SPERM COMPETITION

IN THREE SPECIES OF GROUND-DWELLING SQUIRRELS: INTRASPECIFIC AND INTERSPECIFIC DIFFERENCES

By

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Abstract

Sperm competition is a post-copulatory evolutionary process that can affect sperm production, and consequently reproductive success. Successful males may not necessarily be the males with the highest number of sperm. Other traits such as sperm motility or abnormality may also be factors that impact fertilization success, when there is selection for traits that improve the ability of the sperm to reach the egg. Ground squirrels are an ideal group of organisms to address questions of reproduction and fertility because of the large species diversity in the group, a welldocumented phylogeny, a high variety of mating systems and their diversity of life history traits. The main objective of my thesis was to investigate sperm competition in ground squirrels, since it is biologically, ecologically, and evolutionary important to recognize what traits influence the fertilizing efficiency of males and how different species maximize their reproductive success through post-copulatory sexual selection. I sampled free-ranging adult male Cape ground squirrels in the Northern Cape and SA Lombard Nature Reserve, Bloemhof, South Africa, Richardson's ground squirrels in Assiniboine Park Zoo, Winnipeg, Canada, and Barbary ground squirrels of an invasive population on Fuerteventura Island (Canarian archipelago), Spain. Key findings demonstrated: 1. A positive relationship (interspecifically) between operational sex ratio (OSR) and relative testes size (RTS); 2. A negative relationship (intraspecifically) between RTS and sperm concentration in Cape and Richardson's ground squirrels; 3. Cape ground squirrel males in better body condition produced more sperm and had larger Cowper glands; 4. Sperm length and speed were related in Cape and Richardson's ground squirrels; 5. Selective pressures may act to decrease sperm head variability among males, or possibly favor different sizes and morphologies of sperm within an ejaculate; 6. Barbary ground squirrels had higher sperm concentration, lower sperm abnormality and larger RTS compared to many other species,

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suggesting there is no evidence of inbreeding depression on sperm traits in this highly inbred population. This research contributes to advance our understanding of post-copulatory sperm competition in mammals as viewed from an ecological and evolutionary perspective.

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Now, I will be out of the protocol and use this space may be more than expected to express a little bit of myself and my happiness for this moment.

I am now finishing one more stage of my life, and no doubt about to start new adventures.

I need to say thank you to the people that in some way gave me support to keep my journey as an international student, single mom and Ph.D. student in Canada moving forward.

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Dedication

To my dad, Luiz dos Anjos.

List of Abbreviations

- AIC Akaike information criterion
- CASA computer assisted sperm analysis
- CV coefficient of variation
- GLM general linear model
- GLMM generalized linear mixed models
- HL head length
- HW-head width
- $ICC-intraclass\ correlation\ coefficient$
- OSR operational sex ratio
- PCA principal component analysis
- RTS relative testes size
- SD Standard deviation
- SE standard error
- TFL total flagellum length
- TSL total sperm size
- VAP velocity average path
- VCL curvilinear velovity

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Chapter 1: General Introduction

Sexual selection

Sexual selection is an evolutionary process that influences fertilization success and can result in differential reproductive success (Darwin 1882). What Darwin called sexual selection only pertained to competition before copulation (Bateman 1948). Sexual selection was initially seen as two basic processes: intra-sexual selection (competition for mates) and inter-sexual selection (mate choice; Huxley 1938). However, copulatory success does not guarantee fertilization success (Waterman 2007). Thus, reproductive success can be influenced by intraand inter-sexual selection that can occur before or after copulation (Figure 1; after Pizzari and Parker 2009), and can include post-insemination mechanisms, such as cryptic female choice and sperm competition (post-copulatory sexual selection; Parker 2014).

Cryptic female choice and sperm competition are two different post-copulatory mechanisms of sexual selection (Pizzari and Parker 2009; Roldan 2019). Cryptic female choice includes behavioural, morphological or physiological traits that can be used by a female to bias paternity in favour of some males over others after copulation (Eberhard and Cordero 1995). Those biases can occur, for example, through a female's response to male semen, which is not easily perceived (Eberhard and Cordero 1995). Furthermore, cryptic female choice can influence male mating strategies, particularly their investment in sperm production (Ramm et al. 2014). When a female copulates with two or more males, male-male competition for the fertilization of the egg can happen internally in the female among the sperm of different males, a process called sperm competition (Parker 1970).



Figure 1.1. Main mechanisms of sexual selection (after Pizzari and Parker 2009).

Sperm competition is a strong selective pressure and an evolutionary process that can affect sperm production, and consequently reproductive success (Ramm et al. 2014; Roldan 2019). Successful males may have traits that impact fertilization success besides the highest number of sperm, such as sperm motility or sperm morphology (Snook 2005; Ramm et al. 2014). The quality of sperm, also termed ejaculate fertilizing efficiency (Parker and Pizzari 2010), comprises a group of phenotypic traits, such as sperm morphology, that can be analyzed by measuring the shape of the head, midpiece and tail (Snook 2005). In mammals, sperm design or three-dimensional architecture is predicted to increase the speed of motile sperm under sperm competition (Tourment et al. 2011). For example, some species of rodents have a hook in the sperm head, which results in an aggregation of sperm into chains that can increase their overall speed (Sandera et al. 2013). It is still controversial whether sperm size directly influences the speed of the sperm, as the relationship is positive in interspecific analyses in birds (Lüpold et al. 2009) but not necessarily in mammals (Malo et al. 2006; Tourmente et al. 2011). Intraspecific comparisons of sperm size and speed are even more controversial and less well-supported (Tourmente et al. 2011).

Male traits can also be affected by different levels of sperm competition (Parker and Pizzari 2010). Sperm competition can influence the optimal ejaculate volume or the energy spent on sperm production depending on the degree of risk for sperm competition (defined as the probability that one male's sperm will compete with sperm from other males) and sperm competition intensity (defined as the number of ejaculates that are competing to fertilize an ovum; Engqvist and Reinhold 2005). Different degrees of risk or intensity of sperm competition can affect the evolution of testes and accessory glands (Møller 1998). Two hypotheses have been proposed to explain the evolution of large testes. The first hypothesis contends that large testes give a numerical advantage in sperm competition, while the second hypothesis states that the advantage lies in an increase in ejaculate fluid (and potentially higher number of copulations) independent of sperm concentration (Vahed and Parker 2012).

The mammalian testis has an important non-spermatogenic cell responsible for fostering the success of spermatogenesis, called the Sertoli cell. Sertoli cells help during spermatogenesis by supporting, nurturing, and protecting germ cells such as spermatogonium or spermatids (appendix 1.1), which are located within the seminiferous tubules, in the testis (Borysenko and Beringer 1984). The proportion of the spermatogenetic cells in the testis is variable among species and may be influenced by the intensity and risk of sperm competition (Ramm and Schärer 2014). Testis size in mammals is determined by the proportion of Sertoli and germ cells within the seminiferous tubules, and consequently influences the final number of sperm that are produced (Wistuba et al. 2007).

Semen is a complex fluid containing cells and other molecules produced by the accessory glands (seminal vesicles, prostate and Cowper glands) and these non-sperm components of the ejaculate are critical for successful fertilization (Poiani 2006; Lemaitre et al. 2011). The

accessory glands, such as seminal vesicles, produce a viscous fluid that provides energy for sperm cells (Mann 1946), and prostaglandins, which increase the contraction of smooth muscle in the female reproductive tract (Tripiciano et al. 1998; McKirdy et al. 2013).

Seminal fluid proteins influence post-copulatory selection in a number of ways. Bonanno and Schulte-Hostedde (2009) examined the relationship between sperm competition and the investment in ejaculate volume in red squirrels (*Tamiasciurus hudsonicus*) and found a positive relationship in organ size among the testis, prostate gland, and seminal vesicle. They also found that the size of these accessory glands was positively associated with sperm length in red squirrels. In rodents there is a positive relationship between the risk of sperm competition and the use of copulatory plugs (Poiani 2006), and the size of copulatory plugs is predicted to be related to the size of accessory glands (Ramm et al. 2005). Copulatory plugs (or mating plugs) are a coagulated mass formed through the polymerization of proteins produced by the seminal vesicles and deposited by males after copulation, in the female reproductive tract (Ramm et al. 2005; Poiani 2006).

Seasonality of breeding

The degree to which selection influences the morphology and physiology of a male is dependent on the level of sperm competition in a population (Sandera et al. 2013) which can be influenced by the seasonality of breeding in a particular species. Sperm demand, for example, can be categorized according to seasonality such as highly seasonal breeders, semelparous breeders (i.e., males with only a single breeding season in their lifetime), and continuous breeders. Seasonal breeders are good examples of how environmental conditions can influence testes structure and sperm production. In animals that reproduce through a single mating event, for example, spermatogenesis starts close to the beginning of the breeding season that will allow males produce their sperm in time for mating. Some hibernating species can completely pause spermatogenesis and present testicular regression outside of the breeding season (Wistuba et al. 2007; Tarulli et al. 2012).

Mating systems

Because the reproductive success of males is positively related to the number of mates they acquire (Bateman 1948), sexual selection can also influence mating systems (Emlen and Oring 1977), and consequently levels of sperm competition. A mating system is the manner by which males and females of a population or species mate. There are different mating systems, including monogamy and polygyny, where females mate with a single male or polyandry and promiscuity, where females mate with multiple males (Waterman, 2007). High post-copulatory sexual selection such as sperm competition is expected in systems where, during pre-copulatory sexual selection, two or more males mate with the same female (i.e. polyandry or promiscuity). The operational sex ratio (OSR), defined as the number of sexually active males to receptive females in a population, is a good measure of the level of sperm competition or the intensity of sexual selection (Emlen and Oring 1977; Shuster 2009; Weir et al. 2011). Thus, the operational sex ratio may be a measure of evolutionary pressure on sexual traits, and may influence reproductive success among males (Weir et al. 2011). Different mating systems can be, for example, related to sperm traits and relative testes sizes. Species of Philippine chrotomyine rodents with an absence of sperm competition (monogamous) had smaller testes size and higher

intra-male variation in sperm size. However, Philippine species with a high risk or intensity of sperm competition (promiscuous and polyandrous) had larger testis, and longer sperm with longer apical hooks and less variation in sperm size (Breed et al. 2019).

Other factors can also affect an individual's fitness under intense sperm competition, such as body condition, immunity and parasites. Under sperm competition, the existence of multiple mates can also increase the possibility of sexually transmitted diseases and the transmission of parasites during copulation (Møller 1998). There could be a trade-off among body condition, immunity and sperm production (Manjerovic and Waterman 2012). Animals in better body condition can invest more in sperm production (Schulte-Hostedde et al. 2005). Manjerovic and Waterman (2012) demonstrated that testis size has a negative relationship with spleen size, but a positive relationship with ectoparasite load in Cape ground squirrels (*Xerus inauris*), suggesting that males invest strongly in fertility, but sacrifice their immunity in the process. The trade-offs between traits that aid males successfully compete in post-copulatory sexual selection and male condition are still not well understood, particularly in comparison to our understanding of male condition and the traits needed for successful pre-copulatory sexual selection (i.e., ornaments and weapons; Macartney et al. 2019).

Ground squirrels as a model organism

Ground squirrels are an ideal group of organisms within which to address questions of reproduction and fertility because of the large number of species in the group, their welldocumented phylogeny, the large variety of mating systems in the group, and the diversity of their life history traits (Thorington et al. 2012). Sperm morphology is hypothesized to be diverse

among these species (Roldan 2019) and the morphology and morphometrics of the head and acrosome of sperm vary interspecifically within the squirrel family (Breed et al. 2011).

Cape ground squirrels (*Xerus inauris*) are an African species of ground squirrel that are also promiscuous but breeding occurs year-round. Adults males are scrotal and sexually active all-year (no hibernation), and aggression among males is rare (Waterman 1995). Males are not territorial and live separately from females in all-male bands (Waterman 1995). Almost no pre-copulatory selection occurs in this species (Manjerovic and Waterman 2015). Males in these all-male bands share a home range and will spend much of their day moving from one female group to another, assessing the reproductive status of females (Waterman 1997, 1998). Female Cape ground squirrels have an unpredictable estrus that only lasts a few hours (3.1 ± 0.4 hrs), and they will multiply mate during this estrus (1-10 males; Waterman 1996, Waterman 1997, Waterman 1998; Manjerovic and Waterman 2015). Males have very large testes, large accessory glands, and the operational sex ratios on days of estrus is high (F1: M11), which suggests extremely high levels of sperm competition (Manjerovic et al. 2008; Waterman 1998, 2010).

Richardson's ground squirrels (*Urocitellus richardsoni*) are a hibernating North American ground squirrel with a short, intense, annual mating season, that includes fighting and territorial defense. Their pre-copulatory sexual selection is intense and over 50% of males disappear during the two to four week breeding season and few males live to a second year of breeding (Michener and Lochlear 1990). Females have a short estrus (three to four hours) on a single day, mating with one to four males (Michener 1983, 1984). Both males and females have multiple mates (Hare et al. 2004) and the average operational sex ratio ranges from F1: M2.5 to F1:M3.5 (Michener and McLean 1996). Thus, male reproductive success in Richardson's ground squirrels is influenced by both pre- and post-copulatory sexual selection.

The northern African Barbary ground squirrel (*Atlantoxerus getulus*) is also a nonhibernating species native to Northern Africa (Morocco and parts of Algeria), but was introduced to the Canarian archipelago in 1965 from their native range in Morocco (López-Darias et al 2008). The reproductive biology of Barbary ground squirrels has not been well investigated, but it is expected that this species has high levels of sperm competition, as males have large testes (see chapter 5), a trait seen in males with intense sperm competition (Parker and Pizzari 2010; Tourment et al 2011). While Barbary ground squirrels are active year-round like Cape ground squirrels, they are also seasonal breeders similar to Richardson's ground squirrels, but food availability and temperature are the factors that trigger reproduction, not hibernation (Gouat and Yahyaoui 2001).

Overall, the seasonal differences in sperm demand and in their life histories make these three species of ground squirrel excellent models for investigating how sperm production, sperm traits and other structures vary under different intensities of sperm competition.

Objectives and thesis organization

The main objective of my thesis was to investigate sperm competition in ground squirrels, and how different species maximize their reproductive success through post-copulatory sexual selection. The understanding of sperm competition in mammals is biologically, ecologically, and evolutionary important to recognize which traits influence the fertilizing efficiency of males.

The thesis is divided into four data chapters, which present findings on sperm competition in three different species of ground squirrel as follows:

Chapter 2 investigated the importance of body condition in a promiscuous African ground squirrel (Cape ground squirrels) under sperm competition. The objective was to understand if testis size and the size of accessory glands are indicators of sperm competition, and if males in better physiological condition will have a higher quantity or quality of sperm over other males. The first hypothesis of chapter 2 was that testis size is an indicator of sperm competition in sciurids, the second hypothesis was that accessory glands is an indicator of sperm competition, and finally I hypothesized that male condition would be related to testis size, epididymal sperm concentration, sperm quality, and accessory glands. Chapter 3 investigated patterns of variability of sperm traits (including size, shape and speed) in Cape ground squirrels. The overarching hypothesis in this chapter was that variation in the sperm size and shape amongst males can be linked to sperm velocity (speed). Chapter 4 investigated sperm production and sperm competition in a seasonal breeder with a short and intense breeding season. I hypothesized that testis size is an indicator of sperm competition, that variation in the sperm size will be related to sperm motility and speed in Richardson's ground squirrels, and finally that body condition will influence sperm quantity and quality in males. Finally, Chapter 5 investigated the impact of sperm competition on testis size and sperm traits in a highly inbred population of Barbary ground squirrels on the island of Fuerteventura, Spain. I tested the hypothesis that testes size is an indicator of sperm competition in Barbary ground squirrels. The second hypothesis investigated if inbreeding depression can be considered an important problem to sperm traits (quantity and quality) in this invasive population.

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Chapter 2: Strong post-copulatory selection in a promiscuous African ground squirrel

Abstract

Male traits that represent the quality of an individual are often associated with the condition of the animal and high-quality traits provide a competitive advantage to males in sexual selection, during male-male competition. When females copulate with two or more males, competition for fertilization among the sperm of different males occurs within the female reproductive tract (sperm competition). With intense sperm competition, males are predicted to have larger testes and accessory glands to increase sperm concentration and ejaculate volume. If testis size, and accessory glands are indicators of sperm competition, then male condition will be related to testis size, epididymal sperm concentration, and accessory glands mass. The objective of the research described in this chapter was to understand if testis size and the size of accessory glands are indicators of sperm competition, and if males in better physiological condition will have a higher quantity or quality of sperm over other males. I tested these hypotheses in Cape ground squirrels (*Xerus inauris*), an African species with a promiscuous mating system and high operational sex ratios. Cape ground squirrels had high sperm concentration, vitality, high motility and a low frequency of sperm abnormality compared to other species of ground squirrels. Intraspecifically, males in better body condition (residual index) produced more sperm and had larger Cowper glands. However, males with smaller relative testes sizes had higher epididymal sperm concentrations than males with larger testes. Cape ground squirrels had strong post-copulatory sexual selection with high sperm concentration, vitality and motility, as well as a low frequency of sperm abnormality, compared to other species of mammals, and males in better

body condition produced more sperm and had larger Cowper glands. However, males with smaller relative testes sizes were more likely to produce epididymal sperm at higher concentrations than males with larger testes. This study advances our understanding of the factors influencing fertilization in rodents by providing a comprehensive assessment of postcopulatory sexual selection in an African ground squirrel.

Keywords: ground squirrels, sexual selection, relative testis size, sperm concentration, sperm quality, accessory glands.

Introduction

Sexual selection is an evolutionary process that influences fertilization success and can result in differential reproductive outcomes (Darwin 1882). Male traits that represent the quality of an individual are often associated with the condition of the animal and high-quality traits provide a competitive advantage to males during male-male competition, or during female choice (Andersson and Simmons 2006). Thus, sexual selection explains why the males of many species have conspicuous traits that can be detrimental to survival. For example, males can direct resources to secondary sexual traits more frequently selected by females during mating under sexual selection (Veuille 2010; Hosken and House 2011; Padian and Horner 2014; West-Eberhard 2014).

Sexual selection can have both pre-copulatory, such as competitive searching, and postcopulatory components such as sperm competition (Scantlebury et al. 2008a,b; Pizzari and Parker 2009; Roldan 2019). When females copulate with two or more males, competition for fertilization among the sperm of different males occurs within the female reproductive tract (Parker 1970). Sperm competition is a post-copulatory evolutionary strategy from a male's

perspective, and it is accepted as a notable selective force (Parker 1970; Ramm and Stockley 2016; Roldan 2019).

