the fastest and the average sperm velocity?

There was a significant distinction between the measure of the fastest spermatozoa measured using the SCA machine and the average sperm velocity measured manually from the moving spermatozoa ($t_{18}= -5.32, p< 0.001$) (Figure 2.7). The SCA measurement was only below the manual measure of sperm velocity for three kangaroo spermatozoa samples. The
standard deviations of the manual calculations were larger than the SCA measures due to the accuracy of the measures.

![Graph](image)

**Figure 2.7**: Comparison of fastest spermatozoa using SCA program and the average spermatozoa measured manually with the line representing equal velocity measurement between the two methods. The whiskers on each data point represent the standard deviations with the average sperm velocity having a much larger standard deviation.

### 2.4 Discussion

In this methods-based investigation, potential sperm processing and assessing protocols have been established, which encompasses the optimum processing time post cull, incubation times, concentration and a recommended velocity assessment method. The best time to process the epididymides was within 12 hours of cull to maximise the motility and maintain a high level of normal linear spermatozoa and avoid degradation, as well as incubating for the longer time; the high diluent concentration did seem to produce the highest motility and velocity. The measure of the fastest spermatozoa compared with
average spermatozoa gave significantly higher velocities and is a more accurate measure of sperm competition.

2.4.1 Morphology

The sperm morphology of *Macropus fuliginosus* has not been previously described; however, our observations correspond with those of closely related species the eastern grey kangaroo (McCLean *et al.*, 2006; Taggart *et al.*, 1998). The average total length of the spermatozoa measured was 115.9µm, which is close to that measured for the eastern grey at 112µm (Taggart *et al.*, 1998). The software Motic used to measure spermatozoa was appropriate and provided reasonable accuracy, with the largest error in the midpiece+tail measurement. This source of error would mostly be due to the human error of tracing a longer area, and also distinguishing the starting point of the midpiece from the head, which at some times was difficult. An automated system such as Leica IM50 Image Manager, used in Mossman *et al.* (2013) may have produced more accurate results as the computer program digitally maps the sperm lengths rather than relying on human measurements.

The calculated proportion of normal linear head position compared to the T-shaped orientation was interesting. In marsupials, spermatogenesis includes the flattening of the nucleus in a plane perpendicular to the midpiece axis, which results in the head forming 90 degrees to the tail; this is the T-shaped head orientation (Cummins, 1976; Mate and Rodger, 1996). To achieve the streamlined linear head position of mature spermatozoa, the head must undergo rotation. This process of maturation begins in the testes and is completed during epididymal transit (Bedford and Breed, 1994; Mate and Rodger, 1996). In previous research, marsupial spermatozoa from the caudal epididymis showed 100% normal linear heads (Clulow *et al.*, 1992; Setiadi *et al.*, 1997), while Cummins (1976) found between 71-89 (average 83) mature spermatozoa from the cauda epididymis, and between 33-62 (average 52) mature spermatozoa from the distal corpus epididymis. In the present
study, an average of 14% T-shaped spermatozoa (12% at 12 hours and 15% at 36 hours) was found, which was within the range of the study by Cummins (1976).

There was a trend whereby the smaller individuals had higher numbers of T-shaped spermatozoa, which most likely reflects a sampling error in the actual region of the epididymides sectioned; i.e. that the flush sample included a greater proportion of immature spermatozoa from the body of the epididymis, or that in the smaller individuals, the spermatozoa were on average more immature. There also may have been some degradation of samples after flushing, as seen as Mate and Rodger (1996) who stated that there is rarely any evidence of spermatozoa undergoing the reorientation of the spermatozoa head to T-shaped, and if it is seen after incubation it is likely due to a degenerative change. As the time increased from 12 hours to 36 hours post cull, the amount of T-shaped spermatozoa increased, this may be due to degradation. To minimise the chance of degradation it is important to assess the spermatozoa as soon as possible after flushing. This T-shaped percentage may be reduced to close to 0% if the spermatozoa are assessed immediately after death and ensuring that only the caudal epididymis is flushed.

One additional possibility for the number of T-shaped spermatozoa found in the sample from the caudal epididymis may have be due to the incubation imitating the temperature change in the female reproductive tract (from the isthmus to fertilisation site), during which the spermatozoa rotate to the T-shaped position before fertilisation. However, this seems unlikely due to other factors in the female reproductive tract interacting with the spermatozoa to causing the capacitation and head rotation (Bedford and Breed, 1994) that would not have been present in the experimental conditions. Capacitation has only been achieved in vitro in the brushtail possum, tammar wallaby and American marsupials (who have different capacitation environments). The recent research suggests the oviduct plays a
critical role in the capacitation in Australian marsupials, and the successful study on brushtail possums and tammar wallaby used oviduct epithelial cells (Bedford and Breed, 1994; Lin et al., 2002; Mate, 1996; Mate and Rodger, 1996; Mate et al., 2000).

2.4.2 Incubation

Motility was not seen immediately after flushing; however, after 10 min incubation motility was observed. These observations are consistent with the motility assessments by Boere et al. (2011) and Clulow et al. (1992), where no motility was observed initially, though motility was seen after the sperm samples were diluted and incubated for 15 min.

The methods of incubation times were altered between the summer and winter seasons in this study partly due to differences in ambient temperature. The spermatozoa assessed in winter experienced high rates of mortality when placed in already warmed 37°C EquiPro solution apparently due to a temperature shock. Modifications of the protocol to place the spermatozoa in room temperature (22°C) EquiPro and then to warm in the incubator for an extended period of time improved the survivorship and motility.

The optimum incubation time in this study was 30 min (group 2) and 15 minutes (in group 1). This length of incubation allowed the sample to warm to the appropriate temperature for the spermatozoa at a slow enough rate that there was minimal temperature shock causing death or very low motility. Previous studies had incubated their diluted sample for 15 min at 36°C (Boere et al., 2011), although we found that at 15 min, the motility was not at a maximum and still showing altered motility patterns (bent tails). These motility patterns were similar to what was seen in the eastern grey kangaroo in Holt et al. (1999) when they cooled the spermatozoa down to freeze the sample, though when re-warmed to about 35°C the straight line progressive motility re-established. Our study established that 15 min (in group 2) was an insufficient length of time to incubate. Correspondingly, the
study by Holt et al. (1999) may have underestimated the incubation time and spermatozoan motility. Clulow et al. (1992) investigated the motility of the tammar wallaby spermatozoa was collected from the rete testis, caput, mid-corpus and distal caudal epididymides and incubated the samples at 32°C for 10 hours, with assessments over the course of time. The spermatozoa collected from the caudal epididymis peaked motility at 3 hours incubation and remained at this peak until 6 hours where the motility significantly decreased to less than half after 10 hours. This is a much longer incubation time than this study examined, and also a lower incubation temperature, which will increase the time for motility to reach its optimum temperature. Kangaroos maintain their testes at 2 – 5°C below their body temperature, which equates to approximately 32 – 34°C, so the incubation temperature in Clulow et al. (1992) was likely to be more appropriate for marsupials.