The evolution of testis size and sperm concentration in the ejaculate are affected by the degree of sperm competition (Gomendio and Roldan 1991; Møller 1998). Testes are important organs from an ecological and evolutionary perspective, as they can quickly respond to selective pressures (Schärer et al. 2008) and their size is used as a measure of sperm competition intensity among many species (Møller 1998; Parker and Pizzari 2010; Tourmente et al. 2011; Roldan 2019). Presumably in species under intense competition, males evolve larger testes because they result in a numerical advantage (i.e., more sperm) in post-copulatory sexual selection (Gomendio and Roldan 1991; Vahed and Parker 2012). However, there are other factors that can influence the relationship between testis size and sperm concentration. The male mating rate hypothesis suggests larger testes can be responsible for smaller ejaculates, but more frequent copulations (Vahed and Parker 2012).

To understand the relationship between testis size and sperm concentration, it is important to know about how spermatogenesis and sperm production are related. Testis size in mammals is determined by the proportion of Sertoli and germ cells within the seminiferous tubules, and testis size consequently influences the final number of sperm that are produced (Wistuba et al. 2007). Thus, seminiferous tubule diameter is an important comparative measurement of spermatogenesis (Anderson and Thliveris 1986; Verza and Esteves 2012). In humans, larger diameter seminiferous tubules increase the probability of a male having active spermatogenesis and consequently leads to more sperm in the testes (Verza and Esteves 2012). Successful males may not necessarily be those males with the highest numbers of sperm; other traits, such as sperm quality, may be factors that impact fertilization success when there is

selection for traits that improve the ability of sperm to reach the ova (Snook 2005; Bonanno and Schulte-Hostedde 2009; Ramm et al. 2014). Sperm quality includes phenotypic traits characterized by morphology (i.e., sperm abnormality), size, longevity, vitality, motility and sperm velocity (Snook 2005; Ruiz-Lopez et al. 2010).

Sperm are crucial for successful fertilization. However, healthy sperm also need the nonsperm components of the ejaculate, produced by the accessory glands, to successfully reach the female ova (Poiani 2006; Lemaitre et al. 2011; Perry et al. 2013). Accessory glands (i.e., seminal vesicles, prostate and Cowper glands) are responsible for producing fluids with important components for sperm survival. For example, fructose provides energy to sperm (Mann 1946; Tripiciano et al. 1998; McKirdy et al. 2013), prostaglandins increase the contraction of smooth muscle (Borysenko and Beringer 1984), and clear fluids with carbohydrates clean and lubricate the urethra prior to ejaculation (Williams-Ashman 1984; Ramm et al. 2005). In red squirrels (*Tamiasciurus hudsonicus*), accessory glands correlate positively with testes size and sperm production (Bonanno and Schulte-Hostedde, 2009). In bank voles (Myodes glareolus), seminal vesicles show phenotypic plasticity in response to levels of sperm competition (Lemaître et al. 2011). Seminal fluid proteins are also known to influence sperm competition. For example, male fruit flies (Drosophila melanogaster) with larger accessory glands and greater allocation in seminal fluid were able to increase their reproductive success under sperm competition (Wigby et al. 2009). Sperm competition in rodents may select for larger accessory glands, although, it is still unclear how the non-sperm components of semen are related to reproductive success under sperm competition across species (Ramm et al. 2005).

Another important factor affecting reproductive success is a male's physiological condition, for example infection can be associated with predisposition to low condition

(Beldomenico et al 2008; Boonstra et al. 2017). All these factors can affect male fitness under intense sperm competition. Animals in better condition may be able to invest more in primary reproductive traits, such as increasing the number of sperm and the production of semen (Andersson and Iwasa 1996; Schulte-Hostedde et al. 2005), and increasing investment in other reproductive structures, such as accessory glands (Burness et al. 2008; Blukacz et al. 2010). The relationship between post-copulatory sexual selection traits and condition dependence is still not well understood compared to ornaments and other pre-copulatory sexual selection traits (Macartney et al. 2019). Male condition can be measured in a number of ways, including size/mass indices, percentage of red blood cells in a total blood-cell mixture (%RBC), and neutrophil-lymphocyte ratios. The neutrophil-lymphocyte ratio is an easy measure that can indicate inflammatory response (Faria et al. 2016). Neutrophil-lymphocyte ratio also can be used as an indicator of the stress-axis response (i.e., the higher the neutrophil-lymphocyte ratio, the greater the stress response), and to identify the condition of males during breeding (Boonstra et al. 2017). However, investment in reproductive traits can be costly for males and lead to decreased immunity (Zahavi 1975; Bell 1978; Roberts et al. 2004; Schulte-Hostedde and Elsasser 2011).

The assumption that sperm production and testis size are related to sperm competition has not been well studied in rodents (Ramm and Stockley 2016). To investigate this assumption, and to determine if male condition and sperm production are related, it is critical that studies focus on species with potentially high levels of sperm competition, where selection could be intense. Cape ground squirrels (*Xerus inauris*) are an African species with a promiscuous mating system and high operational sex ratios (F1: M11; Waterman 1998). Operational sex ratios (OSR), defined as the number of sexually active males to oestrous females, are considered good

measures of level of sperm competition or sexual selection intensity (Emlen and Oring 1977; Shuster 2009; Weir et al. 2011). Pre-copulatory sexual selection appears to be weak in this species, as males are non-territorial, fighting and acts of aggression are rare, and in central South Africa, there is no dominance hierarchy amongst males (Waterman 1995; Waterman 1998; Manjerovic 2010). However, sperm competition appears to be strong, as males have very large testes and accessory glands (Manjerovic et al. 2008; Waterman 2010). As litter sizes are small (1-2 offspring; Waterman 1996), most copulating males will not have fertilization success. The rate of reproductive success in this species is typically low, with only 28% of males siring offspring, yet the factors affecting which male sires offspring are poorly understood (Manjerovic and Waterman 2015). Testis size is negatively related to spleen size, but positively related to ectoparasite infection in Cape ground squirrels, suggesting that males invest strongly in fertility, but at the cost of sacrificing their immunity in the process (Manjerovic and Waterman 2012). In a study of free-ranging Cape ground squirrels, O'Brien et al. (2018) investigated the trade-offs between immunity and testosterone and found that testosterone can be negatively affected by an immune response.

As Cape ground squirrels appear to have strong post-copulatory selection (Waterman 1998; Manjerovic et al. 2008; Waterman 2010), this species is a good model to address questions on the relationship between body condition and sperm competition. First, I tested the hypothesis that testis size is an indicator of sperm competition in sciurids (H1). If operational sex ratio is considered a good measure of the level of sperm competition or sexual selection intensity (Emlen and Oring 1977; Shuster 2009; Weir et al. 2011), I predicted a positive relationship between operational sex ratio and relative testes size in sciurids (P1.1). I also predict that intraspecifically, there will be a positive relationship between testis size and sperm quantity in
Cape ground squirrels (P1.2). If accessory glands are an indicator of sperm competition (H2), I predict a positive relationship between testis size and accessory glands mass (P2.1). My final hypothesis is that male condition, represented by the residuals of body mass on spine length (body condition index), the percentage of red blood cells, the neutrophil-lymphocyte ratios, will be related to testis size, epididymal sperm concentration, sperm quality and accessory glands mass (H3). Males with better condition are predicted to have larger testes (P3.1) and higher sperm concentration, higher sperm motility and lower sperm abnormality (P3.2). I also predict that males in better condition will have larger accessory glands (P3.3).

Methods and Analysis

Biology of the study animals

Cape ground squirrels are a non-hibernating southern African species that are promiscuous, with breeding occurring year-round. Adults males are scrotal and sexually active all year (Waterman 1998). Fighting and aggression are rare in this species, and in South Africa there is no dominance hierarchy or territoriality amongst males (Waterman 1998; Manjerovic 2010; Manjerovic and Waterman 2015). Males live separately from females in all-male groups, although some males delay dispersal and remain in their natal group (Waterman 1995). Female Cape ground squirrels are spontaneous ovulators (Bouchie et al 2006) with an unpredictable estrus that only lasts a few hours $(3.1 \pm 0.4 \text{ hrs})$, and they will multiply mate during this estrus (1-10 males; Waterman 1996, Waterman 1997, Waterman 1998; Manjerovic and Waterman 2015). The number of copulations per male within an estrous (repeated copulation) was positively related to disrupted copulation by other males and the number of males that females copulate. Repeated copulations can be an important strategy to compete with other males, increasing sperm competition (Waterman 1998, 2010). Despite small litter sizes, multiple paternity is high in litters of two (Manjerovic and Waterman 2015).

Trapping and handling

I sampled 20 free-ranging adult male Cape ground squirrels in May 2014 from Kimberley Airport (28° 47'S, E24° 46'E) in the Northern Cape and SA Lombard Nature Reserve, Bloemhof, South Africa (27° 60'S, 25° 48'E), where animals were being removed for control measures. Both areas are in the savanna biome, within the vegetation unit described as Eastern Kalahari Bushveld, Kimberley Thornveld (Rutherford et al. 2006). I received 20 squirrels immediately after euthanization with chloroform, the best method to collect parasites (Manjerovic et al. 2008), which were the focus of a concurrent study. Blood samples from a cardiac puncture were immediately collected and I recorded external measurements, including body mass, and spine length, using a spring scale (Pesola AG, Baar Switzerland), and a tapemeasure, respectively.

I calculated a body condition index using the residuals of a regression of body mass against spine length (Schulte-Hostedde et al. 2005; Manjerovic and Waterman 2012). This body condition index considers not only fat reserves, but also lean mass and water content (Schulte-Hostedde et al. 2005). I recorded as additional measures of male condition, including the percentage of red blood cells (hematocrit), from a microhematocrit centrifuge (International Medical Assistance, Inc., Indianapolis, USA), and I made thin blood smears to quantify white blood cells. Differential counts of white blood cells in these smears were made at the Manitoba Veterinary Laboratory (Winnipeg, Manitoba) and I used these counts to calculate neutrophillymphocyte ratios (Boonstra et al. 2017). Operational sex ratios and testes sizes from different species of sciurids were obtained from published data.

All methods were in accordance with the American Society of Mammalogist Guidelines (Sikes et al. 2011) and the University of Manitoba Animal Ethics Committee (Protocol F14-032).

Relative testes size (RTS), seminiferous tubule diameter, and accessory glands

I measured testis mass to ± 0.01 g using an MXX-212 Denver digital scale (Bohemia, NY, USA). I used RTS to compare testis size within and among species (Kenagy and Trombulak 1986; Parker 2016), calculated using the equation for rodents (Y = $-0.031X^{0.77}$; Kenagy and Trombulak 1986). I also calculated the somatic body mass for each individual (body mass – testis mass and accessory gland mass, Bonnano and Schulte-Hostedde 2009; Parker 2016).

I collected and fixed both testes in Bouin's fixative for 24 hours. After 24 hours, samples were washed, cut into smaller pieces and placed in 70% ethanol and I then processed these samples in paraffin embedding medium. I stained cross-sections (5-7 μm) with hematoxylin and eosin stain (H&E; Carson and Hladik, 2009). I measured seminiferous tubule diameter with ImageJ 1.50i (Wayne Rasband National Institutes of Health, USA; Schneider et al. 2012) following Anderson and Thliveris (1986).

I also measured the mass of the prostate glands, Cowper glands and seminal vesicles with the digital scale. The mass of prostate glands, Cowper glands and seminal vesicles was calculated as relative masses corrected by body mass.

Quality and quantity of sperm

To investigate the quality and quantity of sperm, I collected sperm from the cauda epididymis within 5 minutes of euthanasia. The epididymis was immediately cut while in a small

petri dish with 190µL of warm diluent (coconut water) placed on top of a Thermo-plate (MAT-U55, Tokai Hit, Olympus, Japan) set at 37°C. Coconut water has been used as a diluent or for cryopreservation of sperm with other mammals (Cardoso et al. 2005; Luzardo et al. 2010; Mollineau et al. 2011). Using a compound microscope (20X, Carl Zeiss, West Germany), I immediately assessed and recorded the percentage of sperm with progressive motility as 1 (a range of 1 to 20%), 2 (21 to 40%), 3 (41 to 60%), 4 (61 to 80%), or 5 (81 to 100%) following the World Health Organization (2010).

I made two sperm smears for each animal, which were stained with Hancock Stain[®] (Chino, CA, USA) to evaluate sperm vitality (the percentage of live:dead sperm; World Health Organization 2010). I assessed sperm vitality by counting the first 100 sperm from each two smears (100 cells from each replicate, total of 200 cells counted/ male) using a phase contrast microscope (40X, Model CX41, Olympus American Inc[©]., USA), and taking the mean percentage of unstained (live) sperm counted in the two replicates. With the remaining fixed sperm (in 10% in phosphate-buffered formalin). I assessed the morphology of sperm. One sample was not fixed correctly and was excluded from analysis (N=19). The percentage of abnormal sperm was determined and quantified according to World Health Organization (2010) by making two additional smears from the fixed sperm and counting the first 200 cells from each replicate (total of 400 cells counted/ male) under a phase contrast microscope (40X, Model CX41, Olympus American Inc[©]., USA). Sperm abnormalities were quantified as head. neck/ midpiece and tail (see more information Appendix 2.1). As the sperm samples were collected directly from the epididymis, an important place for sperm maturation, the sampled sperm were still involved in the last phases of spermiogenesis, including releasing part of the cytoplasm from the cell. For this reason, I did not consider a cytoplasm droplet as an abnormality in this study

(Cornwall and von Horsten 2007). To assess sperm concentration, I placed 10µL of the epididymal fluid in a Sefi-Medical Makler[®] counting chamber (Haifa 31070, Israel) and I counted the number of sperm in 10 squares (padronized as the same 10 squares over the entire research) under a compound microscope at 40X (Carl Zeiss, West Germany) to determine epididymal sperm concentration (number in millions per mL) according to the World Health Organization (2010).

Statistical analysis

I ran all analyses in the R program (R Core Team 2017, version 3.4.1), with an α of 0.05 set as statistically significant. Unless otherwise indicated, data are described as mean \pm standard error (SE). As no statistical differences were found between animals collected in the two sites, such as body condition index (Wilcoxon rank sum test, W = 40, p = 0.70) or RTS (Wilcoxon rank sum test, W = 23, p = 0.08), I pooled the data from both sites. I tested for normality using a Shapiro-Wilk normality test. Among-male variation was quantified by the coefficient of variation (CV) for each trait (Sokal and Rohlf 1981; Immler et al. 2008) using 20 sperm from each male. Pearson's product-moment correlations were used to check the association amongst variables (Sokal and Rohlf 1981; Dytham 2011). Data with a non-normal distribution were tested using non-parametric statistics. Distributions were fitted by fitdistrplus in the R program (Delignette-Muller and Dutang 2015). A general linear model (GLM) gamma regression was used to identify how much the explanatory variables (predictions 1.1, and 2.1: RTS; predictions 3.1, and 3.2: male condition (e.g., residuals of body mass on spine length, percentage of red blood cells, and neutrophil-lymphocyte ratios) explained variation in the response variable (prediction 3.1: RTS, predictions 1.1, 1.2, and 3.2: sperm quantity; predictions 2.1, and 3.3:

accessory glands mass). As there is no R^2 in GLM models, I quantified how much the explanatory variable explains response variable using the analysis called "explained deviance" or pseudo R^2 (Zuur et al. 2009). The dispersion parameter (overdispersion) for gamma family was considered acceptable at less than 1. The Akaike information criterion was tested using the function AIC in the MuMIn package in R, (Barton 2018) to compare and identify the best-fitting model (Symonds and Moussalli 2011).

Results

Interespecifically, I found a positive relationship between RTS and operational sex ratio in different species of sciurid (Spearman's rank correlation: rho = 0.93, p < 0.001, N = 10, Figure 2.1).

Intraspecifically, descriptive statistics of reproductive measures (epididymal sperm concentration, sperm quality, RTS, and relative accessory gland mass) in Cape ground squirrel males (N = 20) can be found in Table 2.1, and appendix 2.2. I found a negative relationship between RTS and sperm concentration ($F_{1,18} = 4.93$, P = 0.04, R² = 0.22, N = 20; Figure 2.2a), and a positive relationship between RTS and seminiferous tubule diameter ($F_{1,18} = 6.93$, P = 0.02, R² =0.34, N = 20; Figure 2.2b). No significant relationship was found between RTS and accessory glands. RTS was not significantly related to the mass of the Cowper glands ($F_{(1,18)} = 0.96$, P = 0.34, R² = 0.05, N = 20), prostate gland ($F_{(1,18)} = 0.33$, P = 0.57, R² = 0.02, N = 20), nor seminal vesicles ($F_{(1,18)} = 0.16$, P = 0.69, R² = 0.01, N = 20; see appendix 2.3).

There was no significant relationship between male condition and RTS ($F_{(1,18)} = 0.07$, P = 0.79, R² = 0.48, N = 20; see appendix 2.3). However, epididymal sperm concentration was positively related to the body condition index (residuals: $F_{(1,18)} = 11.07$, P < 0.01, R² =0.38, N =

20; Figure 2.5a), but not significantly related with %RBC (Spearman's rank correlation: rho = 0.13, P = 0.58, N = 20), nor neutrophil-lymphocyte ratios (rho = 0.27, P = 0.27, N = 20; see appendix 2.4).

Sperm abnormality in Cape ground squirrels (mean \pm SE = 3.4 \pm 1.29,) were most often found on the neck and midpiece (54%). No significant relationship was found between sperm abnormality and any condition measure (body condition index (residuals): rho = -0.22, P = 0.36; %RBC: rho = 0.19, P = 0.45; and neutrophil-lymphocyte ratio: rho = -0.18, P = 0.48, N = 19). Nor did I find any significant relationship between sperm vitality and any measure of condition (body condition index (residuals): rho = -0.18, P = 0.45; %RBC: rho = -0.03, P = 0.92); and neutrophil-lymphocyte ratio: rho = 0.14, P = 0.57; N = 20). In addition, sperm motility and any condition measure were not significantly correlated (body condition index: rho = 0.09, P = 0.71; percentage of red blood cells: rho = 0.05, P = 0.85; and neutrophil-lymphocyte ratio: rho = 0.22, P = 0.38, N = 20). see appendix 2.5.

Body condition index was positively related to Cowper gland mass ($F_{(1,18)} = 8.78$, P < 0.01, $R^2 = 0.33$, N = 20, Figure 2.3b), but the relationship was not significant to prostate gland ($F_{(1,18)} = 3.94$, P = 0.06, $R^2 = 0.18$, N = 20, Figure 2.3c), nor seminal vesicles mass ($F_{(1,18)} = 0.002$, P = 0.97, $R^2 = 0.00$, N = 20, Figure 2.3d).

To understand the factors that explain the sperm concentrations in Cape ground squirrels I used model selection (Table 2.2). Epididymal sperm concentration was best explained by RTS and the body condition index (lowest AIC value, and highest weight). Despite a positive relationship between RTS and seminiferous tubule diameter, our AIC analysis did not support seminiferous tubule diameter as a strong explanation of sperm concentration in Cape ground squirrels.

Discussion

I found a positive relationship (interspecifically) between operational sex ratio and RTS, and a negative relationship (intraspecifically) between RTS and sperm concentration in Cape ground squirrels. Males with larger testes had larger seminiferous tubule diameters but not a higher concentration of sperm and there was no evidence that testis size and accessory gland mass were related. However, mass/size measures of body condition (condition index) appeared to be an important factor influencing sperm production.

Sperm competition theory predicts that males will adjust their ejaculate volume based on levels of competition (Delbarco-Trillo and Ferkin, 2006). Operational sex ratios are recognized both as a measure of sexual selection and as a measure of evolutionary pressure on sexual traits (Emlen and Oring 1977; Shuster 2009; Weir et al. 2011). Interspecifically, a comparison of the operational sex ratios of different squirrel species indicated the expected positive relationship between levels of sperm competition (measured by operational sex ratio) and testis size. Cape ground squirrels have high operational sex ratios and a female mates with multiple males (mean of 4; Waterman 1998), but high OSR was also observed in two other species (Figure 2.1), Eastern gray squirrels (Sciurus carolinensis) and California ground squirrels (Otospermophilus *beecheyi*) who had smaller testes sizes. The larger RTS in Cape ground squirrel compared to the latter two sciurids may be related to litter size, as Eastern gray squirrel and California ground squirrel both have large litters (Eastern gray squirrel: 2-4, maximum 8 offspring; California ground squirrel: 5-11 offspring; Thorington et al. 2012) compared to Capes ground squirrels (1-2 offspring; Waterman 1996). Small litter sizes like Cape ground squirrels may constrain the ability of males to have any opportunity to fertilize ova during a reproductive event, and thus

could increase the post-copulatory competition amongst males. Certainly very small litter sizes could decrease the probability of a male successfully siring offspring (Abebe et al. 2019). Future studies should focus on how males, under selective pressure, have their testis size adjusted by natural selection in species of sciurid with different litter sizes and operational sex ratios.

Contrary to expectations, intraspecifically, male Cape ground squirrels with largest testis size did not have the highest sperm concentrations. The negative relationship between relative testes size and sperm concentration could be explained by the degree by which sperm competition influences the morphology and physiology of a male (Sandera et al. 2013). The sperm competition risk model (Parker et al. 1997) and the sperm competition intensity model (Parker et al. 1996) make different predictions on how males will allocate sperm investment under different levels of sperm competition (Parker 2016). The risk of sperm competition (the possibility that sperm competition will happen) is defined as the probability that the sperm of any one male (low probability of female double mating) will compete with sperm from other male (i.e., if there is a competitor present or not). A positive relationship between RTS and ejaculate volume is expected for species with a mean maximum of up to 2 competing ejaculates (sperm competition risk model, Parker et al. 1997; Parker 2016). For example, in cichlids (*Julidochromis ornatus*) testis size increased only under risk of potentially sharing in paternity (Awata et al. 2006).

In Cape ground squirrels, with a mean operational sex ratio of 11:1, it would be rare to have only 2 males competing during estrus (Waterman 1998) and therefore the level of sperm competition risk would always be high. Such a high number of potential mates suggests sperm competition intensity is more relevant to sperm competition in this species. The intensity of sperm competition, defined as a high probability of two or more ejaculates competing within the

female's reproductive tract, predicts a negative relationship between RTS and ejaculate volume (Engqvist and Reinhold 2005; Parker and Pizzari 2010; Simmons and Fritzpatrick 2012; Parker 2016). In intensity models, when more than two competitors are expected, the probability of success in reproduction is reduced and, in some species, males respond with a decrease of sperm investment (Parker et al. 1996; Delbarco-Trillo and Ferkin 2006). A male competing against one male is more likely to be successful than competing against multiple males. Thus, in this model, males will be less likely to produce higher numbers of sperm with high numbers of competitors or distribute the sperm concentration in different ejaculates. Low ejaculate volume for each copulation may be more frequent or repeated copulations.

Intraspecifically, changes in number, size or motility of sperm produced (phenotypic plasticity) can be affected by social cues (Ramm and Stockley 2009; 2016), such as presence of rival males and the response may be the conservation of sperm supplies according to the level of future mating opportunities (Vahed and Parker 2012). For example, under high intensity of sperm competition male copulatory behaviour can change, such as increasing the frequency of copulations or decreasing inter-ejaculatory intervals. This change can be related to increasing sperm concentration inside a specific female or by decreasing her receptivity to another competitor male (Delbarco-Trillo and Ferkin 2006).

In Cape ground squirrels, where females mate with an average of four males (Waterman 1998), intensity would be very high and a negative relationship between testis size and sperm concentration would be predicted by the intensity model. Although there are few examples supporting this model in the literature, a pattern of decreased sperm production under high intensity of sperm competition has been found in crickets (*Gryllus veletis*, Schaus and Sakaluk 2001; *Teleogryllus oceanicus*, Simmons et al. 2007) across bush cricket species in the

Tettigoniidae (Vahed et al. 2011), and in two species of gobiid fishes (Zosterisessor

ophiocephalus and *Gobius niger*; Pilastro et al. 2002). The production of ejaculates can be costly and as such, different species may use different strategies to invest in sperm production (Ramm and Stockley 2016). Primates, ungulates, and bats optimize their sperm production according to rates of mating and the level of sperm competition, but this idea is still controversial in rodents (Ramm and Stockley 2016). In meadow voles (*Microtus pennsylvanicus*), males respond differently depending of levels of sperm competition (such as presence of cues of 1 or 5 competitors) and invest more in sperm production at low intensities of sperm competition (Ramm and Stockley 2016).

The second possible explanation for the negative relationship between testes size and sperm concentration in Cape ground squirrels can be explained by the male mating rate hypothesis. This hypothesis suggests that larger testes can be responsible for smaller ejaculates (i.e. the transfer of fewer sperm per ejaculate), but more frequent copulations to guarantee a greater number of females inseminated or for repeated mating with the same female (Vahed and Parker 2012). Thus, according to the male mating hypothesis, my results suggest that Cape ground squirrel males may have the strategy with larger testes and larger seminiferous tubules ready to produce a large number of sperm for successive and repeated copulations (Waterman 1998, 2010; Vahed and Parker 2012). Thus, the negative relationship between RTS and sperm concentration in Cape ground squirrels supports both the high intensity model (Parker et al. 1996), and the male mating rate hypothesis (Vahed and Parker 2012). Testis efficiency or successful males may not necessarily mean larger testes producing more sperm, but the capacity to produce sperm well under competitive pressure, possibly in smaller ejaculates, and more frequent copulations (Firman and Simmons 2010; Vahed and Parker 2012).

If testis size alone does not always mean higher concentration of sperm, then other factors, such as the proportion of spermatogenetic tissue may explain our results (Firman et al 2015; Ramm and Stockley 2016; Roldan 2019). In this study, larger testes had larger-diameter seminiferous tubules, similar to humans (Verza and Esteves 2012), large-headed rice rats (*Hylaemys megacephalus*, Melo et al. 2013), and Wistar rats (*Rattus novergicus*, da Silva et al. 2006) but not greater sperm concentrations. Sperm concentration can be influenced by changes in the testicular structure that do not necessarily influence testicular size, such as changes in spermatogenesis, or even the combination of these factors (DelBarco-Trillo et al. 2013). As well, the proportion of spermatogenetic tissue in the testis varies among species and may be influenced by sperm competition (Ramm and Schärer 2014).

Despite the negative relationship between sperm concentration and testis size, Cape ground squirrel males still produced high concentration of sperm (400.75 ± 108.37 million/ mL) compared to other rodents: Muridae such as *Mus musculus* (9.5 million/ mL) and *Rattus norvegicus* (43.8 million/ mL), Dasyproctidae such as *Dasyprocta leporine* (74.69 million/ mL), Caviidae such as *Hydrochoerus hydrochaeris* (17.21 million/ mL, Lüpold and Fitzpatrick 2015). Different factors such as female ovulation can be important to explain differences in sperm concentration. For example, species with spontaneous ovulation had higher sperm concentration than species with induced ovulation (Soulsbury and Iossa 2010; Soulsbury 2010), possibly because males cannot predict the timing of ovulation in spontaneous ovulators so they are always prepared (Soulsbury 2010). As female Cape ground squirrels are spontaneous ovulators (Bouchie et al. 2006) that can breed year-round, and estruses are totally unpredictable, males need to be prepared for reproductive events at any time (Waterman 1995; Waterman 1997; Waterman 1998; Manjerovic and Waterman 2015).

However, successful males may not necessarily be those males with the highest concentrations of sperm; other traits such as sperm vitality, abnormality, motility and differences in rates of semen production may be factors that impact fertilization success when there is selection for traits that improve the ability of sperm to reach the ova (Snook 2005; Bonanno and Schulte-Hostedde 2009; Ramm et al. 2014). My results indicated that Cape ground squirrels have fewer abnormalities, higher sperm motility and vitality compared to other mammal species. In human the lower reference limit must be considered as 4-48% to normal cells, 40% of cells with progressive motility and 58% of live sperm (WHO 2010), and domestic bulls 30% of normal cells (*Bos taurus*; Menon et al. 2011). Iberian red deer was observed with 70.02% of normal cells, 90% sperm vitality and 62% of sperm motility (*Cervus elaphus hispanicus*, Malo et al. 2005).

The strongest influence on intraspecific variability in sperm in Cape ground squirrels was male condition (residuals). Body condition and testis size are positively related in bushy-tailed woodrats (*Neotoma cinerea*), deer mice (*Peromyscus maniculatus*) and red-backed voles (*Clethrionomys gapperi*), with the assumption that larger testis produce more sperm (Schulte-Hostedde et al. 2005). In northern watersnakes (*Nerodia sipedon*) variation in sperm morphology was negative associated with male condition, suggesting that males in better condition have more stable spermatogenesis, however no information about testis size related to body condition (Schulte-Hostedde and Montgomerie 2006). An increase in ejaculate investment and a reproductive advantage over other individuals is positively associated with body condition in lake whitefish (*Coregonus clupeaformis*, Blukacz et al. 2010). In my study, Cape ground squirrel males with a higher condition index produced higher sperm concentrations. Ejaculates are costly and perhaps males in better condition are able to afford higher sperm production relative to other

males (Bonanno and Schulte-Hostedde 2009). Body condition associated with sperm concentration has also been found in domestic cattle (Beran et al. 2011). In zebrafish (*Danio rerio*) a range of condition measures were related to sperm concentration and this information was used to facilitate the choice of males in case of *in vitro* fertilization (Hagedorn and Carter 2011). The cost of sperm and semen production can vary in different levels of sperm competition and in different species (Macartney et al. 2019). Cape ground squirrels in better condition may, for example, be able produce more sperm, possibly increasing the amount they can allocate to repeated copulations and to compete with sperm from rival males. Competitive searching for mates by male Cape ground squirrels is an important component of reproductive success, and males in better condition index may be able to roam over larger areas, encompassing more potential mates (Scantlebury et al. 2008a,b).

Levels of sperm competition can also be related to the size of accessory glands, since accessory glands will contribute to the non-sperm components of the ejaculate (Ramm et al. 2005). Increases in sperm concentration also may require an increase in the non-sperm components of the seminal fluid (Ramm et al. 2005, Bonanno and Schulte-Hostedde 2009). In this study, Cape ground squirrel males with a better body condition index had larger Cowper glands, which may increase in the non-sperm components of the ejaculate, increasing seminal fluids. A positive relationship between the size of accessory glands, the quantity of seminal protein and sperm transferred to the mated females occurs in *Drosophila bipectinata* (Santhosh and Krishna 2013). In stalk-eyed fly (*Cyrtodiopsis dalmanni*) a positive correlation was observed between accessory glands size, sexual maturity timing and mating frequency. Also, despite the importance of the testis in this species, the accessory glands were the main determinant of sexual maturity and mating frequency (Baker et al. 2003). There is evidence

across taxa, mainly in mammals, that the semen production (sperm and non-sperm components) can be related to dietary nutrients and can be condition dependent (Macartney et al. 2019). The positive correlation between the body condition index and the size of the Cowper glands in Cape ground squirrels could also be an important factor for repeated copulations. The Cowper gland is important for producing fluids that can neutralize the pH of the urethra prior ejaculation and to prepare the female tract to receive sperm (Arnqvist and Rowe 2005). In such a case, fluids produced by Cowper glands would also be important for cleaning or neutralizing the environment inside the female, to establish conditions that are favourable for the sperm in an ejaculate (Arnqvist and Rowe 2005).

In summary, Cape ground squirrels had high sperm concentration, vitality and motility, as well as a low frequency of sperm abnormality, compared to other species of mammals. Intraspecifically, males in better body condition (residuals) produced more sperm and had larger Cowper glands. However, males with smaller RTS were more likely to produce epididymal sperm at higher concentrations than males with larger testes. Because of low pre-copulatory selection and high post-copulatory selection, the Cape ground squirrel is a good model to address whether RTS is an indicator of sperm quantity. This species is also a good model to understand how male body condition is related to testis size, sperm concentration, and accessory glands mass. This study advances our understanding of mechanisms that may account for successful fertilization in rodents by providing a comprehensive assessment of post-copulatory sexual selection in an African ground squirrel.

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Table 2 1. Descriptive statistics of epididymal sperm concentration, quality (motility and vitality), relative testes size, and relative accessory glands mass in Cape ground squirrel males.

Character	
Sperm concentration (million/mL)	400.75 ± 24.23
Sperm motility (range*)	4.65 ± 0.15
Sperm vitality (%)	99.25 ± 0.20
Relative testes size**	2.83 ± 0.10
Relative Cowper glands mass (g)	0.01 ± 0.001
Relative prostate mass (g)	$0.003 \pm 8.84 \text{E-}05$
Relative seminal vesicle mass (g)	$0.0004 \pm 2.33E-05$

Mean \pm SE(N = 20); * Progressive motility recorded in ranges of 1 (1 to 20%), 2 (21 to 40%), 3 (41 to 60%), 4 (61 to 80%), and 5 (81 to 100%), following the World Health Organization (2010). ** More detailed information about RTS see appendix 2.2.

Table 2 2. Model selection using Akaike information criterion (AIC) to compare and identify which model best explains sperm concentration (response variable).

			Response variable (y)					
			Sperm concentration					
Model	Explanatory variables (x)	df	Δ AIC	weigh	(df)F	Р		
#				t				
5	Relative testes size + Body condition	4	0.00	0.83	(2,17) 14.75	<0.01		
4	Relative testes size + Body condition +	5	3.60	0.14	(3,16) 9.25	< 0.01		
	Seminiferous tubule diameter							
2	Body condition	3	7.35	0.02	(1,18) 11.07	< 0.01		
6	Body condition + Seminiferous tubule	4	8.38	0.01	(2,17) 6.78	< 0.01		
	diameter							
1	Relative testes size	3	12.09	0.00	(1,18) 4.93	0.04		
7	Relative testes size + Seminiferous tubule	4	14.79	0.00	(2,17) 2.58	0.10		
	diameter							
3	Seminiferous tubule diameter	3	16.59	0.00	(1,18) 0.31	0.58		

AIC: df, delta AIC and weight; Linear regression: F and P values. Bold indicates the best-fitting model selected by AIC.



Figure 2. 1. Interspecific comparison of relationship between operational sex ratio (OSR, M: F1, Emlen and Oring 1977), and relative testes size (RTS, Kenagy and Trombulak 1986) in ten sciurid species (Spearman's rank correlation: rho = 0.93, p < 0.01). Cape ground squirrel (*Xerus inauris*, this study; Manjerovic et al. 2008; Waterman 1998). Other points represent Richardson's ground squirrel (*Urocitellus richardsonii*, Chapter 4 and Michener and McLean 1996), Barbary ground squirrel (*Atlantoxerus getulus*, chapter 5 and van der Marel *unpubl. data*), California ground squirrel (*Otospermophilus beecheyi*, Dobson 1984; Boellsstorff et al. 1994; Kenagy and Trombulak 1986), Eastern gray squirrel (*Sciurus carolinensis*, Koprowski 1993a; Kenagy and Trombulak 1986), Eastern fox squirrel (*Sciurus niger*, Koprowski 1993b), Eurasian red squirrel (*Urocitellus beldingi*, Hanken and Sherman 1981; Sherman and Morton 1984; Ramm et al. 2005), woodchuck (*Marmota monax*, Armitage 1986; Kenagy and Trombulak 1986), and black-tailed prairie dog (*Cynomys ludovicianus*, Hoogland 1995; Ramm et al. 2005).



Figure 2. 2. A) Relative testes size (RTS) was negatively related to sperm concentration (linear regression, $F_{1,18} = 4.93$, P = 0.04, $R^2 = 0.22$); B) RTS was positively related to seminiferous tubule diameter in Cape ground squirrels (linear regression, $F_{1,18} = 6.93$, P = 0.02, $R^2 = 0.34$).



Figure 2. 3. A) Body condition index (mass/size residuals) was positively related to sperm concentration (linear regression, P < 0.01). B) Body condition index was related to Cowper gland (linear regression, P < 0.01); C and D) No relationship was found between body condition index and prostate gland (linear regression, P = 0.06), nor seminal vesicles (linear regression, P = 0.97) in Cape ground squirrels. Accessory glands calculated as proportion of the total body mass.

Chapter 3: Within- and among-male variation under sperm competition: form and function of Cape ground squirrel sperm

Abstract

Male-male competition can occur as post-copulatory sexual selection when sperm from two or more males compete for fertilization within the female reproductive tract (sperm competition). Moreover, sperm competition can be an important selective pressure that regulates variation in sperm traits. While sperm concentration can be important, the quality of sperm may also be critical for fertility success under conditions of sperm competition, particularly when there is selection for traits that improve the ability of the sperm to reach the ova. Cape ground squirrels (Xerus inauris) are an African species with a promiscuous mating system and a high intensity of post-copulatory sexual selection. I tested the hypothesis that variation in sperm size and shape (sperm form) amongst males is linked to sperm velocity (sperm function). I found that both within and among-male sperm variation can be important to sperm speed, and there is a positive relationship between sperm form (head size) and sperm function (speed) in Cape ground squirrels. The results also suggest that it is not sperm length but sperm head width and sperm head shape that are correlated with and probably influencing sperm speed. My study brings a better understanding about intraspecific variation in Cape ground squirrel sperm and how it can influence sperm speed in a species with intense sperm competition.

Keywords: Xerus, sperm speed, sperm design, sperm size, CASA.

Introduction

Male-male competition can occur as post-copulatory sexual selection when sperm from two or more males compete for fertilization within the female reproductive tract, known as sperm competition (Parker 1970; Pizzari and Parker 2009). Sperm competition can be an important selective pressure on variation in sperm traits (Blengini et al. 2014). Sperm size, shape and speed among mammals varies widely, and it is important to understand how these differences influences fertilization success (Roldan 2019). While sperm concentration can be important, the quality of sperm may also be critical for fertility success under conditions of sperm competition, particularly when there is selection for traits that improve the ability of the sperm to reach the ova (Snook 2005; Ramm et al. 2014).