We believe that this is the first time that a comparison has been made between times after cull to processing. Our results conclude that to optimise the motility and viability of the spermatozoa, the spermatozoa need to be flushed and processed within 12 hours, however we understand that this many not always be a possibility. A small sample size can easily be processed within this time frame, but a large sample size at once may not be possible to process within the 12 hours. The collection site may also be long distances from the processing laboratory, so future studies could investigate different storage options to maximise spermatozoa viability if a longer processing time is needed.

2.4.3 Diluent concentration

There were no significant differences between the three concentrations tested for velocity and total motility. Progressive motility, however, did show a significant difference, with the high concentration (~60 million spermatozoa/mL) having the highest motility. It was expected for the recommended concentration to have the highest motility as this was the
concentration that was used in numerous studies for the assessment of motility (Love et al., 2003; Varner, 2008). The high concentration was predicted to have too many spermatozoa in the sample and effect the movement of the spermatozoa. The low concentration was set at 15million/mL and according to the WHO laboratory manual; this concentration has too few spermatozoa to accurately assess the motility (WHO, 2010). Sperm concentration also depends on the nature of the assessment that is being made. Often high concentrations are used for assessments with the SCA machine, as the machine requires more spermatozoa per frame to assess the motility; however, Boschetto et al. (2011) used between 10-20 million/mL for assessment of velocity using the SCA.

Although the higher concentration (approximately 60million/mL) produced the highest motility, the recommended concentration is still the best option in my opinion. There may be a significant human error associated with the higher concentration, as observations of the large number of spermatozoa can sometimes be misleading, leading to a higher assessment of motility. This was the first study to our knowledge that compared three different concentrations, with the conclusions being inconclusive, as many of the experiments showed no significant difference between the concentrations.

2.4.4 Velocity

The two velocity methods (manual and machine specific) were not strictly comparable. The average measures (calculated by measuring distance divided by time) produced significantly slower velocities than the fastest velocity (calculated by the SCA computer program). Statistically, there was no correlation between the two methods (p= 0.762). These two methods represented different measures of velocity; the fastest sperm velocity is perhaps the most appropriate measure when considering sperm competition. The fastest spermatozoon in the ejaculate has a higher probability of achieving fertilisation compared with the slower spermatozoa, by reaching the site of fertilisation more quickly (Helfenstein
et al., 2010). The fastest velocity measure calculated by the SCA computer program is also more accurate and repeatable measure compared the manual calculation; however, both techniques were very dependent on the sperm incubation time. The SCA program interestingly took approximately 5 min for the spermatozoa to reach a maximum velocity due to the sample having to adapt to the slide they were placed in and the temperature on the warming slide. This observation has not previously been reported and may just be a feature of kangaroo spermatozoa.

The SCA program was much more accurate at measuring the velocity compared with the manual method. The manual velocity was calculated by estimating how far across the screen the spermatozoa travelled while timing it to calculate the velocity. The length and width of the video were known by using the calibration technique in the Motic program, which was used to take the videos. There is a large human error present in the manual calculations; in order to minimize these, all measurements and samples were undertaken by the same person.

This study only assessed the VSL (straight line velocity); however, many other studies using computer programs have also measured the VAP (average path velocity) and VCL (curvilinear velocity) (Boschetto et al., 2011; Del Olmo et al., 2013; Kleven et al., 2009). Lifjeld et al. (2012) only measured the VCL when investigating Passerine birds. We chose to only look at VSL, straight line velocity because the spermatozoa travelling forward are the progressive spermatozoa that are more likely to reach the fertilisation site.

In conclusion, this study provides an overview for the sperm analysis techniques and optimum processing methods for *Macropus fuliginosus*. To maximise motility and velocity a incubation time of 30 min (or potentially longer) with a recommended to high concentration should be used and spermatozoa processing occur as close to the cull time as possible, as the motility and velocity significantly decreased as time post cull increased. The
maximum velocity of spermatozoa gave the best measure of the sperm's ability to fertilise and was a better representative of sperm competition compared to average velocity, so it is recommended to use the SCA system for spermatozoa velocity assessment.
Chapter 3: Sperm competition between male western grey kangaroos (*Macropus fuliginosus*) of different body forms

3.1 Introduction

The significance of sexual selection to drive the biology of the different sexes within animal species was formally discussed by Darwin (1871). Sexually dimorphic appearance, often in extreme forms of colouration, plumage or body size are now well recognised and often cited examples of evolution driven by sexual selection (Cyrus Chu and Lee, 2012; McPherson and Chenoweth, 2012; Packer, 1983; Ralls and Mesnick, 2009). More recently, sexual selection has been linked to post copulatory sperm competition including factors such as increased testes size and spermatozoa with faster velocities (Fitzpatrick *et al*., 2012; Kelly, 2008; Malo *et al*., 2005a). Sexual selection on gross characteristics and sperm competition have, until relatively recently, been considered as separate entities; however, it is now recognised that there are significant relationships between these evolutionary mechanisms (Parker *et al*., 2013). Further, there is now evidence across a range of diverse animal groups of trade-offs between sexually selected traits and traits that represent sperm competition (Fitzpatrick *et al*., 2012; Gage *et al*., 1995; Galeotti *et al*., 2012; Kelly, 2008; Malo *et al*., 2005b; Parker, 1990b; Parker *et al*., 2013; Puniamoorthy *et al*., 2012; Simmons and Emlen, 2006; Warner *et al*., 1995). The ‘trade-off’ between sexually selected traits such as augmented weaponry and investment in the production spermatozoa is predicted to occur due to the limited energy budget of animals (Lupold *et al*., 2014).

Kangaroos exhibit hierarchical promiscuity, in which males of higher ranking have a disproportionately higher number of matings than the lower ranked subdominant males (Hynes *et al*., 2005; Miller *et al*., 2010). In order to achieve a high ranking, a male kangaroo must compete in physical combat with the higher ranked males to move his way up the
hierarchy (Croft and Snaith, 1990; Glanslosser, 1989; Miller et al., 2010). As such, male kangaroos invest heavily in secondary sexual characteristics, including a larger body mass and larger forelimb muscles compared with the female. The largest macropod, the red kangaroo, has the second largest sexual dimorphism of all mammals (Jarman, 1989, 1991). It has also been shown that males invest in differential muscle development (Warburton et al., 2013). These sexually selected traits aid in male-male competition by emphasis on muscles that function in grasping and pulling (Warburton et al., 2013) as well as (potentially) increasing the male attractiveness to the female (female choice) (Birkhead and Møller, 1998).