Sperm quality comprises a group of phenotypic traits characterized by morphology, size, longevity, vitality, motility, and sperm velocity. The presence of different sperm phenotypes within the same ejaculate can influence the possibility of fertilization success (Snook 2005; Pizzari and Parker 2009; Ruiz-Lopez et al. 2010). Since head elongation can decrease drag and consequently the energy required for sperm movement, leading to better hydrodynamics, it is also important to understand if the form - i.e., shape and size - and speed (considered in this thesis as function because it can improve to reach the ova) are associated (Tourmente et al. 2011). Variation is sperm size is usually associated with sperm function, such as speed (Pitnick et al. 2009). Such a relationship is expected as sperm motility or speed is predicted to increase with longer sperm tails (Pizzari and Parker 2009; Godwin et al. 2017). Interspecifically, species under intense sperm competition are predicted to have longer sperm, presumably because they swim faster and would arrive at the egg sooner (Gomendio and Roldan 1991). For example, a high variability in sperm traits was an important strategy for two species of lizards under sperm
competition, as flagellum length was positively associated with speed in *Tupinanbis rufescens* while sperm head length was positively associated with sperm speed in *Tupinanbis merianae* (Blengini et al. 2014). A similar pattern occurs in different species of New World blackbirds (Icteridae, Lüpold et al. 2009). Sperm size is predicted to increase the speed of sperm in birds and mammals (Lüpold et al. 2009; Tourmente et al. 2011; Godwin et al. 2017), and rodent and primate species under sperm competition (polyandrous or promiscuous species) have longer and faster sperm compared to monogamous species (Gomendio and Roldan 1991). The diversity in the relationship between sperm form and function suggests that the selective forces shaping the size and speed of sperm are complex (Godwin et al. 2017).

Intraspecific comparisons of the relationship between sperm size and speed are controversial and not well-supported (Tourmente et al. 2011; Blengini et al. 2014). Sperm size (length) can be related to sperm velocity, although this relationship is not ubiquitous (Gomendio and Roldan 1991), and different species may respond differently to selective pressures (Blengini et al. 2014). Although longer flagellum is often related to increased sperm speed in some species, this relationship does not necessarily mean that these fastest sperm reach the egg first. In external fertilizers, for example, longer sperm may not necessarily mean more fertilization efficiency (Snook 2005). Likewise, the hook present on the sperm head of some rodents can be helpful during sperm transport as the hook can attach sperm to the walls of the female tract and increase sperm speed by aggregating sperm into chains (Sandera et al. 2013; Varea-Sánchez et al. 2014).

Under strong selective pressure, low variation in sperm traits among males is expected and it may favor an ideal sperm size in rodents with more uniform sizes in head and flagellum of the sperm (Varea-Sánchez et al. 2014). In red deer (*Cervus ephalus*), low intramale variation in sperm size is related to faster sperm (Ros-Santaella et al. 2015). Inversely, it is also possible that in a competitive and challenging environment, selective pressures can favor higher sperm size variation instead (Blengini et al. 2014). In Manchega sheep (*Ovis aries*), the heterogeneity of ejaculates possibly represents an adaptation to optimize fertilization by the differential performance and survival of sperm (Maroto-Morales et al. 2015).

The intraclass correlation coefficient (ICC; the proportion of among-male variation) is a commonly used estimate for repeatability or the expression of the genuine variability among males (Sokal and Rohlf 1981; Leushuis et al. 2010; Wolak et al. 2012). Since male variation in sperm, such as size and shape, can be important to reproductive success (Immler et al. 2008, 2014), it is important to understand sperm variation. For example, in zebra finch (*Taeniopygia guttata*), there is no evidence of a correlation between variation in sperm size and speed of sperm (Humphries et al. 2008), while in sea urchin (*Heliocidaris erythrogramma*) variation between the speed and size of sperm was highly correlated (Fitzpatrick et al. 2010). Variation in sperm traits interspecifically may be linked to differences in the strength of selection (Blengini et al. 2014). To understand selection on sperm form and function, it is important to examine sperm traits in species under very strong selection by sperm competition (Roldan 2019).

Cape ground squirrels (*Xerus inauris*) are a good model species to investigate the importance of variation in sperm traits because they have very high levels of sperm competition (Waterman 1998; Manjerovic et al. 2008; Waterman 2010). Cape ground squirrels are an African species that has a promiscuous mating system (Waterman 1998). Pre-copulatory sexual selection appears to be weak, as males are non-territorial, fighting and aggression are rare, and in South Africa, there is no dominance hierarchy amongst males (Waterman 1995; Waterman 1998; Manjerovic 2010). However, sperm competition appears to be strong, as males have very large testes, large accessory glands, and a high average operational sex ratio (F1: M11) (Waterman

1998; Manjerovic et al. 2008; Waterman 2010; Chapter 2). Since litter sizes are small (1-2) and competition is high, most copulating males do not have any reproductive success (Manjerovic and Waterman 2015). Only 28% of males sire offspring, yet the factors influencing who sires offspring are poorly understood (Manjerovic and Waterman 2015). My goal is to understand the variability of sperm traits (including size, shape and speed) in Cape ground squirrels. I hypothesized (H1) that variation in sperm size and shape amongst males will be linked to sperm velocity. I predicted that the size of sperm (total sperm length, flagellum and head length, and head width, P1.1), and head shape (P1.2) will influence sperm speed in Cape ground squirrels.

Methods and Analysis

Trapping & handling

Free-ranging adult male Cape ground squirrels (N = 20) were sampled in May 2014 from the Kimberley airport (28° 47'S, E24° 46'E) in the Northern Cape and SA Lombard Nature Reserve, Bloemhof, South Africa (27° 60'S, 25° 48'E), where animals were being removed for control measures. Both areas are in the Savannah Biome, within the vegetation unit described as Eastern Kalahari Bushveld, Kimberley Thornveld (Rutherford et al. 2006). Males were received immediately after euthanization with chloroform, the best method to collect parasites (Manjerovic et al. 2008), which were the focus of a concurrent study. External measurements were recorded including spine length, body mass, and scrotum length, using a tape-measure, precision scales (Pesola AG, Baar Switzerland), and a Digimatic Plastic Caliper (Mitutoyo Corporation, Kawasaki, Japan), respectively. All methods were approved by the University of Manitoba Animal Ethics Committee (Protocol F14-032) and followed the American Society of Mammalogist Guidelines (Sikes et al. 2011).

Quality of sperm

To investigate the quality of sperm, I collected sperm from the cauda epididymis within 5 minutes of euthanasia. The epididymis was immediately cut while in a small petri dish with 190µL of warm diluent (coconut water) placed on top of a Thermo-plate (MAT-U55, Tokai Hit, Olympus, Japan) set at 37°C. Coconut water has been successfully used as a diluent or for cryopreservation of sperm with other mammals (Cardoso et al. 2005; Luzardo et al. 2010; Mollineau et al. 2011). Videos were recorded using a compound microscope (10, 20 and 40X, Carl Zeiss, West Germany) and camera Aptina MT9F002 CMOS (Color) 1/2.3"(MU1403, AmScope, Irvine, EUA), with 53 frames per second (FPS), and with objectives ph1 10x/0.22, 25/0.45, and 40/0.65X of magnification. The remaining sperm were fixed within 10% in phosphate-buffered formalin to investigate sperm morphology.

Sperm morphology (i.e. form) was analyzed by measuring the shape and size of the sperm head, and length of the tail (Breed et al. 2011, Varea-Sánchez et al. 2013). I used the remaining fixed sperm to assess morphology by collecting digital images of at least 20 sperm from each male (World Health Organization 2010; Montoto et al. 2011) under a differential interference microscope (40X, Zeiss Axio Imager A1). One sample was not fixed correctly and was excluded from analysis (N=19). Sperm measurements were recorded using Image J 1.50i (Schneider et al. 2012). Sperm measurements recorded and included in this study were: head width (HW), head length (HL), total flagellum length (TFL), total sperm length (TSL), as described in Tournment et al. (2011).

A geometric morphometric method was used to measure sperm head shape (Rohlf and Slice 1990). This method is based on two dimensional coordinates (X, Y), called landmarks

(Figure 3.1A), showing directions in the coordinate space, called relative wraps (Rohlf 1993; Bookstein 2015; Figure 3.1B). This geometric morphometric method allowed me to analyze details of the shape that cannot be considered using the traditional method of analysis (Adams et al. 2004). Landmarks in two dimensions were created (N = 22, Figure 3.1A) using TPSdig2 (Version 2.20; Rohlf, 2005, 2015) as specific points on the sperm according to Varea-Sánchez et al. (2013). Landmarks can be represented in a thin-plate spline grid to better see their regional organization. Variation among the specimens within a sample can be expressed as a bending energy matrix (Figure 3.1B). Bending energy is the embodiment of all information related to landmark spacing (Bookstein 2016). I used the TPSrelw32 software (version 1.65) to generate representations of sperm head shape (Figure 3.1B). The software TPSrelw32 performed a linear regression between the variation of the landmarks with the bending energy of the thin-plate spline grid generated with my landmarks (Rohlf 2003, 2015). I then used the slope value from this linear regression to compare sperm head shape with other variables (Bookstein 2015, 2016).

I analyzed videos using ImageJ 1.50i (Wayne Rasband National Institutes of Health, USA, Schneider et al. 2012). I used Image J CASA plugin (Wilson-Leedy and Ingermann 2006, 2007) modified by Purchase and Earle (2012) to determine sperm velocity (i.e. function). I recorded the time-average velocity along the total point to point distance traveled by the sperm, velocity average path (VAP, μ m/s), and the velocity along the total point to point distance traveled by the sperm, curvilinear velocity (VCL, μ m/s) (Wilson-Leedy and Ingermann 2006, 2007). I focused on VAP and VCL because they best represent the total velocity of the sperm as sperm function (speed), and best correlate with size and shape (Fitzpatrick et al. 2010). Only videos from 17 males were usable with the software, most likely because of differences in lighting during recording. In total samples from 16 individuals had all information necessary to

be included in correlation and generalized linear mixed models (GLMM) between sperm size and speed.

Statistical analysis

I ran all analyses in R Program (R Core Team 2017; R version 3.4.1), with an α of 0.05 considered to be statistically significant. I used 20 sperm from each male to identify within-male variation on sperm size and shape, and quantified coefficients of variation (CV, Sokal and Rohlf 1981; Immler et al. 2008). As no statistical differences (Wilcoxon rank sum test) were found between animals collected in the two sites, such as sperm size (HW: W = 57, P = 0.23; HL: W =44, P = 0.90; TFL: W = 65, P = 0.06; TSL: W = 62, P = 0.10), shape (slope regression: W = 41, P = 0.97), and speed (VAP: W = 24, P = 0.21; VCL: 22, P = 0.37), I pooled the data from both sites. I used a relative wraps method that represents principal component vectors in a thin-plate spline grid to describe the major trends in sperm head shape variation (Rohlf 1993). I used MorphoJ software (version 1.06d) to analyze principal component analysis (PCA) results, and to identify which principal component (eigenvalues, % of the total variance and cumulative % of total variance), and landmark (landmark coordinate #, loading) could best represent head shape variation (Figure 3.1C, Klingenberg 2011). Distributions were fitted by fitdistrplus (Delignette-Muller and Dutang 2015). PCA, Pearson correlations and Spearman's rank correlations were used to check collinearity amongst variables (Sokal and Rohlf 1981; Dytham 2011).

Repeatability or ICC (intraclass correlation coefficient; Nakagawa and Schielzeth 2010, Nakagawa et al. 2017) was calculated using an ICC package in R (ICCest, Package ICC version 2.3.0 (Wolak et al. 2012, see more appendix 3.4). The phenotypic constancy or repeatability (i.e. ICC, Nakagawa and Schielzeth 2010; Wolak et al. 2012; Nakagawa et al. 2017), among and

within-male variation are both important to understand which sperm traits are being selected under sperm competition (Blengini et al. 2014). ICC values close to 1 (high) represents a more heterogeneity in a population, and that differences among males are genuine, while ICC close to 0 (lower) means more homogeneity in a population and that within-male variation is due to fluctuations in samples of the same male or error (Leushuis et al. 2010).

Using male identity (ID) as a random factor, I used generalized linear mixed models (GLMM, lme4 package, Bates et al. 2015) fit by maximum likelihood (Laplace Approximation, Harrison et al. 2018), to understand within-male (intra-male variation) and among- male (intermale variation) effects, and how within-male variation can influence sperm form-function correlations. As explanatory variables, I used sperm length (TSL; prediction 1.1), and sperm head width (HW; prediction 1.1). As response variables (predictions 1.1, and 1.2) I included velocity average path (VAP, μ m/s) and curvilinear velocity (VCL, μ m/s), both of which had gamma distributions.

Results

Sperm form (shape and size, see appendices 3.1), and function (speed, see appendix 3.2) were positively related in Cape ground squirrels' sperm. Descriptive statistics can be found in Table 3.1. Within-male variation (CV) in sperm head width was positively correlated with within-male variation in VCL (Spearman's rank correlation: rho = 0.80, P < 0.01, N = 16, Figure 3.2A), and within-male variation in VAP (Spearman's rank correlation: rho = 0.80, P < 0.01, N = 16, Figure 16, Figure 3.2B). Variation in total sperm length was not significantly correlated with within-male variation in VAP nor with within-male variation in VCL (Spearman's rank correlation: rho = 0.25, P = 0.36, N = 16, see appendices 3.1 and 3.2). Shape (slope value) was also positively

correlated with within-male variation in VCL (Spearman's rank correlation: rho = 0.52, P < 0.04, N = 16, Figure 3.2C), and within-male variation in VAP (Spearman's rank correlation: rho = 0.58, P < 0.02, N = 16, Figure 3.2D). Within-male variation in sperm head width was positively correlated with sperm head shape (slope value; Spearman's rank correlation: rho = 0.57, P = 0.01, N = 19, Figure 3.3). Within and among-male variation can be found in figures 3.4 (HW and TSL) and 3.5 (VAP and VCL).

Among-males, I found sperm head width (GLMM: t = 2.25, N= 16 individuals, P < 0.03) was positively related to VAP but not to VCL (GLMM: t = 1.31, N= 16 individuals, P = 0.19). Higher among-male variation was consistent with higher within male repeatability in VAP (ICC = 0.87), and VCL (ICC = 0.90; Table 3.1, see also appendix 3.2). I found low among-male variation and low repeatability in sperm head width (ICC = 0.07) and total sperm length (ICC = 0.18; Table 3.1).

Discussion

I found a positive relationship between sperm form (shape and size) and sperm function in Cape ground squirrels. Sperm head shape and sperm head width were positively correlated, and sperm with wider heads were faster than sperm with more narrow head widths. Within-male variation (CV) and among-male variation in sperm head width were both positively correlated with VAP. In addition, within-male variation (CV), but not among-male variation, in sperm head width was positively correlated with VCL. Among males, sperm head shape was positively correlated with both speed measures (VCL and VAP). Variation among males was higher in sperm speed than sperm size. A positive relationship between sperm length and speed has been described in different species of passerine birds (Lüpold et al. 2009) and in a number of different species of eutherian mammals (Tourmente et al. 2011), such as red deer (Malo et al. 2006; Humphries et al. 2008), polyandrous species of primates (Gomendio and Roldan 1991), and murine rodents (Gomendio and Roldan 1991; Sandera et al. 2013). However other sperm traits, not just sperm length, can influence sperm speed (Roldan 2019). Contrary to my expectations, while I found a significant relationship between sperm head width and speed, I did not find a relationship between sperm length and speed in Cape ground squirrels. Head shape is probably associated with the movement of sperm, such as in muroid rodents where sperm head area influenced hydrodynamic efficiency, or increasing sperm head curvature that allows the formation of sperm chains that increase their speed inside the female tract (Montoto et al. 2011). Indeed, my results suggest that sperm head width is more important than sperm length in Cape ground squirrels to increase sperm speed, and sperm competition can affect sperm head design to improve speed (Varea-Sánchez et al. 2016; Roldan 2019).

Other species of squirrels with larger testes and expected high levels of sperm competition such as Indochinese ground squirrels (*Menetes berdmorei*), iornate squirrels (*Callosciurus inornatus*), and red-cheeked flying squirrels (*Hylopetes spadeus*) had longer sperm than Cape ground squirrels (Breed et al. 2011). Pallas's squirrel (*Callosciurus erythraeus*) that also multiply mate (Thorington et al 2012), had similar results to Cape ground squirrels in TSL, HW and HL (Breed et al. 2011). Comparing my results to an investigation with three species of *Mus* with low (*Mus musculus*), intermediate (*Mus spretus*), and high (*Mus spicilegus*) levels of sperm competition, Cape ground squirrels had larger sperm head (longer and wider), shorter sperm length (TSL), and faster sperm (VAP and VCL) than all three *Mus* species (Delbarco-

Trillo et al. 2018). My results showed low among-male variation on sperm size (HW and TSL) in Cape ground squirrels and low repeatability, suggesting more homogeneity (see appendix 3.3) in the population, related to those traits. The higher within-male variation could be due to either fluctuation in samples of same male or measurement error (Leushuis et al. 2010). It also suggests that under intense sperm competition, selective pressures may act in favor an "ideal sperm phenotype", decreasing the sperm head variability among males (Pitnick et al. 2009; Blengini et al. 2014; Varea-Sánchez et al. 2014; Ros-Santaella et al. 2015). In Philippine chrotomyine rodents with high risk or intensity of sperm competition (promiscuous and polyandrous mating systems), species had larger testes, longer sperm with longer apical hook, and less variation in sperm size (Breed et al. 2019). In Muroid rodents, selective forces are also thought to have impacted sperm head design, possibly affecting fertilization ability (Varea-Sánchez et al. 2016).

Comparing within-male variation among Cape ground squirrels with 26 species of muroid rodents with different levels of sperm competition (see supporting material of Varea-Sánchez et al. 2014), Cape ground squirrels had the lowest within-male variation in sperm head length and head width. The within-male variation in Cape ground squirrels was lower than species with multiple mating and large relative testes sizes, such as the wood mouse (*Apodemus sylvaticus*; RTS = 2.35; Kenagy and Trombulak 1986; Wolff and Sherman 2007; Varea-Sánchez et al. 2014). The RTS of Cape ground squirrels is 2.83 (see Chapter 2). My results suggest that Cape ground squirrels are under intense selective pressure that results in more uniform sperm heads.

Variation in sperm speed in Cape ground squirrels was higher among males than within males and had highly consistent repeatability, suggesting a heterogeneous population with genuine differences among males (Leushuis et al. 2010). This variation can be an important

source for selection on sperm speed under intense sperm competition, suggesting that some Cape ground squirrel males may be more competitive in sperm speed than others (Pitnick et al. 2009; Leushuis et al. 2010). The proportion of motile sperm and variations in sperm speed can increase fertility success in some species (Malo et al. 2005). For example, fertilization rates in populations of red deer were positively associated with sperm speed and percentage of normal sperm (sperm morphology), even with constant sperm concentration (Malo et al. 2005). Differences in sperm speed can, for example, increase the possibility of different sperm reaching the egg at different times, possibly increasing the chance of fertilization, such as in bluegill (*Lepomis macrochirus*, Burness et al. 2004) and house mice (*Mus domesticus*; Firman and Simmons 2010). It is also important to note that male age can be a possible factor influencing the form and function of sperm, and the importance of the age in sperm traits still needs to be investigated in Cape ground squirrels (Helfenstein et al. 2010).