As female kangaroos are mostly polygamous, sperm competition is also likely to occur (Johnson, 1983). Studies on the tammar wallaby determined that the dominant male will secure the initial copulation, but this does not necessarily secure the parentage, and this is due to subdominant males mating and promoting sperm competition (Hynes et al., 2005). Taggart et al. (1998) and Rose et al. (1997) indicated there is a relationship between total testes mass and total body mass in marsupials and monotremes, along with epididymis mass and spermatozoa numbers in the epididymis. This indicates there is investment in sperm competition traits.

This chapter investigates sperm competition and sexual selection in *Macropus fuliginosus* to determine whether there is a trade-off in the investment between the traits of male size and musculature against investment in traits that may result in more competitive sperm. Adult male kangaroo cadavers were used to assess the relationship between body size, forelimb musculature and spermatozoa traits. Forelimb muscle masses were used to calculate an index of musculature, which is an indication of the level of muscle investment against the total body mass. This study investigated the genitalia and spermatozoa traits that were determined to be important measures from the literature including total testes
mass, epididymis mass, relative testes mass (RTM), motility (total and progressive), spermatozoa lengths (head, midpiece, tail and total length), spermatozoa velocity, the percent of morphologically normal spermatozoa and the percent of linear compared with T-shaped heads.

I tested the hypothesis that less muscular (subdominant) male kangaroos will invest more into spermatozoa traits compared with the dominant males who invest more heavily in muscle development and body mass.

### 3.2 Methods

#### 3.2.1 Animal collection

Male *Macropus fuliginosus* of varying body sizes were sourced from professional kangaroo shooters between March 2014 and August 2014 as part of commercial harvest. Kangaroo 10, 19 and 32 (Table 2.1) were removed from the analysis due to small size, apparent lack of sexual maturity and little to no spermatozoa present in the epididymis. Scrotums (intact with testes) were removed from the carcass within an hour of death and cooled to 4°C and stored in a Styrofoam box to maintain refrigeration. The complete left forelimb with skin and muscles intact was removed from the body by making a large circular cut through the skin around the scapla and transaction of the extrinsic muscles. The right femur was also removed. All specimens were transported back to Murdoch University for analysis. Testes were analysed the following day (see chapter 2). Forelimbs were either refrigerated and dissected fresh (within 3 days of death) or stored frozen, prior to being defrosted for dissection.
3.2.2 Spermatozoa recovery

Testes were removed via a long incision in the scrotum and weighed individually with and without the epididymis attached. The epididymis was cleaned of connective tissue and blood vessels so it could be cut such that only the vas deferens and caudal epididymis remained. Using a syringe loaded with 5mL of room temperature EquiPro (Equine Semen Extender), a blunt 25G needle was inserted into the vas deferens (secured with tweezers) aided by a dissecting microscope. The epididymis was then flushed by slowly pulsing the EquiPro through the epididymis. When necessary, extra cuts using scissors were made to the cauda epididymis to allow the fluid to flow out with ease and be collected in a 10mL plastic tube. Due to the small size and tight compaction of the epididymis, not all the spermatozoa present were flushed. See chapter 2 for a description of the experimental methods trialed to determine the best spermatozoa analysis methods.

3.2.3 Muscle dissection

Forelimb specimens were skinned and excess connective tissue and muscle were removed immediately before the dissection to avoid desiccation. Twelve muscles were isolated, removed from the limb and weighed immediately after removal on a digital balance (±0.01g): m. deltoideus, m. supraspinatus, m. infraspinatus, m. teres minor, m. teres major, m. subscapularis, m. coracobrachialis, m. biceps brachii, m. brachialis, mm. triceps group. The antebrachium muscles were grouped into a dorsal division (extensors) and ventral division (flexors) following methods of Warburton et al. (2013). For statistical analyses, the mass of m. teres minor was combined with that of m. infraspinatus, while m. coracobrachialis was removed from the data set due to the high variability in mass of these very small muscles.
3.2.4 Statistical Analyses

Statistical analysis was performed using Statistica 7.1 for Windows and Excel 2007 for Windows; an alpha level of $p = 0.05$ was used to determine significance. All variables were assessed for normality, and variables were transformed if required. Relative testes mass (RTM, with and without epididymis) and relative epididymis mass (REM) were transformed using the Box Cox transformation (Statistica); muscle masses and body mass were log-transformed.

The index of muscularity (IM) was a representation of the male’s proportional investment in forelimb muscle mass against total body mass. The relationship between the mass of each of the individual muscle masses against total body mass was estimated using a reduced major axis (RMA) regression. The residuals were expressed as a percentage of the muscle mass to standardise across the different muscle groups. The percentage residuals were entered into a factor analysis (varimax normalised rotation) along with REM, RTM, spermatozoa motility, velocity and length (head, mid-piece and tail lengths separately). Body mass was also included to determine if there were additional size-dependent measures. Relationships between the index of muscularity and sperm competition measures were graphed and a significant relationship tested for using linear regression.

3.3 Results

3.3.1 Isometric relationships with body mass

Log total testes + epididymis mass ($R^2 = 0.84$, $p < 0.001$; Figure 3.1) showed a positive isometric relationship with log total body mass. Log total testes mass ($R^2 = 0.83$, $p < 0.001$)
and log epididymis mass ($R^2 = 0.77$, $p < 0.001$) also showed positive isometric relationships with log body mass.

![Graph showing log total testes + epididymis mass vs log total body mass.](image)

**Figure 3.1:** Line of best fit to calculate the average log testes and epididymis size for log body size for male *M. fuliginosus* ($n=32$). The line of best fit was calculated using Statistica least square regression $\log \text{Total Testes + Epididymis Mass} = 0.3061 + 0.9698 \times (\log \text{Total Body Mass})$.

Table 3.1 summaries the isometric relationships between the individual muscle masses of the forelimb and log body mass. The teres major and biceps crachii muscles had the largest positive slope with body mass.

**Table 3.1:** Summary of relationship between individual forelimb log muscle masses and log total body mass. Muscles are ordered in descending order of the value of the slope of the regression between the muscle mass and the body mass.

<table>
<thead>
<tr>
<th>Muscle</th>
<th>$R^2$</th>
<th>$p$-value</th>
<th>Equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log M. teres major</td>
<td>0.95</td>
<td>$&lt; 0.001$</td>
<td>$y = -2.2024 + 2.2497 \times x$</td>
</tr>
<tr>
<td>Log M. biceps brachii</td>
<td>0.96</td>
<td>$&lt; 0.001$</td>
<td>$y = -1.9366 + 2.2402 \times x$</td>
</tr>
<tr>
<td>Log Flexor (medial)</td>
<td>0.97</td>
<td>$&lt; 0.001$</td>
<td>$y = -1.6444 + 2.2276 \times x$</td>
</tr>
<tr>
<td>Log M. subscapularis</td>
<td>0.94</td>
<td>$&lt; 0.001$</td>
<td>$y = -1.8326 + 2.21 \times x$</td>
</tr>
<tr>
<td>Log M. intraspinatus &amp; M. teres minor</td>
<td>0.95</td>
<td>$&lt; 0.001$</td>
<td>$y = -1.8165 + 2.1084 \times x$</td>
</tr>
<tr>
<td>Log M. deltoideus</td>
<td>0.95</td>
<td>$&lt; 0.001$</td>
<td>$y = -1.7868 + 2.0817 \times x$</td>
</tr>
<tr>
<td>Log Extensor (lateral)</td>
<td>0.95</td>
<td>$&lt; 0.001$</td>
<td>$y = -1.6153 + 1.9879 \times x$</td>
</tr>
<tr>
<td>Log M. Supraspinatus</td>
<td>0.94</td>
<td>$&lt; 0.001$</td>
<td>$y = -1.679 + 1.967 \times x$</td>
</tr>
<tr>
<td>Log M. brachialis</td>
<td>0.92</td>
<td>$&lt; 0.001$</td>
<td>$y = -1.7158 + 1.9533 \times x$</td>
</tr>
<tr>
<td>Log Mm. triceps group</td>
<td>0.94</td>
<td>$&lt; 0.001$</td>
<td>$y = -0.7778 + 1.7773 \times x$</td>
</tr>
</tbody>
</table>
3.3.2 Muscularity vs sperm competition traits

Sixteen of the sperm competition and muscle investment variables could be summarised into a factor analysis (Table 3.2). The first factor (FA1 forelimb muscularity) was determined by those variables indentified to represent the male’s investment in forelimb muscles mass (besides m. brachialis) and no sperm traits. The second factor (FA2 RTM and REM) was determined by the amount of spermatozoa-producing tissue each animal invested in, as well as the size of the epididymis which is directly related to the amount of spermatozoa that can be stored. This factor is clearly a reflection in the amount of spermatozoa-producing and storing tissue the male invests in. Factor 3 (FA3 Sperm motility) was determined only by the total and progressive motility which is a representation of the males spermatozoa viability and spermatozoa movement. Lastly, factor 4 (FA4 Sperm velocity, head and tail length) was determined by the sperm velocity as well as the head and tail lengths which are all direct measures of the males sperm competition competiveness. Figure 3.2- 3.4 show the significant relationships that were indicated by the factorial analysis.

Figure 3.2 shows factor 1 (Muscularity) against factor 2 (REM & RTM). The grey lines on the figures represent the strength of the relationships, with males with large REM and RTM negative on the y-axis, and males investing highly in muscle mass positive on the x-axis. There is no relationship between factor 1 and factor 2 with the entire group of kangaroos having a spread in all 4 quadrants for all the six populations.
Table 3.2: Summary of the factorial analysis. Factor 1- Muscularity, Factor 2- RTM & REM, Factor 3- Spermatozoa Motility and Factor 4- Spermatozoa velocity, Head and Tail length. Factor loadings greater than 75% of the largest loading were considered to contribute significantly to the PC factor (Mardia et al., 1979).

<table>
<thead>
<tr>
<th></th>
<th>Factor 1</th>
<th>Factor 2</th>
<th>Factor 3</th>
<th>Factor 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log Total Body Mass</td>
<td>-0.2</td>
<td>0.0</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>BoxCox RTM (without Epi)</td>
<td>-0.1</td>
<td>-0.9</td>
<td>0.1</td>
<td>0.0</td>
</tr>
<tr>
<td>BoxCox REM (without Testes)</td>
<td>0.0</td>
<td>-0.7</td>
<td>-0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>Total Motility</td>
<td>-0.1</td>
<td>0.0</td>
<td>-0.8</td>
<td>0.4</td>
</tr>
<tr>
<td>Progressive Motility</td>
<td>-0.3</td>
<td>0.1</td>
<td>-0.8</td>
<td>0.0</td>
</tr>
<tr>
<td>Average Velocity</td>
<td>-0.1</td>
<td>0.0</td>
<td>-0.2</td>
<td>0.9</td>
</tr>
<tr>
<td>Average Head Length</td>
<td>0.0</td>
<td>0.4</td>
<td>0.1</td>
<td>-0.7</td>
</tr>
<tr>
<td>Average Midpiece Length</td>
<td>-0.2</td>
<td>0.1</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Average Tail Length</td>
<td>0.0</td>
<td>0.5</td>
<td>0.1</td>
<td>-0.7</td>
</tr>
<tr>
<td>Proportion normal (vs. T-shaped)</td>
<td>0.0</td>
<td>-0.5</td>
<td>0.2</td>
<td>0.5</td>
</tr>
<tr>
<td>% Residual Log M. deltoideus</td>
<td>0.9</td>
<td>0.2</td>
<td>0.1</td>
<td>0.0</td>
</tr>
<tr>
<td>% Residual Log M. supraspinatus</td>
<td>0.9</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>% Residual Log M. intraspinatus &amp; M. teres minor</td>
<td>1.0</td>
<td>0.0</td>
<td>0.0</td>
<td>-0.1</td>
</tr>
<tr>
<td>% Residual Log M. teres major</td>
<td>0.9</td>
<td>0.2</td>
<td>0.2</td>
<td>0.0</td>
</tr>
<tr>
<td>% Residual Log M. subscapularis</td>
<td>1.0</td>
<td>0.0</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>% Residual Log M. biceps brachii</td>
<td>0.9</td>
<td>0.1</td>
<td>0.1</td>
<td>-0.1</td>
</tr>
<tr>
<td>% Residual Log M. brachialis</td>
<td>0.7</td>
<td>-0.1</td>
<td>0.0</td>
<td>-0.3</td>
</tr>
<tr>
<td>% Residual Log Mm. triceps group</td>
<td>0.9</td>
<td>-0.1</td>
<td>0.0</td>
<td>-0.1</td>
</tr>
<tr>
<td>% Residual Log Extensor (lateral)</td>
<td>0.9</td>
<td>-0.1</td>
<td>0.0</td>
<td>0.1</td>
</tr>
<tr>
<td>% Residual Log Flexor (medial)</td>
<td>0.9</td>
<td>0.1</td>
<td>0.1</td>
<td>-0.1</td>
</tr>
<tr>
<td>Expl.Var</td>
<td>8.2</td>
<td>2.1</td>
<td>1.7</td>
<td>2.5</td>
</tr>
<tr>
<td>Prp.Totl</td>
<td>0.4</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
</tbody>
</table>
Figure 3.2: Factor 1, Muscularity compared to factor 2, REM & RTM. Below the x-axis indicated a large RTM and REM while right of the y-axis indicates the kangaroo had a large investment in muscle mass. The six different kangaroo populations are indicated with the different icons.