My data supports the prediction that sperm form and function are related in Cape ground squirrels. Sperm with wider heads were faster than others. The results also suggest that it was not the length of the sperm that was the most important factor influencing sperm speed. Sperm head width and sperm head shape had the most influence on sperm speed, and both, within and among-male variation can be important to sperm speed in Cape ground squirrels. My study brings a better understanding about intraspecific variation in Cape ground squirrel sperm and how it can influence sperm speed in a species with intense sperm competition.

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Table 3. 1. Descriptive statistics and among-male variation (CV) of sperm size (measured in μ m, using ImageJ) of Cape ground squirrels from South Africa 2014.

Sperm Measure	Mean ± SE	CV (%)
Head Width (HW)	7.13 ± 0.04	2.65
Head Length (HL)	10.16 ± 0.05	2.30
Total Flagellum Length (TFL)	54.41 ± 0.27	2.16
Total Sperm Length (TSL)	64.60 ± 0.27	1.85

 $\frac{1001 \text{ Sperm Length (13L)}}{\text{Males: N} = 19, \text{ Sperm measured for each male: N} = 20.}$

	Ν	Ν	ICC	Lower CI	Upper CI	varw	vara
	(males)	(sperm)					
VAP	17	257.92*	0.87	0.79	0.95	7741.98	51654.09
VCL	17	257.92*	0.90	0.83	0.96	90604.89	770173.80
HW	19	20**	0.07	0.00	0.14	0.29	0.02
TSL	19	20**	0.18	0.06	0.30	5.19	1.13

Table 3. 2. Intraclass correlation coefficient (ICC) from Cape ground squirrel males.

VAP: velocity average path; VCL: curvilinear velocity; HW: sperm head width; TSL: total sperm length. *Average of sperm tracked/ male. **16 sperm were measured from male ZA19. Alpha = 0.05. Lower CI: lower confidence interval limit, upper CI: upper confidence interval limit, varw: the within individual variance, vara: the among individual variance (ICCest package version 2.3.0, Wolak et al. 2012).



Figure 3. 1. A: Morpho J software (version 1.06d, Klingenberg. 2011) output. A procrustes fit, and consensus sperm head shape variation of Cape ground squirrels (male ID: ZA20). Blue dots = mean landmark positions; small black dots = all landmark positions, within-male variation (N = 19 males); red numbers = numbers of the landmark. B: TPSrelw32 software (Rohlf 2015) output. Variation among males of 22 landmarks expressed as a bending energy matrix. C: Morpho J software (version 1.06d, Klingenberg. 2011) output. Visualization of the principal component (PCA analysis) by transformation grid (dot represents mean shape/ landmark location; line length and direction represent the movement of the respective landmark).



Figure 3.2. Spearman's rank correlation (N=16) between: A) Within-male variation of sperm head width (CV of the HW) and within-male variation of curvilinear velocity (CV of the VCL); B) Within-male variation of sperm head width (CV of the HW) and within-male variation of velocity average path (CV of the VAP); C) Shape (slope value) and within-male variation of curvilinear velocity (CV of the VCL); D) Shape (slope value) and within-male variation of velocity average path (CV of the VAP); C) Shape (slope value) and within-male variation of velocity average path (CV of the VAP); D) Shape (slope value) and within-male variation of velocity average path (CV of the VAP) in Cape ground squirrels.







Figure 3. 4. Ggplot2 output (Wickham 2016) in R program (R Core Team 2017, version 3.4.1). Within and among-male variation of: A) sperm head width (HW, μ m); B) total sperm length (TSL, μ m) in Cape ground squirrels. ID: male identification.



Figure 3. 5. Ggplot2 output (Wickham 2016) in R program (R Core Team 2017, version 3.4.1). Within and among-male variation of: A) velocity average path (VAP, μ m/s); B) curvilinear velocity (VCL, μ m/s) in Cape ground squirrels. ID: male identification.

Chapter 4: Sperm competition in a seasonal breeding ground squirrel with short and intense pre- and post-copulatory sexual selection

Abstract

When females copulate with two or more males, competition to fertilize ova (sperm competition) can occur among sperm from different males within the female reproductive tract. Successful males may not necessarily be the males with the highest number of sperm (i.e., quantity), since other sperm traits such as speed or morphology (i.e., quality) may also impact fertilization success. The objective of my study was to investigate sperm production in a species with both intense pre and post-copulatory selection. I hypothesized that relative testis size is an indicator of sperm competition, that variation in the sperm size will be related to speed, and that body condition will influence sperm quantity and quality in males. I tested these hypotheses in a North American hibernating species, the Richardson's ground squirrel (Urocitellus richardsoni). I found a negative relationship between relative testes size and sperm concentration. Also that Velocity average path (VAP) was positively related to total sperm length and flagellum length. Results suggest that males with larger testis did not have higher sperm concentration in their epididymis, and they have lower sperm concentration than other species of rodents. However, sperm length and speed were positively related, supporting longer sperm are faster in this species. Body condition did not explain variation in sperm traits in Richardson's ground squirrels. My data support that quality (faster sperm) may be an important strategy for Richardson's ground squirrel reproductive success. Thus, Richardson's ground squirrel males

with longer and faster sperm, may have an advantage over other males in competition for fertilization success.

Keywords: ground squirrels, relative testes size, sperm concentration, sperm speed, sperm size, male condition, CASA.

Introduction

Post-copulatory sexual selection (Bateman 1948; Pizzari and Parker 2009) can affect sperm production, and consequently reproductive success (Ramm et al. 2014). When females copulate with two or more males, competition to fertilize ova can occur among sperm from different males within the female reproductive tract (sperm competition; Parker 1970). Successful males may not necessarily be the males with the highest number of sperm (i.e., quantity), since other sperm traits such as motility or morphology (i.e., quality) may also be factors that impact fertilization success. The impact of sperm quality on fertilization success is particularly apparent when there is selection for traits that improve the ability of the sperm to reach the ova (Snook 2005; Ramm et al. 2014).

Sperm can vary widely in many animal species (Parker and Begon 1993; Roldan 2019), and selective pressures from sperm competition can influence sperm phenotype and ejaculate traits in different ways (Pizzari and Parker 2009; Varea-Sánchez et al. 2016; Roldan 2019). For example, variation is sperm size is usually associated with sperm function, such as speed (Pitnick et al. 2009). Variation among males or among sperm from the same male (within-male variation) under strong selective pressure can be low, selecting for an optimal sperm size or speed (Blengini et al. 2014; Ros-Santella et al. 2015). On the other hand, it is also possible in a competitive and challenging environment that sperm competition can favor higher sperm size

within-male variation increasing the possibility of fertilization success (Blengini et al. 2014). Variation in sperm speed and the presence of different sperm phenotypes within same ejaculate can bring a wider range of advantages covering different stages of the fertilization process (Pizzari and Parker 2009; Blengini et al. 2014). The relationship between size and speed may be under different selective forces intraspecifically in different species and more species need to be investigated to understand this relationship (Gomendio and Roldan 2008). The relationship between sperm size and speed was positive in an interspecific analysis conducted in birds (Lüpold et al. 2009), but not in mammals (Malo et al. 2006; Tourmente et al. 2011). However, in a meta-analysis of rodents and primates, Gomendio and Roldan (1991) found longer and faster sperm in species under sperm competition.

Species under high sperm competition are expected to have larger testes, assuming that larger testes size result in more sperm being produced (Harcourt et al. 1981; Ramm et al. 2014; Roldan 2019). The final testes size in mammals is determined by the proportions of Sertoli and germ cells within the seminiferous tubules, which consequently influence the final number of sperm produced (Wistuba et al. 2007). A positive correlation between the seminiferous tubule diameter and the amount of spermatogenetic tissue has been found in Wistar rats (*Rattus novergicus*, da Silva et al. 2006), in human testes (Verza and Esteves 2012), and in different species of New World blackbirds (Icteridae; Lüpold et al. 2011). Larger seminiferous tubule diameters increase the possibility of active spermatogenesis and consequently lead to more sperm (Verza and Esteves 2012; Roldan 2019). Seminiferous tubule diameter is therefore an important measure that can be used to understand testis size and sperm production.

A male's condition (e.g., body condition, or immunity) is another important factor that can affect male fitness under intense sperm competition. Animals in better body condition may

be able to invest more resources towards sperm production (Schulte-Hostedde et al. 2005). A male's condition can be essential, particularly in species where the reproductive season is short and with intense pre-copulatory sexual selection. Consequently, the morphology and physiology of sperm can be influenced by breeding seasonality (Ramm and Schärer 2014). Male condition is also important in animals with short or single breeding season, because cell mitosis can occur continually but spermatogenesis and spermiogenesis stages may start close to the moment of mating (Ramm and Schärer 2014). Commencing spermatogenesis close to the beginning of the breeding season allows males to produce fertile sperm in time for mating, and this timing of spermatogenesis needs to result in sperm production without compromising sperm vitality (proportion of sperm cells that are alive in a sample of semen, World Health Organization 2010) or fertilization success (Ramm and Schärer 2014). For example, the Djungarian hamster (*Phodopus sungorus*), a hibernating rodent, has a complete pause in spermatogenesis and a testicular regression outside of the breeding season (Wistuba et al. 2007; Tarulli et al. 2012).

Richardson's ground squirrels (*Urocitellus richardsoni*) are a North American hibernating species that may be a good model for understanding sperm competition in a seasonal breeder. They have an annual mating season that is short (two to four weeks), with intense precopulatory sexual selection (i.e., territoriality and fighting), yet both sexes also mate with multiple partners (Michener and Locklear 1990; Hare et al 2004). Multiple mating suggests that sperm competition is an important fitness component in this species (Hare et al 2004). Females have short estruses (three to four hours) on a single day, mating with one to four males (Michener 1983, 1984) and producing multiply sired litters (Hare et al. 2004). On the day of a female's estrus, the operational sex ratio (OSR) for Richardson's ground squirrels ranges from F1: M2.5 to F1:M3.5 (Michener and McLean 1996). A dark descended scrotum usually is visible

when males emerge from hibernation, and Richardson's ground squirrel males are scrotal annually for only a few weeks after emergence (Michener 1983). Over the breeding season, testis size declines and at the end (of the breeding season) the testes regress into the abdominal cavity (Michener 1983). During the breeding season, fighting and territorial defense by males often results in the loss of fat reserves, injuries, and potential death (Michener and Locklear 1990). Over 50% of males disappear during the breeding season and only a few males live to a second year of breeding (Michener and Locklear 1990).

Seasonal breeders, like Richardson's ground squirrel, are good species to examine and understand how a short and intense breeding period can influence sperm production, such as the quality and quantity of sperm. The hibernation physiology of male Richardson's ground squirrels differs from females (Michener 1992). Males need to enter into euthermy before females, and this will make possible males being ready and with sperm weeks before emergence from hibernation, similar to golden-mantled ground squirrels (Callospermophilus lateralis; Barnes et al. 1986; Barnes 1996). Richardson's ground squirrel males spend more time in euthermy and emerge up to two weeks before the females, and they usually store food in their hibernaculum (i.e., the underground chamber). This food storage allows the recovery of fat lost during the winter and coincides with the onset of sperm production prior to emergence (Michener 1992). Spermatogenesis in Richardson's ground squirrels begins before emergence from hibernation, as the testes descend and both spermatogenesis and spermiogenesis commence (Michener 1992). In the golden-mantled ground squirrel, spermatogenesis and steroidogenesis in the testis are temperature-dependent and male arousal during hibernation is longer compared to females (Barnes et al. 1987). This arousal time allows some increase in testis mass and the presence of spermatogonia and spermatocytes before the end of hibernation (Barnes et al. 1987). However,

during this time, both luteinising hormone (LH) and follicle-stimulating hormone (FSH) are inhibited from binding with gonadal receptors, and this inhibition is most likely why, despite the presence of spermatocytes, the completion of spermatogenesis occurs only during euthermy (Barnes et al. 1987). Testosterone levels are also elevated over the first few weeks of euthermy (Barnes 1996). Sperm can be observed in the seminiferous tubules before emergence in Richardson's ground squirrels, indicating that spermatogenesis begins during euthermy, and only after 11.5 days after the end of heterothermy is sperm found in the epididymis and the male becomes competent reproductively (Michener 1992; Barnes 1996). The complete presence of sperm in seminiferous tubules are only seen after terminating hibernation (Michener 1992). Thus, like golden-mantled ground squirrels, Cascade golden-mantled ground squirrel (*Callospermophilus saturatus*), and Arctic ground squirrel (*Urocitellus parryi*), Richardson's ground squirrel males awaken weeks before emergence to be reproductively prepared with functional sperm before the mating season (Barnes et al. 1986; Michener 1992; Barnes 1996).

The objective of my study was to investigate sperm quantity and quality in Richardson's ground squirrels. I hypothesized that testis size is an indicator of sperm competition (H1), which predicts a positive relationship between testis size and seminiferous tubule diameter (P1.1), and testis size and sperm concentration (quantity, P1.2). My second hypothesis (H2) is that variation in sperm size will be related to sperm speed in Richardson's ground squirrels. Under intense sperm competition, I predicted that the size of the sperm (total sperm length and flagellum length) will influence sperm speed (P2.1). My third hypothesis (H3) is that body condition will influence sperm quantity and quality. We expect that males with better body condition will have higher sperm concentration, large testes size, and faster sperm (P3.1).

Methods

Trapping & handling

I used live traps (Tomahawk Live Trap Inc., WI, USA; 15 X 15 X 50 cm) baited with peanut butter (see Waterman et al. 2014) to capture Richardson's ground squirrel males (N = 20) from the Assiniboine Park Zoo in Winnipeg, Canada (N49 52 5 – W97 14 26) at the beginning of the breeding season from March 28th to April 5th, 2014. The first male observed was seen on March 12th 2014. Richardson's ground squirrels are routinely removed from the Assiniboine Park Zoo through their pest management program. Males were euthanized with isoflurane followed by an intracardiac injection of potassium chloride by zoo veterinarians (see Waterman et al. 2014).

I assessed male body condition by regressing body mass (spring scale Pesola AG, Baar, Switzerland) against spine length (measured from the occipital condyles to the base of the caudal vertebrae; Schulte-Hostedde et al. 2005; Manjerovic and Waterman 2012). This body condition index considers not only fat reserves, but also lean mass and water content (Schulte-Hostedde et al. 2005). Immediately following euthanasia, blood samples were collected from a cardiac puncture, and as an additional measure of body condition, I recorded the percentage of red blood cells (%RBC) from a microhematocrit centrifuge (International Medical Assistance, Inc., Indianapolis, USA). I made thin blood smears to quantify white blood cells (Boonstra et al. 2017). To assess neutrophil-lymphocyte ratio, differential counts of white blood cells in these smears were made at the Manitoba Veterinary Laboratory (Winnipeg, Manitoba). Neutrophillymphocyte ratio can be used as a measure of male condition as it is indicative of stress axis response (higher NRL indicates greater stress response; Boonstra et al. 2017).

All methods were approved by the University of Manitoba Animal Ethics Committee (Protocol F14-032) and in accordance with the American Society of Mammalogists Guidelines (Sikes et al. 2011).

Relative testes size (RTS), and seminiferous tubule diameter.

I recorded total testes mass using a MXX-212 Denver digital scale (Bohemia, NY, USA) to ± 0.01 g. I calculated relative testes size using an equation for rodents (Y = -0.031X^{0.77}; Kenagy and Trombulak 1986).

I fixed both testes in Bouins fixative for 24 hours after which I cut the testes into smaller parts. After 24 hours, I washed samples, placed them in 70% ethanol and finally processed them in paraffin embedding medium. I stained cross sections (5-7 μm) with hematoxylin and eosin stain (H&E, Carson and Hladik 2009). I took images using a CX41 phase contrast compound microscope (Olympus American Inc[©]., USA), and camera Aptina MT9F002 CMOS 1/2.3"(MU1403, AmScope, Irvine, EUA). I measured seminiferous tubule diameters with ImageJ 1.50i (Wayne Rasband National Institutes of Health, USA, Schneider et al. 2012) according to Anderson and Thliveris (1986).

Quantity and quality of sperm

Sperm quality includes phenotypic traits characterized by morphology (i.e., sperm abnormality), size, vitality, motility and sperm velocity (Snook 2005, Ruiz-Lopez et al. 2010). In this study I analyzed sperm size and speed as quality traits. To investigate the quality and quantity (sperm concentration) of sperm, I collected sperm from the cauda epididymis within 5 minutes of euthanasia and immediately cut it open into a small plate with 190μL of warm diluent (coconut water) using Thermo-plate (MAT-U55, Tokai Hit, Olympus, Japan) set at 37°C. Coconut water has been successfully used as a diluent or for cryopreservation of sperm with other mammals (Cardoso et al. 2005; Luzardo et al. 2010; Mollineau et al. 2011). Using a CX41 phase contrast compound microscope (Olympus American Inc[®]., USA). I immediately recorded videos of sperm movement using a camera (QImaging MicroPublisher 3.3 RTV) with 30 frames per second (FPS). I analysed videos with ImageJ computer assisted sperm analysis (CASA; Wilson-Leedy and Ingermann 2007; Kime et al. 2001; Schneider et al. 2012), with the modified ImageJ CASA_automated plugin (Purchase and Earle 2013) to determine the time-average velocity along the total point to point distance travelled by the sperm, the velocity average path (VAP, μm/s, Wilson-Leedy and Ingermann 2006, 2007).

To assess the quantity of sperm collected from the cauda epididymis, I placed 10µL of epididymal fluid in a Sefi-Medical Makler[®] counting chamber (Haifa 31070, Israel). I counted the number of sperm in 10 squares to determine sperm concentration in million per mL (World Health Organization 2010) under a compound microscope (40X, Carl Zeiss, West Germany). I fixed the remaining sperm in 10% phosphate-buffered formalin to collect digital images of at least 20 sperm from each male (World Health Organization 2010; Montoto et al 2011) using a differential-interference microscope (Zeiss Axio Imager A1, Jena, Germany). Sperm measurements were adapted from Tourmente et al. (2011) using a free software Image J (NIH Image, Schneider et al. 2012), and included: head width (HW), head length (HL), total flagellum length (TFL), and total sperm length (TSL).
Statistical analysis

I ran all analyses in R Program (R Core Team 2017, version 3.4.1), with an $\alpha = 0.05$ considered to be statistically significant. I used 20 sperm from each male (N = 17 squirrels) to identify intra-male variation on sperm size quantified by a calculation of coefficients of variation (CV; Sokal and Rohlf 1981; Immler et al. 2008). One male was considered azoospermic (lack of sperm), and two others were oligospermic (low sperm count), thus these three squirrels did not have enough fixed sperm to be analysed for size. Within-male variation in sperm size can be important to male fitness (Immler et al. 2008, 2014). CV variables were log-transformed to obtain a normal distribution and compared with male condition. I fitted distributions with fitdistrplus (Delignette-Muller and Dutang 2015). Data that were not normally distributed were compared using nonparametric statistics. I used Pearson and Spearman's rank correlations to examine association and collinearity amongst variables (Dytham 2011; Sokal and Rohlf 1981).

Phenotypic constancy or repeatability among males (i.e. ICC, Nakagawa and Schielzeth 2010; Wolak et al. 2012; Nakagawa et al. 2017), and within-male variation (Immler et al. 2008, 2014) are both important to understand which sperm traits are being selected under sperm competition, and relevant to understanding the evolution of sperm (Ros-Santella et al. 2015). The intraclass correlation coefficient (ICC), the proportion of among-male variation, is used to estimate repeatability or the expression of the genuine variability among males (Sokal and Rohlf 1981; Leushuis et al. 2010; Wolak et al. 2012). Very high ICC (from 0.80 to 1) and high ICC (0.61 to 0.79) represents a more heterogeneous population, and that differences among males are genuine, while 0.41 to 0.60 represent only moderate among male variance (Landis and Koch 1977; Leushuis et al. 2010). Lower ICC (from 0 to 0.40) means that within-male variation is due to fluctuation in samples of the same male or error (Leushuis et al. 2010). Sperm size (TFL and

TSL) and sperm speed (VAP) repeatability or ICC (Nakagawa and Schielzeth 2010; Nakagawa et al. 2017) were calculated using the ICCest package in R (ICCest, Package ICC version 2.3.0), and ICC will be reported as (ICC= the intraclass correlation coefficient, maximum of 1; % of confidence interval from the analysis, CI: lower and upper confidence interval limits; Wolak et al. 2012).

Using male identity (ID) as a random factor, I used a GLMM(Ime4 package, Bates et al. 2015) fit by maximum likelihood (Laplace Approximation, Harrison et al. 2018) to understand within-male (intra-male variation) and among-male (inter-male variation) effects, and how the size of sperm relates to sperm speed (form-function, prediction 2.1). I considered total flagellum length (TFL), total sperm length (TSL), sperm head length (HL) and sperm head width (HW) as explanatory variables with velocity average path (VAP, µm/s) as the response variable.

Only videos from 11 males were usable with the software, most likely because of differences in lighting during recording. In total samples from 10 individuals had all information necessary to be included in a GLMM analysis between sperm size and speed.

Results

Seminiferous tubule diameter (Spearman's rho = -0.21, P = 0.42, N = 20) and sperm concentration (Table 4.1) were not significantly related, nor were seminiferous tubule diameter and relative testes size (Spearman's rho = 0.24, P = 0.37, N = 20, Table 4.1, appendix 4.2, P1.1). However, I found a negative relationship between relative testes size and sperm concentration (Spearman's rho = -0.64, P < 0.01, N = 20, Figure 4.1a).

I compared sperm traits with velocity average path (VAP) and found that total sperm length (GLMM: t = 2.28, N = 10 individuals, P = 0.02), and flagellum length (GLMM: t = 3.68,

N = 10 individuals, P < 0.001) were positively related to velocity average path (Figure 4.2). No significant results were found between velocity average path and head length (GLMM: t = -0.26, N = 10 individuals, P = 0.80) nor sperm head width (GLMM: t = -0.69, N = 7 individuals, P = 0.49). For descriptive statistics and Image J results in sperm size and speed see Table 4.2, and more details about intra-male variation in sperm size can be seen in appendix 4.1. Male velocity average path (VAP) had high repeatability (ICC = 0.86), while repeatability for total flagellum length (ICC = 0.52) and total sperm length (ICC = 0.56) was only moderate (Table 4.3).

I found no significant relationship (Spearman's rank correlation) between male condition (P3.1) and RTS (body condition residuals: rho = -0.27, P = 0.25; %RBC: rho = -0.159, P = 0.53; N:L: rho = -0.06, P = 0.80, N = 19), sperm concentration (body condition: rho = 0.30, P = 0.21; %RBC: rho = 0.26, P = 0.26; N:L: rho = 0.10, P = 0.60, N = 17), nor VAP (body condition residuals: rho = 0.10, P = 0.75; %RBC: rho = 0.27, P = 0.41; N:L: rho = -0.45, P = 0.16, N = 10).

Discussion

Contrary to my predictions, I found a negative relationship between RTS and sperm concentration in Richardson's ground squirrels. Males with larger testis did not have more sperm in their epididymis, and I did not find an association between RTS and seminiferous tubules diameter. Velocity average path (VAP) was positively related to total sperm length and flagellum length, demostrating that longer sperm are faster in this species. Higher among-male variation was consistent with high repeatability, and lower within male variation in velocity average path but no relationship was found between male condition and RTS, sperm concentration or sperm speed.

Interspecifically, the relative testes size of Richardson's ground squirrels was similar to relative testes sizes of the solitary woodchuck (Marmota monax, Armitage 1986; Kenagy and Trombulak 1986; Allainé 2000) and harem-living Black-tailed prairie dogs (Cynomys ludovicianus, Hoogland 1995; Ramm et al. 2005), and smaller than Cape ground squirrels (Xerus *inauris*, Waterman 1998) and Barbary ground squirrels (*Atlantoxerus getulus*, Chapters 2 and 5). The OSR of Cape ground squirrels is higher than that of Richardson's ground squirrels while the OSRs of woodchucks (Armitage 1986; Kenagy and Trombulak 1986; Allainé 2000) and blacktailed prairie dogs (Hoogland 1995; Ramm et al. 2005) are lower. The OSR of Richardson's ground squirrels is suggestive of a lower level of post-copulatory sexual selection (sperm competition) compared to Cape and Barbary ground squirrels, and close to the two polygynous species (woodchucks and black-tailed prairie dogs). There is evidence in Philippine chrotomyine rodents that different levels of sperm competition are related to sperm (size of the tail and head) and relative testes sizes. Monogamous species (no sperm competition) had smaller testes size and higher variance in sperm traits. Promiscuous and polyandrous (high risk or intensity of sperm competition) species had larger testes, longer sperm with longer apical hooks and less variation in sperm size (Breed et al. 2019). Richardson's ground squirrel had total flagellum lengths closest to Apomys (Megapomys) abrae (mean of RTS: 0.56; HL: 6.6 µm; mean TFL: 109 µm), which have a low level of sperm competition, and the sperm head length closest to *Chrotomys* whiteheadi (mean of RTS: 1.43; HL: 9.1 µm; mean TFL: 135 µm), which have a higher level of sperm competition relative to other species of Philippine chrotomyine rodents (Breed et al. 2019).

My findings did not support the assumption that testis size is an indicator of sperm production intraspecifically, as I did not find a positive relationship between testis size and

seminiferous tubule diameter, and testis size and sperm concentration were negatively related. This negative relationship has also been observed in crickets (*Gryllus veletis*, Schaus and Sakaluk 2001; *Teleogryllus oceanicus*, Simmons et al. 2007; and across bush cricket species in the Tettigoniidae, Vahed et al. 2011), in species of gobiid fishes (*Zosterisessor ophiocephalus* and *Gobius niger*; Pilastro et al. 2002), and in Cape ground squirrels (Chapter 2). The production of ejaculates can be costly and as such, different species may use different strategies to invest in sperm production (Ramm and Stockley 2016). The strategy in Richardson's ground squirrels may be to decrease sperm production under a high intensity of sperm competition (Parker 2016) or transfer fewer sperm per ejaculate, but with more frequent copulations to guarantee a greater number of females inseminated (Vahed and Parker 2012).

Quantity of sperm is just one way that species can respond to sperm competition. Another way is through the quality of sperm. There are different factors that can contribute to sperm speed such as the length of sperm (Roldan 2019), and sperm competition can influence sperm head curvature or design, improving sperm speed (Varea-Sánchez et al. 2016). Longer sperm are predicted to arrive at the egg first, presumably because they swim faster (Gomendio and Roldan 1991; Lüpold et al. 2009; Tourmente et al. 2011). Rodent and primate species under sperm competition had longer and faster sperm than monogamous species (Gomendio and Roldan 1991). The speed and size of sperm were also highly correlated under sperm competition in an external fertilizing sea urchin (*Heliocidaris erythrogramma*, Fitzpatrick et al. 2010), 21 different species of eutherian mammals (Gomendio and Roldan 2008), and in two seasonal breeding species of lizards (*Tupinambis merianae* and *Tupinambisrufescens*, Blengini et al. 2014). Gomendio and Roldan (2008) suggest that interspecifically, sperm competition favors longer and faster sperm, that speed can be consider as a good indicator of the ability to compete by males,

and that speed can determine fertilization success in a competitive environment. My results supported the hypothesis that sperm size and speed were related intraspecifically in Richardson's ground squirrels.

The analysis of sperm morphology by measuring the size of the sperm head, the length of sperm and the sperm tail (Breed et al. 2011; Varea-Sánchez et al. 2014), can also provide important information about variation in sperm traits, and the proportion of among-male variation is the most used estimation for repeatability or the expression of the genuine variability among males (Sokal and Rohlf 1981; Leushuis et al. 2010; Wolak et al. 2012). Although, the idea that variation can be an important strategy to be successful in fertilizing the ovum, this idea is still controversial (Blengini et al. 2014). High variation in sperm size in an ejaculate (withinmale) was an important strategy to successfully compete and maximize the fertilization ability in sperm competition in lizards (Blengini et al. 2014). In Richardson's ground squirrels, I found higher among-male variation in sperm tail and total sperm length compared to within-male variation (Table 4.3), but a moderate repeatability, suggesting that among males there are some heterogeneity in sperm length (Leushuis et al. 2010). Richardson's ground squirrels had longer and faster sperm than Cape ground squirrels who have high levels of sperm competition (Chapter 3), and lizards (Blengini et al. 2014) with sperm competition. Arguably the length of sperm contributes to sperm speed in Richardson's ground squirrels. The highly consistent repeatability in sperm speed supports phenotypic constancy among males, suggesting some males may have more competitive sperm than others (Leushuis et al. 2010). Size and speed of the sperm may also be influenced by male age, and the importance of the age in sperm traits still needs to be investigated in Richardson's ground squirrels (Helfenstein et al. 2010). However, strong precopulatory selection results in over 50% of males disappearing during the breeding season and

only a few males live to a second year of breeding. Thus, the majority of males competing for reproductive opportunities are of a similar age (Michener and Locklear 1990).

In this study I investigated males in the first week of their breeding season, and contrary to my expectations, I did not find a relationship between sperm traits (quantity or quality) and male condition. There is evidence across taxa, including mammals, that nutrient limitation can decrease the production of sperm and non-sperm components of the ejaculate (Macartney et al. 2019). Richardson's ground squirrel males start spermatogenesis at the end of hibernation and close to the beginning of the breeding season (Michener 1992; Barnes 1996; Ramm and Schärer 2014). While males store food in their hibernaculum and consume food before emerging from hibernation, they do not spend much time spent foraging during the short breeding season (Michener and Locklear 1990; Hare et al. 2004). Both their testes size and body condition decline during the breeding season and male condition, as well nutrition limitation, may be important factors affecting sperm production later in the season (Michener 1983; Michener and Locklear 1990; Macartney et al. 2019). The relationship between body condition and sperm traits still needs to be investigated throughout the breeding season in Richardson's ground squirrels.

Richardson's ground squirrel males have a short time period (seasonal breeding) to compete for mates (pre-copulatory sexual selection) and to compete with sperm from other males inside the female tract (post-copulatory sexual selection). The intensity of the breeding season, including fighting and aggression among males, means that males have short reproductive tenures (most will only have a single year to breed) and they may put everything into reproduction despite varying body condition. To be a successful male, they need to be able to fertilize females as soon as the female becomes receptive, and our data suggest that quality

(faster sperm) may be an important strategy for Richardson's ground squirrel sperm to ascertain fertilization.

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Table 4. 1. Descriptive statistics and intraspecific variation (CV) of sperm concentration (N = 17), relative testes size, and percentage of red blood cells in Richardson's ground squirrel males (N = 20).

Character	Mean ± SE	CV (%)
Sperm concentration (million/mL)	59.25 ± 12.51	94.42
Relative testes size*	0.61 ± 0.06	40.69
Red blood cells (%)	45.93 ± 1.24	12.06

Detailed information about RTS see appendix 4.2.

	Sperm trait	Mean	SE	CV (%)	
Size (µm)	Head Width (HW)	8.18	±0.13	6.75	
	Head Length (HL)	11.17	± 0.28	10.52	
	Total Flagellum Length (TFL)	110.61	± 1.47	5.49	
	Total Sperm Length (TSL)	121.76	±1.65	5.58	
Speed (µm/s)	Velocity average path (VAP)	1512.93	±91.41	21.74	
	Curvilinear velocity (VCL)	3810.43	± 270.86	24.33	

Table 4.2. Descriptive statistics and Image J results for sperm size (N = 17) and speed (N = 10, CASA_automated plugin) of Richardson's ground.

The sperm speed had 677 observation generated by CASA program (average of 6.94 ± 1.24 sperm observed by male).

Table 4. 3. Intraclass correlation coefficient (ICC) from Richardson's ground squirrel males.

	ICC	Lower CI	Upper CI	varw	vara
VAP	0.86	0.72	1.00	26044.78	162862.00
TSL	0.56	0.38	0.74	34.14	43.42
TFL	0.52	0.34	0.71	31.17	34.49

VAP: velocity average path, N = 10; TSL: total sperm length, N = 17; TFL: total flagellum length. Alpha = 0.05. N = 17. Lower CI: lower confidence interval limit, upper CI: upper confidence interval limit, varw: the within individual variance, vara: the among individual variance (ICCest package in R version 2.3.0, Wolak et al. 2012).



Figure 4.1. Relationship between relative testes size (RTS) and A: sperm concentration (Spearman's rho: P < 0.01). B: seminiferous tubule diameter in Richardson's ground squirrels (Spearman's rho: P = 0.37).



Figure 4. 2. Relationship between total sperm flagellum length (TFL) and sperm velocity average path (VAP) in Richardson's ground squirrels.

Chapter 5: Barbary ground squirrel: sperm competition in an invasive population

Abstract

Reproductive success can be influenced by sexual selection that can occur before or after copulation, and can include post-insemination mechanisms, such as sperm competition (postcopulatory sexual selection). Inbreeding depression is a consequence of reproduction between close kin leading to an increase in homozygosity, an important problem to small populations, as it is marked by a decrease in reproductive success often due to negative effects on fertility, including sperm traits. Understanding the possible consequences of inbreeding depression on fertility in wild populations, and how sperm competition can affect fertilization success is important to understand inbreeding in wild populations. The objective of my study was to explore the impact of sperm competition on testis size and sperm traits in a highly inbred population. I hypothesized that testes size is an indicator of sperm competition, and that inbreeding depression will negatively impact sperm traits (quantity and quality) in a highly inbred invasive population of Barbary ground squirrels (Atlantoxerus getulus), a non-hibernating species native to Northern Africa. Barbary ground squirrels had large testes sizes but there was no relationship (intraspecifically) between relative testes size (RTS) and sperm concentration. Contrary to many other species that have experienced strong genetic bottlenecks, I did not find reduced quantity or quality of sperm traits in the island population of Barbary ground squirrels. Barbary ground squirrels had higher sperm concentration, lower sperm abnormalities and larger RTS compared to many other species of rodents, suggesting no negative effects of inbreeding on testis size and sperm traits in this species. This study brings a better understanding of the reproductive success of an invasive population of small mammals.

Keywords: sexual selection, relative testes size, sperm concentration, sperm quality, inbreeding depression, Fuerteventura Island.

Introduction

Reproductive success can be influenced by intra- and inter-sexual selection that can occur before or after copulation (Pizzari and Parker 2009), and can include post-insemination mechanisms, such as sperm competition (post-copulatory sexual selection; Parker 2014). When a female copulates with two or more males, competition for the fertilization of ova can happen internally among the sperm from different males, a process called sperm competition (Parker 1970). Sperm competition, and variation in the level of sperm competition, can be an important selective pressure influencing different male traits (morphology and physiology, Parker and Pizzari 2010; Sandera et al. 2013; Roldan 2019).

The evolution of testes can also be affected by different degrees of sperm competition intensity (Møller 1998). In species with a high intensity of sperm competition, males usually have larger testes sizes, most likely to produce more sperm (called the numerical sperm competition hypothesis; Parker and Pizzari 2010; Tourmente et al. 2011; Vahed and Parker 2012). The quality of sperm, termed the ejaculate fertilizing efficiency (Parker and Pizzari 2010), may also be critical to reproductive success. The quality of sperm comprises a group of phenotypic traits, such as vitality, motility, and morphology (Snook 2005), which could affect fertility success differently (Snook 2005; Ruiz-Lopez et al. 2010). Overall, high levels of sperm competition select for higher sperm concentration and enhance sperm form and function (more

sperm, faster sperm and fewer abnormalities; Ros-Santaella et al. 2015; Delbarco-Trillo et al. 2018).

In contrast to high levels of sperm competition, high levels of inbreeding usually have a negative effect on sperm traits (Santymire et al. 2015). Inbreeding depression is a consequence of reproduction between close kin leading to increase in the homozygosity of individuals (Charlesworth and Charlesworth 1987). It is considered an important problem to small populations, as it is marked by a decrease in reproductive success (Barret and Charlesworth 1991; Caro and Laureson 1994; Hasselgren and Norén 2019). When just a few individuals establish a new population (i.e. founder effect, one type of population bottleneck), the new population is expected to have low genetic variability and heterozygosity usually leading to a population decline or even extinction (Charlesworth 2006; Roldan and Gomendio 2009). This low genetic variability is thought to negatively impact reproduction, including mating ability, maturation, female fecundity and sperm traits (Santymire et al. 2015). Inbreeding is expected to negatively impact both sperm concentrations (e.g., oldfield mice, *Peromyscus polionotus*, Margulis and Walsh 2002) and sperm quality (e.g., increased sperm abnormalities found in a small isolated, and inbred population of lions, Panthera leo, Wildt et al. 1987; wild rabbits (Oryctolagus cuniculus, Gage et al. 2006). However, there are also examples where no relationship has been found between inbreeding and the quantity or quality of sperm traits (e.g. mosquitofish, Gambusia holbrooki, Marsh et al. 2017; song sparrows, Melospiza melodia, Losdat et al. 2018).

The Barbary ground squirrel (*Atlantoxerus getulus*) is a non-hibernating species native to Northern Africa. In 1965, two animals were introduced to the island of Fuerteventura, in the Canarian archipelago, from a mainland Moroccan population (Machado 1979; López-Darias et

al. 2008). Since that introduction, the population has increased from two individuals to over a million (López-Darias 2007) and the current population is highly inbred, with an effective population size of 77. Mitochondrial DNA of individuals compared from island and mainland supported that intense genetic bottleneck happened during their colonization of the island (Kratzer et al. *unpubl. data*). Over 75% of adult females mate and successfully raise litters, and litter sizes are relatively large, averaging 3 offspring per litter but can be as large as 8 offspring (van der Marel et al. *Submitted*). Breeding in this species is seasonal, with males fully scrotal over a period of 76 days (van der Marel et al. *Submitted*). Like the closely related Cape ground squirrel (*Xerus inauris*), the Barbary ground squirrel is promiscuous and has a high OSR (approximately F1:M6; van der Marel *unpubl. data*), suggesting that sperm competition could be an important factor in male reproductive success. The successful growth and expansion of this invasive population over 55 years suggest that even with very low genetic diversity, the population is not experiencing inbreeding depression, but it is unknown how inbreeding has influenced their sperm traits.