Figure 3.3 shows the relationship and spread of data between factor 1 (Muscularity) and factor 3 (Spermatozoa motility). There was no significant relationship between the two factors but there was an even spread across the six populations of males that invested heavily in forelimb muscle mass and males that invested less in muscle mass and high and low spermatozoa motility.
Figure 3.3: Relationship between Factor 1, Muscularity and Factor 3, Spermatozoa motility. Below the x-axis indicates the kangaroo had high progressive and total motility, while right of the y-axis that the kangaroos had invested highly in forelimb muscle mass. The six different populations are indicated with the different symbols.

Figure 3.4 shows the non-significant relationship and spread of data between factor 1 (Muscularity) and factor 4 (Spermatozoa velocity, head and tail length). Negative on the y-axis indicates a longer spermatozoa head and tail with slower velocity, while positive indicates smaller but faster spermatozoa.
Figure 3.4: Relationship between Factor 1, Muscularity and Factor 4, Sperm velocity, head and tail length. Below the x-axis indicated a larger sperm head and length, while above the x-axis indicates a higher velocity. To the right of the y-axis indicates that the kangaroos are investing more in forelimb muscle mass. The six different kangaroo populations are indicated with the different symbols.

Positive relationships indicated by associations between variables of the factor analysis are represented in Figure 3.5. RTM without epididymis and REM were significantly positively correlated so the relative size of the testes increased, the relative size of the epididymis also increased. Total motility and progressive motility were also positively correlated so that as the total motility of the sample increased, the progressive motility also increased. Average sperm velocity was inversely correlated with the average sperm head length and tail length. This relationship indicates that the smaller the spermatozoon, the faster the swimming velocity.
Figure 3.5: Significant relationships from the factorial analysis. (A) Positive relationship between RTM and REM, REM = -3.248 + 0.2225*x. (B) Positive relationship between total motility and progressive motility, Progressive motility = 25.7838 + 0.627*x. (C) inverse relationship between spermatozoa velocity and average spermatozoa head length, head length = 7.3378 - 0.01796*x. (D) inverse relationship between spermatozoa velocity and average spermatozoa tail length, tail length = 101.4263 - 0.0576*x.

Of the sperm competition traits measured, none showed a significant relationship with the calculated index of muscularity (Table 3.3). The sperm measures that showed the strongest trends were progressive motility (p= 0.065) and the average midpiece length (p= 0.170). Progressive motility showed a trend for an inverse relationship with the index of muscularity, such that, more muscular males had lower measured values of progressive motility.
Table 3.3: Summary of p-values and linear regression equations. Line of best fit calculated using Statistica least square regression for each sperm competition trait against the index of muscularity.

<table>
<thead>
<tr>
<th>Trait</th>
<th>R²</th>
<th>P value</th>
<th>Equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scrotum Mass (g)</td>
<td>0.01</td>
<td>0.698</td>
<td>y = 157.2181 - 5.1966*x</td>
</tr>
<tr>
<td>Total Testes Mass (g)</td>
<td>0.04</td>
<td>0.250</td>
<td>y = 70.0286 - 5.7359*x</td>
</tr>
<tr>
<td>Log Total Testes Mass</td>
<td>0.07</td>
<td>0.148</td>
<td>y = 1.8087 - 0.0495*x</td>
</tr>
<tr>
<td>Total Testes + epi Mass (g)</td>
<td>0.03</td>
<td>0.331</td>
<td>y = 89.9822 - 6.4858*x</td>
</tr>
<tr>
<td>Log Total Testes Mass + Epi</td>
<td>0.06</td>
<td>0.188</td>
<td>y = 1.9145 - 0.0471*x</td>
</tr>
<tr>
<td>Epididymis mass</td>
<td>0.01</td>
<td>0.686</td>
<td>y = 19.9536 - 0.75*x</td>
</tr>
<tr>
<td>Log Epididymis Mass</td>
<td>0.03</td>
<td>0.345</td>
<td>y = 1.2403 - 0.0417*x</td>
</tr>
<tr>
<td>RTM with epi</td>
<td>0.01</td>
<td>0.678</td>
<td>y = -5.053 - 0.0855*x</td>
</tr>
<tr>
<td>RTM without epi</td>
<td>0.01</td>
<td>0.656</td>
<td>y = -5.7248 - 0.0938*x</td>
</tr>
<tr>
<td>REM</td>
<td>0</td>
<td>0.924</td>
<td>y = -4.522 + 0.0067*x</td>
</tr>
<tr>
<td>Total Motility</td>
<td>0.01</td>
<td>0.674</td>
<td>y = 62.5 - 1.3602*x</td>
</tr>
<tr>
<td>Progressive Motility</td>
<td>0.11</td>
<td>0.065</td>
<td>y = 64.6875 - 6.7198*x</td>
</tr>
<tr>
<td>Average Velocity</td>
<td>0.02</td>
<td>0.473</td>
<td>y = 48.7416 - 3.0492*x</td>
</tr>
<tr>
<td>Average Head Length</td>
<td>0</td>
<td>0.894</td>
<td>y = 6.3633 - 0.0159*x</td>
</tr>
<tr>
<td>Average Midpiece Length</td>
<td>0.06</td>
<td>0.170</td>
<td>y = 10.9329 - 0.2143*x</td>
</tr>
<tr>
<td>Average Tail+Mid Length</td>
<td>0.01</td>
<td>0.548</td>
<td>y = 109.5372 - 0.3192*x</td>
</tr>
<tr>
<td>Average Tail Length</td>
<td>0</td>
<td>0.851</td>
<td>y = 98.6044 - 0.1048*x</td>
</tr>
<tr>
<td>Average Total Length</td>
<td>0.01</td>
<td>0.588</td>
<td>y = 115.9005 - 0.3351*x</td>
</tr>
<tr>
<td>Proportion normal (vs T-shaped)</td>
<td>0</td>
<td>0.846</td>
<td>y = 85.4531 - 0.2835*x</td>
</tr>
<tr>
<td>Morphologically normal (vs defect)</td>
<td>0.04</td>
<td>0.283</td>
<td>y = 83.0625 - 1.1542*x</td>
</tr>
</tbody>
</table>

3.3.3 Sperm characteristics

Average sperm velocity was also correlated with total motility, average midpiece and tail length, and the average total sperm length. Average combined midpiece and tail length ($R^2=0.15$, p= 0.026), and average total sperm length ($R^2=0.22$, p= 0.006) as seen in Figure 3.6 were inversely correlated with the average velocity. Total motility showed a relationship that as the velocity of the spermatozoa increased, the total motility of the sample also increased, however the other two length measures, midpiece+tail and total length showed a negative relationship, with an increase in velocity as sperm size decreased.
3.6: Linear regression to test for the presence of sperm competition in 33 *M. fuliginosus*’s. There was a strong relationship between the average velocity with total motility (A), average midpiece+tail (B), and (C) average total spermatozoa length. (B & C) showed a negative relationship with the average velocity, while (A) had a positive correlation.