The objective of my study was to explore the impact of sperm competition on the testis size and sperm traits in a highly inbred population of Barbary ground squirrels on the island of Fuerteventura, Spain. As an interspecific comparison, I found a positive relationship between relative testes size (RTS) and the operational sex ratio of different squirrel species indicating levels of sperm competition (Chapter 2). I tested this hypothesis, that testes size is an indicator of sperm competition, in Barbary ground squirrels (H1). I predicted that their testes size (RTS) will be larger compared to other species of squirrels (interspecific comparison, P1.1) that have lower OSRs and that there will be a positive relationship between testis size and sperm quantity in Barbary ground squirrels (intraspecific comparison, P1.2). My second hypothesis examined if

inbreeding depression has affected sperm traits (quantity and quality) in this invasive population (López-Darias et al. 2008; H2). I predicted that this highly inbred population will have low sperm concentrations, low levels of live sperm (vitality), low motility, and high levels of sperm abnormalities similar to other highly inbred mammals (P2.1).

Methods

Trapping & handling

I sampled free-ranging adult male Barbary ground squirrels in January 2016 during the mating season (van der Marel et al. *Submitted*). In total, 20 males were collected in La Oliva (N28 37 9 - W13 55 38), Tindaya (N28 35 9 - W14 0 45), Volcano (N28 42 5 - W13 55 11) and Playa Esquinzo (N28 38 11 - W14 1 31) in Fuerteventure, Spain. Barbary ground squirrels were euthanized with an intraperitoneal injection of sodium pentobarbital (see López-Darias et al. 2008a). All handling was in accordance with the American Society of Mammalogists Guidelines (Sikes et al. 2011) and the University of Manitoba Animal Ethics Committee (Protocol F14-032).

Relative testes size (RTS).

I measured total testes mass with an MXX-212 Denver digital scale (Bohemia, NY, USA) to ± 0.01 g. Relative testes size is a good measure to compare testes size within and among species (Kenagy and Trombulak 1986; Parker 2016). I calculated the RTS of Barbary ground squirrels using an equation for rodents (Y = $-0.031X^{0.77}$; Kenagy and Trombulak 1986).

Quality and quantity of sperm

To investigate the quality and quantity of sperm, I collected sperm from the cauda epididymis within 5 minutes of euthanasia. The epididymis was immediately cut while in a small petri dish with 190µL of warm diluent (coconut water) placed on top of a Thermo-plate (MAT-U55, Tokai Hit, Olympus, Japan) set at 37°C. Coconut water has been successfully used as a diluent or for cryopreservation of sperm with other mammals (Cardoso et al. 2005; Luzardo et al. 2010; Mollineau et al. 2011). Using a compound microscope (20X, Carl Zeiss, West Germany), I immediately assessed the percentage of sperm with progressive motility and recorded these in ranges of 1 (1 to 20%), 2 (21 to 40%), 3 (41 to 60%), 4 (61 to 80%), and 5 (81 to 100%), following the World Health Organization (2010).

I made two sperm smears for each animal and stained them with Hancock Stain[®] (Chino, CA, USA) to evaluate sperm vitality (World Health Organization 2010). I assessed sperm vitality (the percentage of live sperm, World Health Organization 2010) by counting 100 sperm from each smear using a phase contrast microscope (40X, Model CX41, Olympus American Inc[®]., USA), and taking the mean percentage from both replicates. I placed 10µL of epididymal fluid in a Sefi-Medical Makler[®] counting chamber (Haifa 31070, Israel) and counted the number of sperm in 10 squares to determine epididymal sperm concentration in million per mL under a compound microscope (40X, Carl Zeiss, West Germany; World Health Organization 2010).

I fixed the remaining sperm in 10% phosphate-buffered formalin and used it to assess sperm abnormalities. I determined the percentage of abnormal sperm according to criteria the World Health Organization (2010) by making two additional smears from the fixed sperm and counting 200 cells from each replicate (total of 400 cells counted/ male) under a phase contrast microscope (40X, Model CX41, Olympus American Inc[©]., USA). As the sperm samples were collected directly from the epididymis, an important place for sperm maturation, the sampled sperm were still involved in the last phases of spermiogenesis, including releasing part of the cytoplasm from the cell. For this reason, I did not consider cytoplasm droplets as an abnormality in this study (Cornwall and von Horsten 2007).

Statistical analysis

I ran all analyses in R Program (R Core Team 2017; R version 3.4.1), with an α of 0.05 set as statistically significant. I tested for normality using a Shapiro-Wilk normality test. Among male variation was quantified by coefficients of variation (CV; Sokal and Rohlf 1981; Immler et al. 2008). Spearman rank test correlations were used to check the association amongst variables (Sokal and Rohlf 1981; Dytham 2011). Data that were not normally distributed were tested using nonparametric statistics. Results are reported as mean \pm SE (% CV)

Results

The mean RTS of Barbary ground squirrels was 3.12 ± 0.09 g (12.48%, N = 20). The relationship between RTS and sperm concentration was not significant (Spearman's rho = -0.06, P = 0.80, N = 20 males, P1.2, see detailed information about RTS appendix 5.1). The descriptive statistics of the sperm of Barbary ground squirrels were: epididymal sperm concentration 584.60 \pm 36.57 (27.97%, N = 20) million sperm cells per mL, percentage of live sperm (sperm vitality) 95.40 \pm 0.44% (2.07%, N = 20), motility 4.25 \pm 0.19 (range from 61 to 80% sperm with progressive motility; 20.02%, N = 20), and sperm abnormality was 1.88 \pm 0.34% of abnormal cells (79.94%, N = 20).

Discussion

Barbary ground squirrels have lower operational sex ratios but larger RTS than Cape ground squirrels (Waterman 1998), Eastern gray squirrels (*Sciurus carolinensis*, Koprowski 1993a; Kenagy and Trombulak 1986) and California ground squirrels (*Otospermophilus beecheyi*, Dobson 1984; Boellstorff et al. 1994; Kenagy and Trombulak 1986). They also have larger RTS, and higher, but similar OSR to that of Eastern fox squirrels (*Sciurus niger*; Koprowski 1993b). Testes size is often considered as a proxy for sperm competition and sperm production (Harcourt et al. 1981; Ramm et al. 2014). Like in the Cape ground squirrel (Chapter 2), I did not find a positive relationship between RTS and sperm concentration intraspecifically, suggesting testis size may not be a good intraspecific indication of sperm concentration or sperm investment.

However, interspecifically, relative testes size does appear to reflect intensity of sperm competition (Ramm et al 2005; Ramm and Schärer 2014; Rowley et al. 2018; Chapter 2) and the large RTS of Barbary ground squirrels suggests that sperm competition is intense. It is predicted that in species with larger litters, sperm competition will be stronger, as there is more potential for multiple paternity in larger litters resulting in higher reproductive success for more males during a single estrus event (Abebe et al. 2019). Litter sizes of Barbary ground squirrels (mean 3.0, range 1-8 offspring; van der Marel 2019) are mid-range amongst sciurids, as they are similar to Eastern fox squirrels (mean 2.5, range 2-3, maximum 7 offspring; Thorington et al. 2012), and Eastern gray squirrel (mean 3, range 2–4, maximum 8 offspring; Thorington et al. 2012), but smaller than California ground squirrels (mean 8, range 5-11 offspring). Barbary ground squirrel litters are much larger than the closely related Cape ground squirrels (mean litter size of 1.6, range 1-2 offspring; Waterman 1996). The large testes size of Barbary ground squirrels, but

moderate-sized litters and operational sex ratios, do not seem to fit the typical patterns seen in other species of mammals (Abebe et al. 2019). Thus, future studies should investigate the relationships between RTS, litter sizes and operational sex ratios in sciurids.

The production of ejaculates can be costly, and males can respond differently depending of levels of sperm competition (Parker et al. 1996; Delbarco-Trillo and Ferkin 2006). Barbary ground squirrels had higher sperm concentrations compared many other rodent species including rats (*Rattus norvegicus*, 43.8 million/ mL), agoutis (*Dasyprocta leporine*, 74.69 million/ mL), and capybaras (*Hydrochoerus hydrochaeris*, 17.21 million/ mL; Lüpold and Fitzpatrick 2015). Compared to three species of Mus with different levels of sperm competition (house mice, Mus musculus, with low; Algerian mice, Mus spretus, with intermediate; and mound-building mice, Mus spicilegus, with high sperm competition), Barbary ground squirrels had higher sperm concentrations than all three species, and similar sperm motility to the two species with intermediate to high sperm competition (Delbarco-Trillo et al. 2018). The sperm concentrations of Barbary ground squirrels were higher, but close to chimpanzees (*Pan troglodytes*, 518.84; Lüpold and Fitzpatrick 2015), which have high levels of sperm competition. In comparison with other ground squirrels from my thesis, Barbary ground squirrels from this highly inbred population had higher sperm concentrations, higher levels of live sperm (vitality), higher motility, and lower levels of sperm abnormality compared to Richardson's ground squirrels (Urocitellus richardsonii, chapter 4) and the closely related Cape ground squirrel (chapter 2). This latter southern African species is considered to be under strong selection via sperm competition (Manjerovic et al. 2008; Manjerovic and Waterman 2015), but its mean concentration of sperm (400.75 \pm 24.23 m/mL; Chapter 2) was lower than Barbary ground squirrels.

Sperm traits are highly variable in mammals (Roldan 2019). Species with a low risk of sperm competition often have low sperm quantity and quality because of a lack of selective pressures (van der Horst et al. 2011; van der Horst and Maree 2014). For example, naked molerats (Heterocephalus glaber) have very low levels of sperm competition and their sperm have degenerative features in the midpiece and tail (van der Horst et al. 2011). In primates, species with a low risk of sperm competition, such as gorillas (Gorilla gorilla) and humans, have higher sperm abnormalities compared to chimpanzees that are promiscuous (van der Horst and Maree 2014). Under more intense sperm competition, intraspecific variation in sperm traits is reduced (Ros-Santaella et al. 2015; Delbarco-Trillo et al. 2018), including sperm abnormalities (Montoto et al. 2011). For example, in a comparison of three closely related species of Mus, sperm abnormalities were much lower in the two species with high levels of sperm competition (Delbarco-Trillo et al. 2018). Sperm abnormalities in the promiscuous bank vole (Clethrionomys glareolus) were approximately 10%, the promiscuous long-tailed field mouse (Apodemus sylvaticus) had 6.5%, and the promiscuous mound-building mouse (Mus spicilegus) had around 15.5% (Wolff and Sherman 2007; Montoto et al. 2011). The large RTS, high sperm concentrations and low percent of abnormalities in Barbary ground squirrels suggest this species is under strong selection from sperm competition.

However, there is evidence in different species that sperm traits can be negatively affected by inbreeding depression (Losdat et al. 2014). Examples of inbreeding effects on sperm traits include increased sperm abnormalities in inbred lions in the Ngorongoro Crater in Tanzania (50.5% of abnormal sperm) and from Sakkarbaug Zoo, India (66.2%; Wildt et al. 1987). Lower sperm motility and higher sperm abnormality has been observed in many endangered mammals with small population sizes (Fitzpatrick and Evans 2009; Roldan et al. 1998). Inbred populations

of cheetah (*Acinonyx jubatus*) had 81% abnormal sperm and low sperm concentrations (61 ±10.6 million sperm cells per mL; Terrell et al. 2016). In zebra finch (*Taeniopygia guttata*), inbreeding was associated with lower sperm motility and higher sperm abnormality (22%) in both wild and captive populations (Opatová et al. 2016). An inbred population of Florida panthers (*Felis concolor*) had lower ejaculate volumes, lower sperm concentrations and higher sperm abnormalities compared to outbred populations (93.5%; Barone et al. 1994). Similarly, 56.2% of sperm had abnormalities in an inbred population of Iberian lynx (Gañán et al. 2010). My study population of inbred Barbary ground squirrels had lower sperm abnormalities than an outbred population of the closely related Cape ground squirrel, which had 3.4% abnormal sperm (Manjerovic and Waterman 2015; Shave and Waterman 2017; Chapter 2).

The interaction of sperm competition and inbreeding on sperm traits is complex and inbreeding depression does not always negatively affect sperm traits (Fritzsche et al. 2006, Bocedi and Reid 2017). In golden hamsters (*Mesocricetus auratus*), despite increased reproductive success in wild-derived vs. laboratory animals, no differences in sperm traits were observed (Fritzsche et al. 2006). Inbreeding in three-spined sticklebacks (*Gasterosteus aculeatus*) did not influence testis size and sperm traits, but a trade-off between sperm quantity and sperm quality was observed when comparing inbred and outbred males (Mehlis et al. 2012). Similarly, no negative effects of inbreeding on sperm traits were found in species with moderate inbreeding, such as song sparrows (*Melospiza melodia*, Losdat et al. 2018), and mosquitofish (*Gambusia holbrooki*, Marsh et al. 2017). Moreover, environmental conditions can affect the magnitude of inbreeding depression (Roldan and Goomendio 2009) and, in some cases, such as cactus finches (*Geospiza scandens*) and medium ground finches (*Geospiza fortis*), changes in

environment conditions (i.e., number of competitors, and food availability) impacted the effects of inbreeding depression (Keller et al. 2002).

The high quality of sperm (quantity and quality), as well as the rapid growth and expansion of the Barbary ground squirrel population across the island of Fuerteventura (López-Darias 2007), suggests that inbreeding depression is not currently impacting reproduction in this species. One possible explanation for this high quality and for the robustness of the population, despite having only two founders, is that there has been an elimination (i.e. purging) of deleterious traits (Hasselgren and Norén 2019). As sperm competition can impose strong directional selection, inbred species with strong sperm competition may eliminate deleterious sperm genes (Robinson et al. 2018).

In conclusion, contrary to what is found in many other of species that have experienced a strong genetic bottleneck, I did not find low quantity or quality of sperm traits in Barbary ground squirrels from Fuerteventura island. Barbary ground squirrels had higher sperm concentration, lower sperm abnormality and larger RTS compared to other inbred species, and even relative to species that were not inbred, suggesting there is little evidence of inbreeding depression on testis size and sperm traits in this species. This study brings a better understanding of the mechanisms that account for successful male reproduction in an invasive population of Barbary ground squirrels.

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Chapter 6: General Conclusion

The results of the research conducted in this thesis bring a new understanding of sperm competition in three species of ground-dwelling squirrels. A comparison in ecology and behaviour among Cape ground squirrel, Richardson's groung squirrel, and barbary ground squirrel can be found in Table 6.1. The chapters over-view and main findings are described in Table 6.2, and results can be summarized by the following findings:

- 1. Testes size can represent levels of sperm competition interspecifically.
- 2. Intraspecifically, larger testes do not necessarily mean more sperm.
- Male condition is an important factor influencing sperm quantity in Cape ground squirrels.
- 4. Sperm competition is an important selective pressure and variation in sperm traits (within and among-male) can be an important factor in post-copulatory sexual selection.
- 5. Both the quality and quantity of sperm are important. However, different species with different levels of pre- and post-copulatory sexual selection can use different strategies in their sperm traits to be competitive.
- 6. Sperm form (size and shape) and sperm function (motility and speed) are related. Sperm speed was not necessarily determined by the sperm length in Richardson's ground squirrels. Sperm head shape was a determinant factor probably helping in the hydrodynamics of the sperm and consequently, speed in Cape ground squirrels.
- Contrary to my expectations, Barbary ground squirrels had higher sperm concentration, lower sperm abnormality, and larger RTS compared to other species of rodents and

compared to Cape ground squirrels and Richardson's ground squirrels. This result suggests that there is little or no evidence of inbreeding depression on testis size and sperm traits in this species.

Hypotheses and predictions tested and their results from each chapter of this thesis are described in Table 6.3. Cape ground squirrels (**Chapter 2**) had high sperm concentration, vitality and motility, as well as a low frequency of sperm abnormality, compared to other species of mammals. Intraspecifically, males in better body condition (residuals) produced more sperm and had larger Cowper glands. However, males with smaller relative testes sizes were more likely to produce epididymal sperm at higher concentrations than males with larger testes. Differences in sperm quality can also be the determinant of fertilization success, and consequently reproductive success.

Despite controversial findings between the form and function of sperm found in the literature (Tourmente et al. 2011; Blengini et al. 2014), my data support the prediction that sperm form and function are related in Cape ground squirrels (**Chapter3**). The results also suggest that it is not the length of the sperm that was the most important factor influencing sperm speed. Sperm head width and sperm head shape had the most influence on sperm speed, and both, within and among-male variation were important to sperm speed in Cape ground squirrels.

Richardson's ground squirrel males (**Chapter 4**) have a short time period (seasonal breeding) to compete for mates (pre-copulatory sexual selection) and to compete with sperm from other males inside the female tract (post-copulatory sexual selection). The intensity of the breeding season with fighting and aggression among males means that males have short reproductive tenures (most will only have a single year to breed) and they may put everything into reproduction despite varying body condition. To be a successful male, they need to be able

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to fertilize females as soon as the female becomes receptive, and my results suggest that quality (longer and faster sperm), may be a more important strategy than sperm concentration for Richardson's ground squirrel sperm to ascertain fertilization. An interspecific comparison between Cape ground squirrel and Richardson's ground squirrel with main results can be found in Table 6.4.

Finally, Barbary ground squirrel males (**Chapter 5**), contrary to what is found in many other species that have experienced a strong genetic bottleneck, do not have low quantity or quality of sperm traits in an inbred population. Barbary ground squirrels had higher sperm concentration, lower sperm abnormality and larger RTS compared to other species of rodents and other inbred species, suggesting there is little evidence of inbreeding depression on testis size and sperm traits in this species.

This research brings a better understanding of the mechanisms such sperm production, function and structure, behaviour, ecology, and evolution that account for successful male competition for fertilization in three species of ground squirrels with different life histories. This study also brought new questions and suggestions to be investigated in the future, with application in different fields of research, including reproduction (medicine and veterinary), conservation, ecology and evolution.

Future directions

The testis in mammals has an important cell responsible for spermatogenesis, called the Sertoli cell. Sertoli cells help during spermatogenesis by supporting, nurturing, and protecting the germ cells such as spermatogonium or spermatids, which are cells located within the seminiferous tubules inside the testis (Borysenko and Beringer 1984). The proportion of the

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spermatogenetic tissue in the testes is variable among species and may be influenced by the intensity and risk (level) of sperm competition (Ramm and Schärer 2014). Moreover, diet also can influence sperm and semen production (Aitken and Roman 2008; Macartney et al 2019). For example, in an experiment performed on rats (*Rattus novergicus*), zinc concentration was critical for spermatogenesis, as zinc-deficient diet was associated with a decrease in testes size and sperm production (Kumari et al 2011). Thus, to understand the constraints on sperm competition, it is important to investigate the relationships between minerals, fatty acids (lipid components from testes) and sperm production.