3.4 Discussion

I found no correlation between the degree of forelimb musculature and any of the measured sperm competition traits. This may suggest that the investment in forelimb muscle mass and sperm traits are independent in *Macropus fuliginosus*. I discuss these findings in the light of other studies that have compared weaponry traits (sexually selected traits) with sperm competition traits.
3.4.1 Isometric relationship between testes mass and body mass

I found a positive isometric relationship between body mass and testes physical measures (scrotum mass, total testes mass and epididymis mass). Similar patterns of body size/testes size were found in the literature or mammals and frogs (Breed and Jason, 2000; Byrne et al., 2002; Rose et al., 1997; Taggart et al., 1998).

These physical measures that are correlated with body mass (testes and epididymis mass) and also body size have been shown to be linked to testosterone (Ligon et al., 1990; McGlothlin et al., 2008; Miller et al., 2010). Miller et al. (2010) determined that in eastern grey kangaroos the dominant male was significantly larger in body mass than the other males, as well as having significantly more circulating testosterone, which may have influenced the animal’s body size/mass. However, this relationship with testosterone was not reported in tammar wallabies (Hynes et al., 2005) where a high testosterone concentration did not ensure dominance or the control of sexual activity.

3.4.2 Sperm characteristics

There was a negative association between sperm length and the average spermatozoa velocity. This relationship suggests that smaller spermatozoa swim faster, with head, midpiece+tail, tail and total length all negatively correlated with the average velocity. The relationship between sperm morphology and sperm velocity is not well understood generally, especially in marsupials. In contrast to my findings, in other mammal species, there is a mostly positive correlation between sperm head length and sperm velocity.

Gómez Montoto et al. (2011b) identified the head shape as the driving force towards faster swimming spermatozoa affecting sperm performance in sperm competition. In fish a strong negative relationship between sperm competition and sperm length has been identified and this is believed to be due to the shorter spermatozoa having a longer survival (longevity...
which is advantageous for external fertilisers) (Stockley et al., 1997). Longevity was not assessed in this study; however, if the same occurs in kangaroo spermatozoa, the smaller and faster swimming spermatozoa would have more of a chance to travel long distance and survive the storage in the female reproductive tract to fertilise the oocyte. The female tammar wallaby ovulates several hours after mating (Rudd, 1994; Taggart et al., 1998; Taggart and Temple-Smith, 1991; Tyndale-Biscoe and Rodger, 1978), and spermatozoa stores can be found within 6 hours of mating in the cervices ready for ovulation to occur (Tyndale-Biscoe and Rodger, 1978). Longevity of the spermatozoa therefore may be important as they need to maintain fertilisation capacity for an extended period of time waiting for the oocyte. There is now evidence that the caudal isthmus oviduct acts as a sperm storage in marsupials and eutherians and the spermatozoa are stored in specialised crypts or attach to the oviduct epithelial or become trapped in the secreted mucus (Bedford and Breed, 1994; Taggart and Temple-Smith, 1991).

In contrast to the hypothesis that larger spermatozoa can swim faster is the ‘fixed resource budget’, where the smaller spermatozoa may allow the males to produce more gametes, which would aid in sperm competition (Parker, 1982). Unfortunately a total sperm count was not able to be determined for all kangaroos due to the difficulty of flushing the spermatozoa through the relatively small epididymides. The female’s unusual reproductive tract may also influence sperm form and function; such that the spermatozoa will travel to the uterus that has not recently given birth (there are two uteri). As we saw this correlation between the sperm lengths and sperm velocity, these measures are possibly the best representations of sperm competition in Macropus fuliginosus.

3.4.3 Sperm competition and sexual selection

My results revealed no significant associations between sperm competitive traits and other traits under presumed sexual selection in Macropus fuliginosus, but rather that sperm traits
vary independently of muscularity; i.e., there was no significant trade-off between the investment in muscle for sexual selection and reproductive traits for sperm competition. Progressive motility had the strongest trend against the index of muscularity (p= 0.065) suggesting an inverse relationship between progressive motility and muscularity. All the other spermatozoa measures were not significant (p>0.148). Thus, rather than there being evidence of a trade-off in investment between muscularity or sperm traits, selection for these traits appears to be independently selected for or are not important for sperm competition in this species. More data/test work may show a significant inverse relationship but at present no relationship can be shown to relate sperm traits to the index of muscularity.

Selection for forelimb muscle investment has previously been reported in *Macropus fuliginosus* Warburton et al. (2013) and then confirmed again in this study. This indicates that male *M. fuliginosus* are sexually selecting for forelimb muscles that aid in male-male competition with emphasis on the muscles that function to grasp and pull. All the muscles besides m. brachialis were involved in the factor analysis (this also had the lowest correlation with body mass), and this muscle may not be involved due to its use in forelimb movement. The m. brachialis is only involved in flexion of the elbow (Harvey and Warburton, 2010), which indicates there is not a high level of sexual selection for this muscle compared to the other muscles including the teres major, which is involved in adduction and retraction of the humerus and the biceps brachii which are used in flexion of the elbow and other rotations (Harvey and Warburton, 2010). These two muscles were also the two highest muscles sexually selected for in Warburton et al. (2013) having the most positive slope against body mass for the male kangaroos indicating they are sexually selected to aid in male-male competition.
The investment in the amount of sperm-producing tissue will determine the efficiency of spermatozoa production (Peirce and Breed, 2001), and therefore has been predicted to influence success in sperm competition. Measures of sperm production, such as testes mass was the closest to significant sperm competition physical measure with the index of muscul arity and indicated that as the index of muscul arity increased, the total testis mass decreased (p= 0.148). This supports the hypothesis that the males that invest most in muscle mass have less energy remaining to invest in sperm competition traits; however the relationship was not significant and testis mass was highly correlated to body mass as discussed above. The relationship with the index of muscul arity had a higher level of scatter compared to body mass, indicating that body mass was a more appropriate predictor of testes size, and that testes mass increases as total body mass increases.

Testes mass has been correlated to sexual selected traits in other species. Fitzpatrick et al. (2012) found a negative relationship between testes mass and pre-copulatory weaponry in pinnipeds, which was interpreted as a trade-off between pre- and post-copulatory sexually selected traits in line with the predictions of Parker et al. (2013). The benefits of producing more spermatozoa when facing sperm competition is almost universal in most species: the animals facing the higher level of sperm competition have larger testes. This has been seen in invertebrates and vertebrates (Gage, 1994; Jennions and Passmore, 1993; Kenagy and Trombulak, 1986; Møller, 1991; Stockley et al., 1997) review see (Parker et al., 1997).

Epididymal mass was also not correlated to the index of muscul arity. Epididymal mass is a relative measure of sperm storage (Jones, 1999), as the larger the epididymis, the more spermatozoa that can potentially be stored to ensure the male is not depleted of spermatozoa while mating with multiple females. A larger epididymis may also lead to higher numbers of spermatozoa ejaculated, which significantly increases the animal’s sperm competition traits. This has been previously reported in primates and monotremes.
(Bercovitch and Rodriguez, 1993; Jones et al., 2007); however, epididymal mass has not previously been compared to the investment in sexually selected for traits in other mammals (antler size or body mass dimorphism).

Relative testis mass is a commonly used as an indirect measure for sperm competition (Byrne et al., 2002; Lüpold et al., 2009; Møller, 1991; Pitcher et al., 2005). Relative testis mass has previously been correlated with a measure of another sexually selected for trait, with the red deer with larger antlers also having the largest relative testes mass (Malo et al., 2005b). This suggests that the more dominant males with the larger antlers are also investing in relative testes mass. Relative testes mass with or without the epididymis was not significantly correlated with my index, which suggests that there is no trade-off between the sexually selected muscle mass and sperm competition traits in *M. fuliginosus* because RTM is such a universal index of sperm competition. I did, however, find a correlation between RTM and REM (relative epididymal mass) from the factor analysis. These measures have not been correlated before to my knowledge; however, epididymis mass and testes mass are positively correlated in rhesus macaques (Bercovitch and Rodriguez, 1993), showing the same relationship as the REM and RTM.

None of the sperm motility traits were significantly correlated with the index of muscularity; however, progressive motility was the closest to being significant and showed a negative relationship with the index of muscularity and was also positively correlated with total motility. Traits of sperm motility have not previously been compared with sexual selection in any species, while spermatozoa velocity has only been researched in a few studies. One of the studies found that fish sperm velocity increased in sneaker males (Smith and Ryan, 2011) while a study on red deer indicated that sperm velocity was positively correlated with the sexually selected for trait (Malo et al., 2005b). In our study spermatozoa length was also not correlated to the index of muscularity.
The velocity and vigour of the spermatozoa will determine the ability to overcome barriers in the female reproductive tract as well as penetrate the oocyte(s) to fertilise. Velocity will also then gauge the competitiveness of the spermatozoa against rival spermatozoa in the race to fertilise the oocyte(s) (Gomendio et al., 2007; Gomendio and Roldan, 2008). There may be no difference between the dominant and subdominant males in sperm velocity, although the dominant males may have some behavioural tactics that they employ to fertilise the ovum regardless of sperm traits. Dominant male Tammar wallabies will often follow the oestrus female and mate with her as soon as she becomes receptive (Rudd, 1994). The male will often guard the female and use the copulatory plug to prevent the subdominant males from mating with the female (Taggart et al., 1998; Tyndale-Biscoe and Rodger, 1978; Tyndale-Biscoe and Renfree, 1987). These are behavioural traits that the dominant males may also use to ensure their paternity of the offspring, and avoid facing sperm competition.

This study compared multiple representative measures of sperm competition from the literature, unlike previous studies who only compared one or two measures with sexually selected trait. My index of muscularity was calculated to represent the investment in muscle mass. It included all investments in the individual muscles, and ensured that the masses of all muscles were included, i.e. the index was not just driven by the large muscles of the triceps but the smaller muscles also effected the index. No previous study has used the amount of muscle data before as a measure of sexual selection; the previous studies have often used measures such as body mass, antler length or the level of sexual dimorphism. Most of these studies have correctly accounted for body mass, as was done in our index of muscularity, however one study did not correct for body mass, they simply separated the males into large investment and small investment by the length of the head piece (Kelly, 2008). Although beetles between these two categories (more than 19mm, but less than 23mm head length) were not included in the study, it is difficult to separate
animals into two distinct groups; this method also did not correct for body mass. The other studies that have compared sexually selected traits against sperm competition traits have either used a phylogenetically controlled regression (Fitzpatrick et al., 2012) or simply corrected for body size and therefore age (Malo et al., 2005b).

This study thoroughly covered all bases of sperm competition. Results from this study may suggest that sperm competition could be occurring due to the sperm length and velocity being highly correlated, but I can confidently conclude from the data collected that there is no trade-off and that in fact the traits of sperm competition and forelimb muscle mass vary independently.
Chapter 4: General Discussion

4.1 Overview of the study

This was the first study to investigate sperm competition in conjunction with sexual selection in *Macropus fuliginosus* or in any other marsupial. Research combining sperm competition and sexual selection in any species is limited, along with research in sperm competition in marsupials, so there is no agreed method to tackle these problems. This study covered an extensive amount of the measures of sperm competition, which has not previously been done in the one species. Often only one or two measures of sperm competition are compared with a sexually selected trait, while we compared at least six measures.

The sperm traits that were assessed were those found to be the most appropriate measures of sperm competition throughout the literature. The sperm traits measured have previously been seen to have a relationship with sperm competition, and some have been correlated with sexually selected traits in some animals (Malo *et al.*, 2005b). In our study these traits that were anticipated to be correlated showed a large scatter and little association (non significant) indicating that muscle mass investment and sperm competition traits are invested in independently of each other. Even though there was no association I believe some of the sperm assessment methods slightly affected the results due to the presence of human error, in measuring the sperm lengths on the program Motic, and human bias in assessing the total and progressive motility rates. To minimise these affects, all these measures were performed by the same person in as similar conditions as possible, but these errors could be significantly reduced in the future by using automated sperm assessing computer programs. Another factor that may have affected the sperm motility measures was that spermatozoa were only assessed within 12 hours after cull. A significant
decrease in sperm motility may have occurred within those 12 hours, especially as we saw a significant decrease from 12 hours to 36 hours.

4.1.1 Sperm preparation methods

The optimal spermatozoa assessing methods were determined for *Macropus fuliginosus* when assessing for sperm competition and then compared with sexually selected traits. The 33 kangaroos assessed produced the highest motility and velocity when incubated for the longest incubation time (15 min in group 1 and 30 min in group 2), in preferably the recommended or high sperm concentration within 12 hours of cull to maximise sperm viability. There was a significant decrease in motility between 12 and 36 hours, therefore to maximise motility, assessment should be done as soon as possible after the time of cull.