Associated with a high number of sperm, the primary strategy used by Cape ground squirrels seems to be the significant presence of sperm agglutination. All slides visualized from all animals presented more than 50 sperm clumped head to head, and despite the decreased motility of clumped sperm, the agglutination could act as a physical barrier to block the sperm of the next possible male to copulate. In addition, sperm from agglutinations can separate after some time while within the female, and possibly move to fertilize the egg (Cohen-Dayag and Eisenbach 1994). Changes in the environmental conditions inside the female reproductive tract or changes in sperm during the trajectory to reach the egg can facilitate or impede the movement of sperm (Roldan 2019). Whether sperm agglutination is an important factor happening inside the female tract, or a by-product of the procedures used during our sperm collection and sample analysis, remain to be determined.

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Table 6. 1. Interespecific comparison in ecology and behaviour among three species of ground-
dwelling squirrels investigated under this research.

	Cape ground	Barbary ground	Richardson's ground
	squirrel	squirrel	squirrel
Hibernating	No	No	Yes
Territoriality	Non-territorial	Non-territorial	Strongly territorial
Breeding season	All year	1 or 2/year	1/year
Operational sex ratio	11/1	6/1	3/1
(M / F)			
Females multiple	Yes (1-10)	Yes	Yes (1-4)
mate			
Litter size range	1-2	1-8	1-14

Chapter number	Ground squirrel species	Main idea
Chapter 1		Rationale and background
Chapter 2	Cape ground squirrels	Intraspecifically, males in better body condition (residuals) produced more sperm and had larger Cowper glands. Males with smaller relative testes sizes were more likely to produce epididymal sperm at higher concentrations than males with larger testes.
Chapter 3	Cape ground squirrels	Sperm form and function are related Sperm head width and sperm head shape had the most influence on sperm speed Within and among-male variation were important to sperm speed
Chapter 4	Richardson's ground squirrel males	Sperm form and function are related Quality (longer and faster sperm), may be a more important strategy than sperm concentration
Chapter 5	Barbary ground squirrel males	Barbary ground squirrels had higher sperm concentration, lower sperm abnormality and larger RTS compared to other species of rodents and other inbred species There is little evidence of inbreeding depression on testis size and sperm traits in this species.
Chapter 6		Overall conclusion

Table 6. 2. Summary of the thesis with chapters over-view and main findings.

Chapter number	Hypothesis	Predictions	Supported
	Testis size is an indicator of sperm competition in sciurids (H1)	A positive relationship between operational sex ratio and relative testes size in sciurids (P1.1)	Yes
		Intraspecifically, there will be a positive relationship between testis size and sperm quantity in Cape ground squirrels (P1.2)	No
2	Accessory glands are an indicator of sperm competition (H2)	A positive relationship between testis size and accessory glands mass (P2.1)	No, negative relationship
	Male condition will be related to testis size, epididymal sperm concentration, sperm quality and accessory glands mass (H3)	Males with better condition are predicted to have larger testes (P3.1)	No
	quality and accessory grands mass (113)	Males with better condition are predicted to have higher sperm concentration, higher sperm motility and lower sperm abnormality (P3.2)	Partially (sperm concentration)
		Males with better condition are predicted to have larger accessory glands (P3.3).	Partially (Cowper glands)
3	variation in sperm size and shape amongst males will be linked to sperm velocity (H1)	The size of sperm (total sperm length, flagellum and head length, and head width, P1.1) will influence sperm speed in Cape ground squirrels	Partially (head width)

Table 6. 3. Hypotheses and predictions of the thesis and if they were supported.

		Sperm head shape (P1.2) will influence sperm speed in Cape ground squirrels	Yes
4	Testis size is an indicator of sperm competition (H1)	A positive relationship between testis size and seminiferous tubule diameter (P1.1)	Yes
		A positive relationship between testis size and sperm concentration (quantity, P1.2)	No, negative relationship
	Variation in sperm size will be related to sperm speed in Richardson's ground squirrels (H2)	The size of the sperm (total sperm length and flagellum length) will influence sperm speed (P2.1)	Yes
	Body condition will influence sperm quantity and quality (H3)	Males with better body condition will have higher sperm concentration, large testes size, and faster sperm (P3.1).	No
5	Testis size is an indicator of sperm competition (H1)	Barbary ground squirrel's testes size (RTS) will be larger compared to other species of squirrels (interspecific comparison, P1.1) that have lower OSRs	Yes
		There will be a positive relationship between testis size and sperm quantity in Barbary ground squirrels (intraspecific comparison, P1.2).	None
	Inbreeding depression has affected sperm traits (quantity and quality) in this invasive population	Highly inbred population will have low sperm concentrations, low levels of live sperm (vitality), low motility, and high levels of sperm abnormalities similar to other highly inbred mammals (P2.1).	No

Table 6. 4. Interspecific comparison of main results between Cape ground squirrel andRichardson's ground squirrel.

	Cape ground squirrel	Richardson's ground squirrel
Testes vs sperm concentration	Negative relationship	Negative relationship
Body condition	Sperm concentration and Cowper glands	Not supported
Quantity or quality	Higher sperm concentration, lower abnormality, and higher vitality	Longer and faster sperm
Variability of sperm traits	Sperm head shape	Sperm length

Appendices



Appendix 1.1. The structure of the spermatogenic epithelium in mammals. Cape ground squirrel testes. Bouin, TS, 40X.

Sperm	Abnormality (WHO 2010)
Head	Tapered Pyriform No acrosome Small Amorphous Vacuolated Two heads
Neck and midpiece	Bent neck Asymmetrical Thick insertion Thin neck* Thin midpiece
Tail	Short Bent Coiled Two tails

Appendix 2. 1. Description of sperm abnormalities investigated in this study with Richardson's ground squirrels.

*Adapted after WHO (2010).

		Total testes	
Male ID	Body mass (g)	mass (g)	RTS
ZA01	689	12.14	2.56
ZA02	745	15.02	2.98
ZA03	655	13.98	3.06
ZA04	710	15.66	3.22
ZA05	695	15.64	3.27
ZA06	720	20.52	4.18
ZA07	685	13.53	2.86
ZA08	865	18.42	3.25
ZA09	660	11.33	2.47
ZA10	625	9.03	2.05
ZA11	745	13.89	2.75
ZA12	745	14.43	2.86
ZA13	595	12.54	2.96
ZA14	665	13.01	2.81
ZA15	845	12.92	2.32
ZA16	580	8.65	2.08
ZA17	675	13.39	2.86
ZA18	700	13.13	2.73
ZA19	705	12.63	2.61
ZA20	690	13.26	2.79

Appendix 2. 2. Body mass (g), total testes mass (g), and relative testes size (RTS) in Cape ground squirrels.

Total testes mass (right + left), N = 20. RTS calculated according Kenagy and Trombulak (1986).



Appendix 2. 3. No significant relationship found between: A) relative testes size (RTS) and Cowper glands (linear regression, P = 0.34); B) relative testes size (RTS) and prostate (linear regression, P = 0.57); C) relative testes size (RTS) and seminal vesicles (linear regression, P = 0.69); D) body condition index (mass/size residuals) and relative testis size (RTS, linear regression, P = 0.79) in Cape ground squirrels.



Appendix 2. 4. No significant relationship was found between % RBC (linear regression, P = 0.16), nor N:L (linear regression, P = 0.18) and sperm concentration in Cape ground squirrels.



Appendix 2. 5. A, B, and C) No relationship was found between sperm abnormality and any condition measure (Spearman's rank correlation), body condition index: rho = -0.22, P = 0.36, percentage of red blood cells: rho = 0.19, P = 0.45, and neutrophillymphocyte ratio: rho = -0.18, P = 0.48). D, E, and F) No relationship was found between sperm vitality and any measure of condition (body condition index: rho = -0.18, P = 0.45; percentage of red blood cells rho = -0.03, P = 0.92); and neutrophil-lymphocyte ratio: rho = 0.14, P = 0.57). J, K, and L). G, H, and I) sperm motility and any condition measure were not correlated (body condition index: rho = 0.09, P = 0.71; percentage of red blood cells: rho = 0.05, P = 0.85; and neutrophil-lymphocyte ratio: rho = 0.22, P = 0.38) in Cape ground squirrels.

Male	Head width (µm)		ale Head width (μm) Head length (μm)				Total Sperm length (µm)		
	Mean	SD	CV (%)	Mean	SD	CV (%)	Mean	SD	CV (%)
ZA01	6.86	0.42	6.07	10.06	0.52	5.12	65.17	1.84	2.82
ZA02	7.07	0.37	5.23	9.86	0.35	3.55	63.42	0.96	1.51
ZA03	7.29	0.42	5.78	10.08	0.46	4.56	64.66	1.68	2.59
ZA04	6.67	0.87	13.01	10.19	0.44	4.33	64.01	2.61	4.08
ZA05	7.01	0.40	5.64	10.31	0.50	4.83	64.89	2.22	3.42
ZA06	7.12	0.43	5.98	10.26	0.51	4.95	65.23	1.93	2.96
ZA07	7.21	0.54	7.43	10.26	0.44	4.26	65.43	1.07	1.64
ZA08	6.92	0.60	8.61	9.93	0.46	4.65	64.86	1.84	2.84
ZA09	7.19	0.48	6.73	10.08	0.41	4.10	65.92	1.11	1.68
ZA11	7.09	0.53	7.49	9.83	0.42	4.29	64.04	0.92	1.44
ZA12	7.04	0.65	9.26	9.98	0.52	5.21	63.23	1.61	2.55
ZA13	7.22	0.52	7.14	10.12	0.59	5.79	61.48	5.66	9.21
ZA14	7.46	0.44	5.87	10.58	0.38	3.60	65.23	2.68	4.11
ZA15	7.13	0.48	6.76	9.96	0.50	5.00	64.12	1.99	3.10
ZA16	7.21	0.49	6.78	9.90	0.49	4.96	64.93	2.32	3.57
ZA17	7.18	0.46	6.45	10.34	0.54	5.18	65.20	1.52	2.33
ZA18	7.09	0.37	5.20	10.16	0.46	4.57	63.14	2.69	4.26
ZA19*	7.49	0.75	9.95	10.59	0.38	3.58	66.58	0.75	1.12
ZA20	7.13	0.50	7.03	10.54	0.36	3.43	65.78	1.42	2.16

Appendix 3. 1. Descriptive statistics and within-male variation (CV) of sperm size (measured in μ m, using ImageJ, N = 20, *N=16) of Cape ground squirrels (N = 19) from South Africa 2014.

MALE	VCL	VAP	VSL	LIN	WOB	PROG	BCF	# sperm tracked
ZA03	1828.82	691.39	259.34	0.37	0.38	13657.51	28.87	571.29
	± 53.97	± 22.30	± 12.00	± 0.01	± 0.00	± 723.17	± 0.10	± 31.77
ZA04	4506.34	1257.52	276.40	0.21	0.28	13598.33	30.27	1.06
	± 434.71	± 237.83	± 161.50	± 0.08	± 0.04	± 11798.95	± 2.02	± 0.24
ZA05	2253.68	813.87	285.21	0.35	0.36	14825.61	28.97	263.67
	± 108.22	± 45.02	± 15.86	± 0.01	± 0.00	± 695.27	± 0.15	± 30.60
ZA06	1738.68	661.22	249.85	0.38	0.38	13256.82	28.91	716.68
	± 36.19	± 15.66	± 7.51	± 0.01	± 0.00	± 519.72	± 0.10	± 31.74
ZA07	1887.78	695.50	231.27	0.33	0.37	12064.08	28.93	351.57
	± 151.82	± 78.30	± 55.18	± 0.05	± 0.01	± 2950.54	± 0.18	± 198.53
ZA08	2901.50	1014.72	328.52	0.33	0.36	16900.60	29.24	200.83
	± 842.73	± 183.72	± 34.42	± 0.02	± 0.03	± 2017.34	± 0.49	± 97.17
ZA09	2829.73	1033.82	335.98	0.33	0.37	17193.73	29.04	166.67
	± 105.46	± 29.20	± 12.66	± 0.01	± 0.01	±745.09	± 0.19	± 20.78
ZA10	3131.58	1125.41	354.05	0.31	0.36	17957.48	29.20	120.35
	± 57.27	± 27.33	± 9.99	± 0.01	± 0.01	± 654.54	± 0.21	± 4.22
ZA11	2653.41	900.84	276.73	0.31	0.34	13940.23	29.35	108.24
	± 225.73	± 50.18	±16.14	± 0.02	± 0.02	± 964.87	± 0.33	± 89.72
ZA12	2528.31	814.70	265.88	0.33	0.33	14075.43	29.67	242.71
	± 506.41	± 124.97	± 51.99	± 0.05	± 0.06	± 3455.62	± 1.27	± 194.03
ZA13	2344.96	846.17	279.68	0.33	0.36	14306.35	29.14	247.37
	± 126.05	± 31.60	± 23.02	± 0.02	± 0.02	± 1392.18	± 0.23	± 103.78
ZA14	4109.55	1334.46	418.73	0.31	0.32	21298.08	30.20	22.89
	± 152.63	± 57.04	± 33.47	± 0.02	± 0.01	± 2447.90	± 0.35	± 6.77
ZA15	1732.66	660.31	241.35	0.37	0.38	12817.77	28.82	598.00
	± 40.51	± 18.80	± 8.22	± 0.01	± 0.00	± 478.48	± 0.09	± 17.13
ZA16	4179.93	1295.30	394.18	0.31	0.31	19889.08	30.21	24.71
	± 495.82	± 134.87	± 50.93	± 0.03	± 0.02	± 3483.10	± 0.64	± 73.17
ZA17	2545.83	924.11	301.25	0.33	0.36	15462.53	29.06	183.52
	± 37.19	± 18.68	± 10.34	± 0.01	± 0.00	± 620.94	± 0.16	± 6.45
ZA18	2331.24	858.45	295.47	0.34	0.37	15203.64	29.09	312.00
	± 35.96	± 15.34	± 10.20	± 0.01	± 0.00	± 486.04	± 0.18	± 9.44
ZA19	3513.52	1175.33	363.22	0.31	0.34	19397.97	30.40	58.91
	± 826.65	± 237.89	± 63.94	± 0.04	± 0.02	± 4201.49	± 1.43	± 74.12

Appendix 3.2. Descriptive statistics of sperm velocity (average \pm SD; CASA_automated plugin) of Cape ground squirrels (N = 17) from South Africa 2014. Curvilinear velocity (VCL, μ m/s), Velocity average path (VAP, μ m/s), velocity straight line (VSL, μ m/s), linearity (LIN, %), wobble (WOB μ m/s), progression (PROG, μ m), and beat cross frequency (BCF, Hz).

							Total Sperm length					
	Head width (µm)			Head width (µm) Head length (µm)			(µm)			Total Flagellum length		
Male			CV			CV						CV
ID	Mean	SD	(%)	Mean	SD	(%)	Mean	SD	CV (%)	Mean	SD	(%)
63M	7.67	0.66	8.64	10.17	0.95	9.36	114.26	13.84	12.12	105.28	12.25	11.64
64M	7.87	0.40	5.04	9.84	0.84	8.51	115.65	3.91	3.38	115.65	3.91	3.38
65M	7.98	0.44	5.55	11.38	0.78	6.82	110.84	6.44	5.81	99.45	6.38	6.41
66M	8.02	0.28	3.51	11.50	0.67	5.79	123.71	2.57	2.08	112.20	2.36	2.10
67M	7.51	0.60	8.02	9.10	1.07	11.80	116.72	5.44	4.66	107.62	5.44	5.05
69M	8.06	0.57	7.06	10.55	0.76	7.23	114.64	5.97	5.21	104.09	5.91	5.68
70M	7.89	0.45	5.71	10.03	0.53	5.24	125.88	3.03	2.41	115.85	3.12	2.70
71M	7.34	0.74	10.03	10.77	0.77	7.15	122.14	2.80	2.30	111.37	2.83	2.54
72M	7.37	1.17	15.91	9.16	1.32	14.43	106.65	6.40	6.00	97.49	6.54	6.71
73M	8.59	0.86	10.06	11.96	0.85	7.09	128.15	3.45	2.69	116.20	3.44	2.96
74M	9.06	0.44	4.89	12.03	0.76	6.32	123.45	5.25	4.25	111.42	5.13	4.60
75M	8.63	0.68	7.89	11.88	1.08	9.11	124.37	5.30	4.26	112.22	5.26	4.69
76M	8.91	0.79	8.81	11.91	0.83	7.01	132.48	5.52	4.16	120.58	5.42	4.49
77M	8.14	0.48	5.93	11.65	0.96	8.26	123.10	4.24	3.44	111.21	3.92	3.53
78M	8.69	0.58	6.66	12.60	0.66	5.23	126.37	3.10	2.45	113.68	2.88	2.54
80M	8.43	0.38	4.47	12.49	0.67	5.37	126.78	2.58	2.03	114.08	2.21	1.94
81M	8.93	0.78	8.71	12.79	0.75	5.90	124.90	6.33	5.07	112.03	6.48	5.78

Appendix 4.1. Descriptive statistics and intra-male variation (CV) of sperm measurements (N = 17): Head width, head length, total flagellum length and total sperm length, in Richardson's ground squirrels.

Male ID	Body mass (g)	Total testes mass (g)	RTS
62M	400	0.72	0.23
63M	380	2.55	0.85
64M	470	0.89	0.25
65M	445	2.52	0.74
66M	340	1.73	0.63
67M	390	2.96	0.97
68M	320	2.02	0.77
69M	435	2.50	0.75
70M	305	2.01	0.79
71M	375	2.03	0.68
72M	355	2.04	0.72
73M	480	2.45	0.68
74M	448	1.98	0.58
75M	413	0.89	0.28
77M	377	2.15	0.72
78M	425	1.29	0.39
79M	357	2.31	0.81
80M	370	1.87	0.64
81M	398	2.14	0.69

Appendix 4. 2. Body mass (g), total testes mass (g), and relative testes size (RTS) in Richardson's ground squirrels.

Total testes mass (right + left), N = 20. RTS calculated according Kenagy and Trombulak (1986).

	Total testes		
Male ID	Body mass (g)	mass (g)	RTS
FV01	265	7.26	3.19
FV02	235	6.75	3.25
FV03	250	8.57	3.94
FV04	243	7.17	3.37
FV05	259	7.8	3.49
FV06	210	6.07	3.19
FV07	225	6.97	3.47
FV08	237	6.77	3.24
FV11	260	7.07	3.15
FV12	255	6.07	2.75
FV13	275	4.98	2.13
FV14	260	6.57	2.93
FV15	250	5.99	2.75
FV16	247.5	6.23	2.88
FV17	232	6.87	3.34
FV18	240	7.15	3.39
FV20	238	5.55	2.65
FV21	232	5.76	2.80
FV22	231	6.58	3.21
FV23	263	7.54	3.33

Appendix 5 1. Body mass (g), total testes mass (g), and relative testes size (RTS) in Barbary ground squirrels.

Total testes mass (right + left), N = 20. RTS calculated according Kenagy and Trombulak (1986).