4.1.2 Sperm characteristics

The relationship between the index of muscularity and all sperm measures was found to be insignificant and poorly correlated, but the spermatozoa lengths were significantly correlated with the average sperm velocity. Sperm lengths were inversely proportional to the velocity indicating that the smaller the sperm, the faster the velocity. This relationship indicates that there is a significant relationship between the velocity and lengths of the spermatozoa in the *M. fuliginosus* mating system and that the male with the fastest spermatozoa have smaller spermatozoa and may be producing more spermatozoa according to the ‘fixed resource’ budget theory (Parker, 1982). The kangaroo can invest the energy in producing many smaller sperm, compared with fewer larger sperm; therefore different males can invest their energy differently.
4.1.3 Sperm competition and sexual selection

There were no sperm competition traits that were correlated to the index of muscularity in this study, which indicates that the *Macropus fuliginosus* male does not have a trade-off between the investment in forelimb muscle mass and sperm competition traits such as testes mass, sperm length and sperm velocity. Therefore these two components of sexual selection, muscle investment and sperm competition traits seem to be independent of each other. Sperm motility measures have not previously been correlated to sexual selection in any species besides the swordtail where they correlated faster sperm velocity with sneaker males (Smith and Ryan, 2011). The physical measures of sperm competition, total testes mass and epididymis mass were not correlated with the index of muscularity; however, they were strongly correlated with the body mass. These results indicate that testes mass and epididymal mass was driven by body mass, rather than the risk of sperm competition or because of a trade-off between muscle investments.

Some of the short comings of this study were mostly due to the relative small number of animals, resulting in a small range of index of muscularities and the simplistic sperm assessing methods. The majority of the animals were of average muscularity with far fewer animals having a very large investment or very small investment in muscle mass. This may have been a good representation of the population due to limited dominant and small males in a mob. Along with the slightly limiting data set, we also had a small number of animals compared with other studies in similar fields but in different species. The number of animals we had, 35, is a high number for a native species, however since most of the research of pre- and post-copulatory sexual selection has been in insects, their sample sizes are higher and therefore are able to produce more accurate results. The last factor affecting the research was the assessments on spermatozoa, which are always difficult due
to numerous factors. Due to logistical limitations involving sample preparation and remote collection sites, the time from cull to spermatozoa assessment was either 12 hours or 36 hours. There was significant deterioration from 12 hours to 36 hours seen in the sperm measures. I suspect that a decrease would also have been seen between time of cull and 12 hours later due to the spermatozoa sensitivity to temperature, environmental effects and the effects of handling.

As such a large sample size is required to effectively detect a significant relationship accurately between sexual selection and sperm competition; the alternative would be to study a captive population similar to Miller *et al.* (2010) or Hynes *et al.* (2005). The captive environment would allow management of the population and observational studies to aid in the research. The mating system of the *Macropus fuliginosus* would be useful in providing valuable information on the male and female behaviour of mating and parentage. A comparison of results from the individuals collected in the field and those from a semi-captive population would help to ascertain if there is a relationship between sperm competition and sexual selection (muscle and body mass investment) and confirm the validity of the methods used in this study. Obviously, different measures of sexual selection would be needed (collection of forelimb muscles cannot be done); however, similar measures such as bicep circumference and forelimb length could be indicators of muscle and forelimb development.

### 4.2 Conclusions, management implications and future direction

Sexual selection and sperm competition in kangaroos are difficult to research due to the limited access to animals and the limited understanding and background into the interaction between these two driving forces of sexual selection (Lupold *et al.*, 2014). The dominant male in the population will both try to monopolise the females in the mob, and therefore try and stop the other males mating with the females, as well as invest in his
large body and muscle mass so that he can maintain this dominant position (able to fight off the opposing males) (Birkhead and Møller, 1998). This is where the subdominant males have the options to invest in muscle and body mass and try to compete with the dominant male for the optimum position, or they can heavily invest in spermatozoa and try and secure some sneaky matings ensuring the female is still on heat. Our findings suggest that the males do not have a trade-off between sexual selection and sperm competition they are independent and that relative testes mass was driven by body mass, rather than sperm competition.

This research has not previously been investigated, especially in marsupials and as extensively as in this study. There is very limited research on M. fuliginosus as most current research has been conducted on tammar wallabies and eastern grey kangaroos. This research will aid in furthering understanding of sexual selection M. fuliginosus. This research has followed on from that by Warburton et al. (2013). This research may also further aid in cryopreservation for marsupial semen. The smaller and shorter spermatozoa were seen to have a higher velocity and motility in our research so this may lead to higher success rates after the freezing and re-warming process. Semen cryopreservation in marsupials will be used to aid in captive breeding programs to preserve endangered species from extinction. Using frozen sperm samples, genetics can be monitored and transported without having to move critical animals. The data collected and conclusions drawn from this research may be transferable to a degree to other marsupial species.

This study was a good starting point into finding the optimum M. fuliginosus processing method. The methods that were used were very basic, with minimal equipment, which is a beginning, with advanced sperm analysis techniques to follow to improve the process. From this study further investigation into the time to processing is needed, and to see if there is a more appropriate way of storing the testes is an extended period of time is
needed between cull and assessment. The $4^\circ$C storage may not be the most appropriate storage method as there was a significant decrease in motility, therefore other methods of transport and storage should be investigated.

Results from chapter 3 conclude that there may not be an interaction between investment in sexually selected traits, and sperm competition traits, which has not been previously documented in marsupials. Very little research has previously been done in this area, which made this study a good beginning into future research with larger sample sizes and different marsupial species. A larger sample size would assist in obtaining a larger range of the indexes of muscularity so that these relationships can be confirmed or determined that they are independent, along with observational studies. Observational studies to investigate the female’s preferred phenotype would further aid in the understanding of the kangaroo mating system as well as how the investment in sexual selection and sperm competition is influenced by female choice. Females may be choosing to mate with the very large dominant males, or they may be choosing the slightly smaller males with larger forelimb muscle mass. Observational studies will also help to determine how many males a female will mate with in one cycle, and paternity tests would determine which male’s spermatozoa is successful in fertilising the released oocyte.

In conclusion, our study does not support the hypothesis that the larger males invest more in muscle mass and less in sperm traits. There was no correlation seen between the spermatozoa and muscle traits; however, testes mass was correlated with body mass indicating that testes size is driven by body mass. Second, sperm length was negatively correlated with spermatozoa velocity, which was unexpected, especially compared with other mammal species. This study leads into future research that will further the understanding of sexual selection, sperm competition and the mating system of *M. fuliginosus* as well as other marsupial species.
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